A Biological and Chemical Comparison of Impacted Subsurface Sediments of the Lower Mahoning River

by

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A Biological and Chemical Survey of Subsurface Sediments of the Lower Mahoning River

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Abstract-Microbial Activity

To examine potential for bioremediation of industrially contaminated sediments in the Mahoning River of northeast Ohio, microbial activity measurements were made by estimating dehydrogenase activity using INT. Dehydrogenases take part in respiration and is fundamental to microbial activity. As INT accepts electrons, it is reduced to a red colored formazan (INTF), which allows for rate of reduction determination by colorimetric analysis. Attempts using previously published methods for this technique were unsuccessful due to background chemical reactions from high levels of PAHs and metals in the sediments. Major modifications were necessary to circumvent these reactions. To find a solvent that did not chemically reduce INT, the following solvents were examined: acetonitrile, methanol, ethanol, N,N-dimethylformamide, dichloromethane, formaldehyde and ethyl acetate. Acetonitrile had the highest extraction efficiency and least chemical interference. Temperature trials were performed at 4, 22, 40, 60 and 95°C. The INT reduction was inhibited at 95°C indicative of biological activity. Various INT concentrations were also tested, with a 1.08 mM solution having the least variance and did not require dilution for analysis. Two and sixty minute incubations were performed. Two-minute incubations showed initial activity and rate, while sixty minutes gave overall activity. The method reported here yields a simple, quick, inexpensive, and precise estimation of microbial activity.

Abstract- River Sediment Survey

The Mahoning River, located in northeastern Ohio, has received direct industrial effluents for over eighty years that have accumulated in the sediments. This study compares the distribution of microbial activities, microbial biomass, toxicity and levels of polycyclic aromatic hydrocarbons (PAHs) in subsurface sediments. Microbial activities were measured via dehydrogenase reduction of iodonitrotetrazolium chloride to a formazan dye. Microbial biomass was determined by lipid phosphate extraction. Toxicity assays were performed using a modified basic solid phase test with MicrotoxOmni from Azur Environmental Inc. The PAHs were extracted using USEPA organic extraction method #3550 and quantified in a gas chromatograph/mass spectrometer as per USEPA method #8270. The results show microbial activities and microbial biomass higher in anthropogenically-contaminated sediments, which is indicative of potential for use of indigenous microbes for bioremediation.

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Introduction

Mahoning River History

The Lower Branch of the Mahoning River begins in Leavittsburg, Ohio and flows in a southeasterly direction through the cities of Warren, Niles, Girard, Youngstown, Struthers and leaves the state past Lowellville (Figure 1). The river travels into Pennsylvania and joins the Shenango River to form the Beaver River (U.S. Army Corps of Engineers, 1999).

The Mahoning River has received industrial effluent and municipal wastewater treatment discharges for over 80 years. At the turn of the 19th century, Youngstown was a booming steel town. The river provided cooling waters for the manufacturing of steel, iron and other metals. The water was returned to the river contaminated and at elevated temperatures. Other factories used the river for dumping industrial wastes such as petroleum, coke quench water, lubricating oils and various chemicals (U.S. Army Corps of Engineers, 1999). At one point in 1977, the river received over 70,000 pounds of oil and grease each day (U.S. Army Corps of Engineers, 1999).

The Ohio Environmental Protection Agency began studying the water quality in 1980, and found significant levels of metals, volatile and semi-volatile organics compounds and polychlorinated biphenyls (PCBs) (Ohio EPA, 1996).

With the advent of the United States Environmental Protection Agency in 1970 the volume of unregulated wastes tapered off. Subsequent testing of the river water chemistry has shown that the conditions have improved and that chemical contaminants in the water column have reached acceptable levels (Ohio EPA, 1996). However, the sediments have not fared as well. Recent surveys by the Ohio EPA indicate that the toxic levels in the

sediment have either remained the same, or in some areas, have become more contaminated since the 1980 survey (Ohio EPA, 1996). Levels of polycyclic aromatic hydrocarbons (PAHs), PCBs and metals are at levels hazardous to human health. The Ohio Department of Health has issued a dermal contact advisory for sediments in the river from Warren to the Pennsylvania border along with a consumption warning for the benthic feeding fish in the river (Ohio Department of Health, 2000).

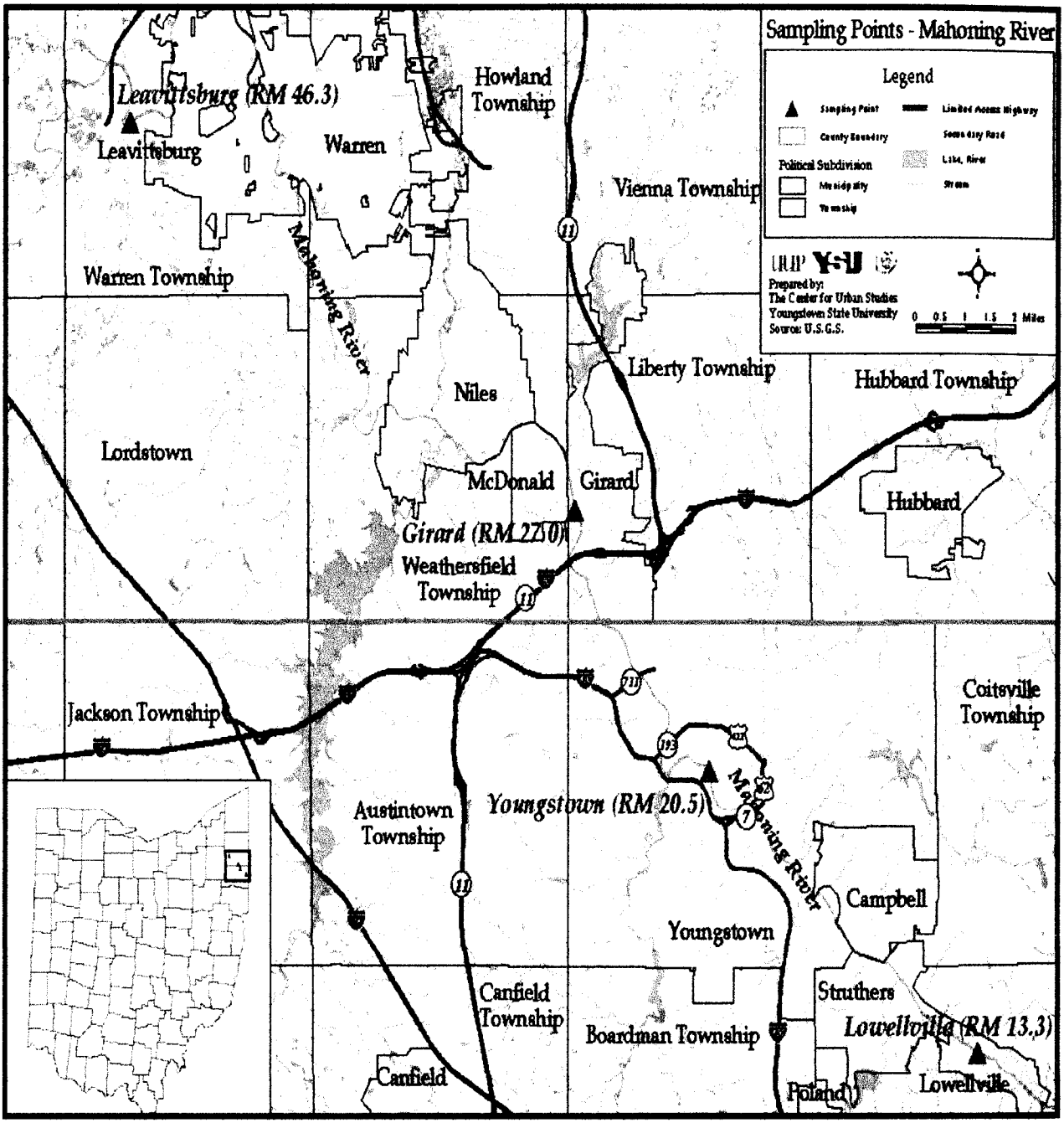

Figure 1: Map depicting Lower Mahoning River. (YSU Center for Urban Studies, U.S. Geological Survey, 2002).

Study Objectives

The objective of this study was to survey microbial activity, microbial biomass, PAHs and toxicity levels of the sediments in the Mahoning River. This assessment was to determine the potential for bioremediation of the sediments using indigenous microbes. These study parameters were estimated in sediments directly beneath the riverbed in four locations and beneath the riverbank at one location. To perform this survey, a method was developed to approximate microbial activity. Measurements of pH, total organic carbon, hexane extractable hydrocarbons, sediment particle size distribution, water holding capacity and temperature were also taken at each location.

Leavittsburg, river mile (RM) 46.3, served as the non-contaminated site as it is located upstream from heavy industrial areas. Girard (RM 27.0) and Youngstown (RM 20.5) were the sites chosen in the midst of the industrial regions of the Mahoning Valley. Lowellville (RM 13.3) is downstream of the heavy industrialized region. Lowellville was also chosen for riverbank sampling as a bend in the river allowed for sediments to tunnel under the bank.

The deep sediments were chosen for this study, as the surface sediments are newer as they undergo mixing with water due to currents and composition is disrupted (Lutz-Arend, 1994; Leff et al. 1994; Leff et al.1992). Therefore, the surface layer (5-8 cm) of sediments was not used for this survey.

The time span for this survey was brief to ensure that climatic conditions would not be a confounding factor as studies have shown that microbes are affected by seasonal changes (Richards, 1999; Findlay and Watling, 1997).

Bioremediation is a process utilizing microorganisms to remove or break down contaminants in environmental substrates (Sylvia et al. 1999). There are several environmental criteria that must be met for bioremediation to occur. The microbes must have enough catabolic energy to successfully degrade the contaminants, the contaminants must be bioavailable (unbound to the substrate), and the substrate conditions must be conducive to enzymatic activities of the microbes (Sylvia et al. 1999). Ideal conditions for bioremediation include the use of indigenous microbes rather than introducing "foreign" microbes. Treatability studies are required to deem the environmental conditions of the substrate. For example, more than one type of organic or inorganic contaminant will require different approaches and remediation techniques. Some environmental contaminants require aerobic conditions for degradation while other types of contaminants are mineralized in the presence of inorganic nutrients (i.e. nitrogen or phosphorus) (Rittmann and McCarty, 2001; McRae and Hall, 1998).

For this study, microbial activity and microbial biomass are measured and how these parameters related to environmental parameters, namely PAHs were addressed. It is important to determine activity of the microbes, as there could be an abundance of microbes present but they are not necessarily "active", as bioremediation using indigenous microbes relies more on metabolic activity than actual microbial biomass (Fonseca et al. 2001; Ringleberg et al. 2001; Stapleton et al.1998; Grosser et al.1991).

Microbial Methodologies

Microbial Activity

Dehydrogenases are oxidoreductase enzymes that take part in the respiration of the microbial cell. These enzymes oxidize organic compounds by the transfer of electron pairs from a substrate to nicotinamide adenine dinucleotide (NAD^+) or nicotinamide adenine dinucleotide phosphate ($NAPD^+$) forming NADH or NADPH, respectively (Smith and McFeters, 1997). This is a vital part of the electron transport system of a cell (Crane et al.1991). The use of a tetrazolium salt is a widely accepted method for measuring redox reactions in cells. The compound, 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) successfully competes with NAD^+ and $NADP^+$ for electrons. INT inserts between ubiquinone and cytochrome *b* in the electron transport chain (Maurines-Carboneill et al. 1998). As INT accepts electrons, it is reduced to a red colored formazan (Figure 2) (Altmann, 1969, Curl and Sandberg, 1961). INT provides an accurate assay of dehydrogenase activities under both anaerobic and aerobic conditions (Bhupathiraju et al, 1999, von Mersi and Schinner, 1990).

Microbes in sediments are usually found adhered to sediment particles, which creates difficulty in quantitating microbes using conventional microbial methods (Richards, 1999; Riis et al.1998; Lutz-Arend, 1994). Reduction of tetrazolium salts has been used as an indicator for dehydrogenase activity (Fonseca et al. 2001). Estimating the dehydrogenase activity of the microbes using INT was the method selected as it could be performed in short incubations and does not require the use of radioactive material. Other types of tetrazolium salts are 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) and triphenyltetrazolium chloride (TTC). CTC and TTC have been used in previous research

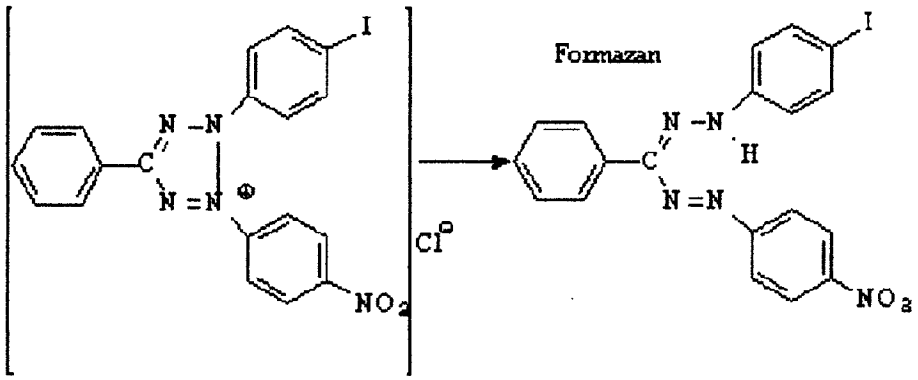

Figure 2: 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) (left) reduced to iodonitrotetrazolium formazan (right) (Sigma-Aldrich, Inc. 1997).

to measure dehydrogenase activity in environmental samples (Bhupathiraju et al. 1999; Wuertz et al. 1998; Lopez-Amoros et al. 1998; Maurines-Carboneill et al. 1997). INT and TTC have been shown to work well in anaerobic systems, while it is still unclear if CTC will work anaerobically (Bhupathiraju et al. 1999). TTC reduction is inhibited by oxygen making it useful in anaerobic conditions only (Camina et al. 1998). It has been shown by Trevors et al. (1982) that INT is effective aerobically and anaerobically.

Sodium azide is used as an abiotic control, as it is useful in determining the extent of background chemical reactions that reduce INT. Sodium azide is a respiratory inhibitor, shutting down microbial respiration at the cellular level (Ning et al.1996). When sodium azide is added to a cellular organism, cytochrome *b* is reduced and the electron uptake ceases, which will terminate electron transport and dehydrogenase activities (Wilson and Chance, 1967). Metabolic reduction of INT will not transpire as the cell has been exterminated without releasing intracellular components. Measurements of samples incubated with sodium azide are subtracted from samples incubated with water to provide an estimation of net microbial activities (Ning et al.1996; Rozycki and Bartha, 1981).

Microbial activities can be estimated numerous ways, many methods require the use of radiolabeled carbon sources (Atlas and Bartha, 1998) Measuring heterotrophic potential is possibly the most accurate using ^{14}C acetate, ^{14}C glucose or other radiolabeled carbon compounds and measuring incorporation into lipids (Stewart and Lovell, 1992; Vestal and White, 1989). Respiration may also be measured by radiolabeled carbon dioxide ($^{14}CO_2$). A substrate labeled with ^{14}C will release $^{14}CO_2$ during mineralization (Konopka et al. 1999; Atlas and Bartha, 1998).

Other methods investigated involve the reduction of dimethylsulfoxide (DMSO) to dimethylsulfide (DMS). This procedure involves incorporation of DMSO into an environmental sample and measuring the DMS produced by gas chromatograph (Griebler, 1997). Phosphatase enzyme assays require the use of p-nitrophenol phosphate as a substrate. Phosphatase enzymes convert the p-nitrophenol phosphate to p-nitrophenol, which is quantitated spectrophotometrically (Atlas and Bartha, 1998). Similar to phosphatate, nitrogenase enzymes are capable of reducing acetylene to ethylene. This reduction can be quantitated via gas chromatograph (Atlas and Bartha, 1998, Wickstrom and Corkran, 1997). Measuring sulfate reduction potential is another method often utilized. This assay requires the use of ion chromatography to quantify metabolic rates. The drawback to this method is sulfate must be present in the samples (Rothfuss et al.1996).

Estimating CO_2 respiration and evolvement through colorimetric assays were examined by Dilly and Munch (1998). These assays concentrate on estimating potential activities rather than actual rates. Other means of measurement are glucose utilization or estimating oxygen consumption (Rich and King, 1999).

Microbial Biomass

Measuring microbial biomass is expressed as mass of microbes per gram of sediment. A useful method of determining biomass of a microbial community is measuring a common cellular component of all microbes, phospholipids, which degrade easily upon cell death thus providing an estimate of viable of biomass (Vestal and White, 1989). Microbial biomass is estimated by extracting phosphate bound lipids with organic solvents and colormetrically analyzing with malachite green in a spectrophotometer

(Pinkart et al. 1998; Dobbs and Findlay, 1993; Findlay and Dobbs, 1993; Vestal and White, 1989). Other methods for determining microbial biomass involve chloroform fumigation and extraction. The extracts are fumigated with chloroform to kill indigenous microbes, then reinoculated with organisms that consume the dead microbes. The consumption is measured by CO_2 production. (Turner et al. 2000; Nunan et al.1998). A less toxic method for measuring biomass involves microwave irradiation of soil. The carbon released is extracted and measured as total organic carbon (Islam and Weil, 1998).

Measuring microbial biomass is simpler than direct counts using microscopy, which involves adding a dye to the samples and manually counting the microbes. Although direct counts would provide a more accurate estimation with less variability in aquatic samples, it is time consuming and in sediments samples it is impossible to visualize microbes that are adhered to the sediment (Richards, 1999; Riis et al. 1997; Findlay, et al.1989). Microbial biomass cannot be directly correlated with direct counts.

Toxicity

The Microtox® system is becoming a common method to measure toxicity in sediments (Sabaliunas et al. 2000; Bispo et al. 1999). Freeze-dried bioluminescent marine bacteria, *Vibrio fisheri* are exposed to sediment and the change in the amount of luminescent output by the bacteria is measured (Guzzella, 1998). Toxic sediments cause cell death, therefore less light is emitted from the bioluminescent bacteria colonies in more toxic sediments. This method provides a relative measurement of toxicity in general; it does not identify specific toxins. This is a convenient, standardized method as the bacteria are provided freeze dried by the manufacturer.

Other assays for measuring toxicity expose eukarotic organisms (i.e. *Daphnia* spp. or *Strongylocentrous* spp.) to sediment and addresses mutagenicity (Weltens et al. 2000; Bispo et al.1999; Wernersson and Dave, 1997). The advantage of these assays is involvement of indigenous organisms as a biomarker. A major disadvantage to this assay is considerable time is required for incubations (days to weeks), whereas the Microtox requires a 30 minute incubation.

Polycyclic Aromatic Hydrocarbons

PAHs are organic compounds with a multiple closed benzene ring structures (Figure 3). PAHs are most commonly the result of incomplete combustion of industrial processes (Kastner et al. 1998). The compounds have low solubility rates and low desorption rates in sediments as they adhere to sediment particles (Poeton et al. 1999). They pose an environmental threat, as they are carcinogenic, estrogenic, and mutagenic (Witt and Trost, 1999). By identifying these compounds and their location in the sediments, a better picture is established of the composition of the sediments. Extractions are possible by using strong organic solvents and utilizing a gas chromatograph/mass spectrometry (Simpson, et al.1995).

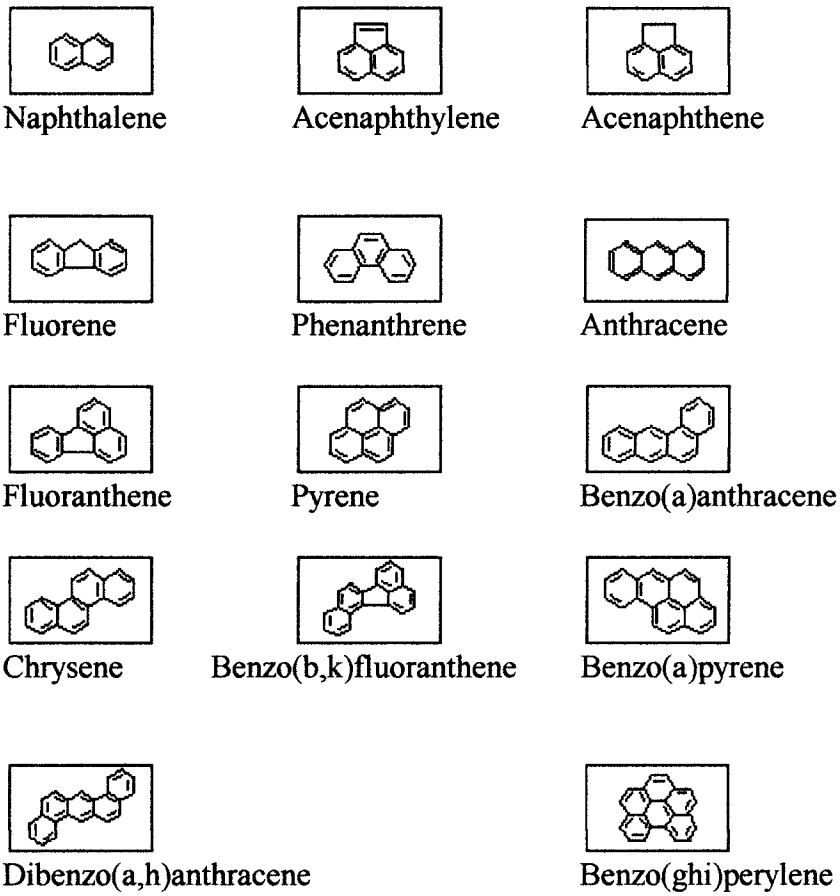

Figure 3: Diagrams of common PAHs (From Jinno Laboratory, 2001).

Methods

Sampling

Sediments were collected with a Wildcat coring device from four locations from the Mahoning River and one bank in triplicate (Figures 4 and 5). The sites selected were Leavittsburg (RM 46.3), Youngstown (RM 20.5), Girard (RM 27.0) and Lowellville (RM 13.3). The bank sediment was collected from Lowellville (RM 13.3) 2.26 M from the river edge and 1.23 M below the surface of topsoil. The survey spanned from late summer to early fall. The corer was fitted with a plexiglass tube measuring 48 cm in length and 5.25 cm in diameter. The sample tubes were capped on both ends and placed in an upright position. Upon returning to the laboratory, the samples were immediately placed in an anaerobic glove bag. The surface layer (10.0-14.0 cm) was removed and discarded. The remaining sediments (15.0-45.0 cm) were placed in glass all-purpose containers, homogenized and labeled. The sediment used for anaerobic microbial activities remained in sealed containers, while the rest of the sediments were allowed exposure to the atmosphere. The sediments were immediately used for microbial activity measurements as timing and handling was crucial for that assay.

Figure 4: Placing the plexiglass tube into the Wildcat corer.

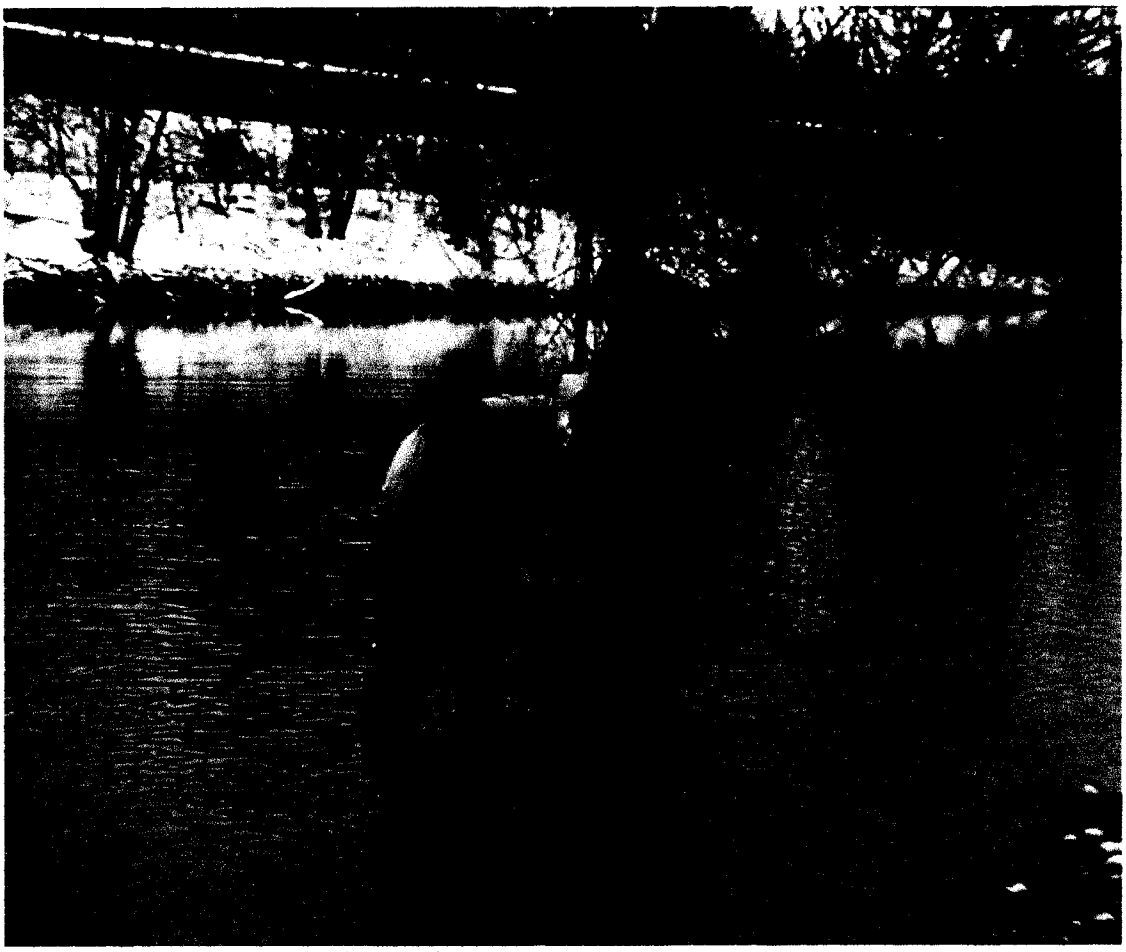

Figure 5: Sampling at Market St. in Youngstown.

Microbial Activity:

Dehydrogenase enzyme activity was measured by the reduction of INT to a red colored formazan and analyzed colormetrically in a spectrophotometer. Due to the organic contamination of these primarily anaerobic sediments, previous methods (Mathew and Obbard 2001; Camina et al.1997; von Mersi and Schinner 1991) were unsuccessful as many solvents (i.e. N,N-dimethylformamide, acetone, and dichloromethane) possibly caused chemical reduction of INT.

Sediment (0.100 g) was placed in scintillation vials, in triplicate. All activity measurements were performed in the dark and/or dark room conditions as the tetrazolium compound is light sensitive. This method was performed anaerobically in a glove bag under nitrogen flow, as well as aerobically.

The samples for metabolic measurements had 0.5 ml of 18 MΩ water added and vortexed. These were incubated at room temperature for thirty minutes. Next, 0.5 ml of 1.08 mM INT solution was added and mixed; these were incubated for two and sixty minutes at room temperature. The INT formazan was then extracted with 3.0 ml of acetonitrile at room temperature for ten minutes. The samples were filtered using Whatman No. 40 filter paper and the sediment washed with 10.0 ml of acetonitrile. The filtrate was read in a Shimadzu UV-260 spectrophotometer at 490nm.

The control samples were killed with 3.0 ml acetonitrile prior to addition of INT to prevent reduction. Then 0.5 ml of 1.08 mM INT was added and allowed to extract for 10 minutes before filtering.

Blank samples were included to determine background sediment interference. These were performed without the addition of INT, but otherwise identically to the above metabolic samples.

INT formazan solutions of in the following dilutions were prepared for a standard curve: 0, 22, 45, 89, 176, and 265 nmol. These solutions were read in the spectrophotometer at 490nm (Appendix 1).

Calculations were done using the linear equation (Appendix 2) from the standard curve in nmol INTF reduced per g^{-1} dry weight. The net microbial activities were calculated by subtracting the control sample data from the samples incubated with milli-pore water. The standard deviations of the metabolic rates and control samples were calculated from the error formula by Kanipe (1977) (Appendix 3).

Lipid Phosphate Biomass:

Lipid phosphate biomass measurements were done using a modified malachite green lipid phosphate method (Findlay et al.1989; Vestal and White, 1989; Fang and Findlay, 1996). Fresh sediment (0.250 g) was weighed and placed in culture tube in quintuplet. The exact amount of sediment in each tube was recorded. The next step involved adding 2.0 ml of 50 mM phosphate buffer, 5.0 ml of methanol and 2.5 ml of dichloromethane (DCM) to each sample. The samples were vortexed until well blended and were allowed to stand in the dark at room temperature for 24h. Then, 2.5 ml of DCM and 2.5 ml of 50 mM phosphate buffer were added. This was mixed well and allowed to separate into layers for 24h at room temperature in the dark. The lower lipid layer was removed from the vial, placed in a graduated cylinder and the volume recorded. The lipid layer was then placed into a scintillation vial and into a Pierce Reacti-Therm III heating/stirring

module and dried down under nitrogen stream at 37°C. Lipids were resuspended with 1.0 ml of DCM and gently swirled. Next, 400 µl of the redissolved lipids were pipetted into a glass ampule, placed in a Pierce Reacti-Therm III heating/stirring module and dried using a nitrogen stream at 37°C. Saturated potassium persulfate (0.9 ml) was added to each ampule and the ampules were flame sealed. The ampules were placed in a Fisher Scientific Isotemp 500 Series drying oven at 105°C for 24h to digest the lipid and release phosphates.

The ampules were broken open and ammonium molybdate (0.2 ml) was added and allowed to stand for ten minutes. Malachite green (0.9 ml) was added and stood for thirty minutes and read in a Shimadzu UV-260 spectrophotometer at 610 nm.

The standard curve for lipid phosphate biomass was made by adding 0, 5, 7, 10,15, 20, and 25 µl of 0.5 µM glycerol phosphate to glass ampules providing amounts of: 0.0, 2.5, 3.5, 5.0, 7.5, 10.0, and 12.5, nmol of glycerol phosphate respectively (Appendix 4). Next, 0.9 ml of saturated potassium persulfate was added to each ampule and the ampules flame sealed. These were also placed in the oven at 105°C for 24h. The ampules were broken open and 0.2 ml of ammonium molybdate was added and stood for ten minutes. Malachite green (0.9 ml) was added and stood for thirty minutes. The samples were read in the spectrophotometer at 610 nm.

Data were extrapolated by finding the amount of phosphate derived from lipid phosphates. The linear equation (Appendix 2) of the standard curve was used. The unknown (x) was found in the equation. A separate equation was used to determine total lipid phosphate biomass (Appendix 5). The figures derived from this equation are a quantitative measure of total lipid phosphates in the sediment.

Toxicity:

Sediment toxicity was determined using a basic solid-phase test on the MicrotoxOmni® M500 analyzer manufactured by AZUR Environmental Incorporated. The method was a modification provided from the manufacturer in which fourteen serial dilutions were used instead of the usual nine dilutions due to high toxicity of the sediments.

A report was generated by the software of the Microtox system indicating the solid phase EC_{50} concentrations of the sediments. The EC_{50} is the concentration of sediments (mg wet weight/L) that exterminate 50% of the bacteria. This figure was used to gauge the toxicity of the sample. The lower the EC_{50} concentration, the higher the toxicity measurement.

Polycyclic Aromatic Hydrocarbon Extractions

PAHs were extracted from sediments using United States Environmental Protection Agency (USEPA) sonication extraction method 3550 (USEPA, 1996a). Approximately 10.0 g of sediment was massed using a Denver Instrument M-120 into a 150 ml beaker, and the sample mass accurately recorded. Duplicate, 10.0 g sediment samples were weighed into 150 ml beakers. Next, 1.0 ml of surrogate solution was added to each sample. A surrogate solution is a compound added to the sample that does not occur naturally in environmental samples. A known amount was added to samples in order to measure extraction efficiency. In this study, the surrogate solution contained 2 µg/ml each of 2-fluorobiphenyl, *p*-terphenyl-d14 and nitrobenzene-d5. Sodium sulfate was added in excess to dry the samples. Dichloromethane (DCM) in the amount of 40 ml was added and mixed well. The samples were sonicated for twenty minutes in a VWR

Scientific Aquasonic 750HT and filtered over sodium sulfate saturated with DCM. Another 30 ml of DCM was added and the samples were mixed well. This was sonicated for 20 minutes and filtered. This process was repeated once more. The DCM extracts from each of the three sonication steps were combined. The funnel was rinsed with 20 ml DCM and the rinsate added to the combined sample extract.

A matrix spike solution (1ml), containing 10μg/ml of naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b,k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, indeno(1,2,3-cd)pyrene, and benzo(ghi)perylene was added to 10.0 g sediment and processed identically as the above extractions. This represents a control as the sediments were spiked with a known amount of PAHs. Another control was performed using the matrix spike solution without the addition of sediments along with a blank control.

Excess solvents were evaporated with simple distillation on a Fisher stirring hotplate to 5-8 ml. The final volume of the extract was accurately recorded, and then 1.0 mL of the sample extract was transferred to an autosampler vial. In accordance with the GC-MS method, internal standards were added to the sample extracts. These internal standards contained 4 μg/ml of each of the following compounds: acenapthene-d10, chrysene-d12, 1,4-dichlorobenzene-d4, naphthalene-d8, perylene-d12 and phenanthrene-d10. These compounds are used as a control for the mass spectrometer.

The samples were quantified in a Hewlett Packard 5890 Gas Chromatograph/ 5970B Mass Spectrometer (GC/MS) in accordance to USEPA method 8270C (USEPA, 1996b). The GC/MS was fitted with a Supelco MDN-5S column measuring 30 M, 0.25 mm ID

and 0.25 μM film. The mass spectrometer was equipped with a Burle Channeltron electron multiplier #5772. The injection port and transfer line temperatures were 270°C. The samples (1.0 μL) were injected and the temperature was held at 50°C for 0.5 minutes. The temperature was ramped at 15°C per minute until 270°C was reached. The temperature was then ramped at 40°C per minute until the temperature reached 310°C. This final temperature was held for 10 minutes. The GC/MS software calculated an on column concentration of PAHs in ug/mL. This concentration was then converted to ug/kg and corrected for moisture present in the sample (Appendix 6). A standard curve was performed with the GC/MS using 0.2, 2.0, 4.0, 6.0 and 8.0 μg/ml PAH standards.

pH

Boiled 18 MΩ water was used to make a 0.1 M NaCl solution. The water was boiled to release CO_2 and give a neutral pH. This NaCl solution was mixed with sediment at a 1:1 concentration to add ionic strength. pH was measured with a Fisher Scientific Accumet portable AP10 pH meter with adjustments according to temperature. USEPA Method's 9040C and 9045C were followed (USEPA, 1996).

Hexane Extractable Hydrocarbons

Hydrocarbons were extracted from sediments by sonication with hexane. Sediments in the amount of 4.0 g were weighed into a glass beaker and dried with excess sodium sulfate. Approximately 20 ml of Hexane was added and sonicated in a VWR Scientific Aquasonic 750HT for 60 minutes. The hexane was decanted into a preweighed flask containing boiling chips. The hexane was distilled off at 60° C and the remaining hydrocarbons were gravimetrically analyzed (USEPA, 1998; Grosser et al. 1991).

Percent Moisture

Percent moisture was determined by massing sediments with a Denver Instrument M-120 scale into aluminum boats and dried in a Fisher Scientific Isotemp 500 oven at 105°C for 24 hours then weighed again.

Total Organic Carbon

For total organic carbon, oven dried sediments were weighed into aluminum boats and combusted at 550°C for 24h then gravimetrically analyzed for carbon content (Tiessen and Moir, 1993).

Water Holding Capacity

Water holding capacity was measured by placing dried sediments (50ml) into a 600 ml beaker and 18 MΩ water was added until saturation (Livingston, 1993).

Temperature

Water and sediment temperatures were taken with a precalibrated Fisher digital thermometer.

Particle Size Distribution

Settling time using a hydrometer (Fisher Environmental) method was analyzed to determine particle size of the sediment (Sheldrick and Wang, 1993). Sediments were combusted at 550°C for 24h and 40 g were placed into a 1000 ml flask. Detergent solution (50 g/L) was added in the amount of 100 ml along with 300 ml of Millipore water. This mixture was stirred and sat overnight. The mixture was homogenized with a Tekmar TR-10 tissue homogenizer and poured into a 1000 ml graduated cylinder. Millipore water was added to bring the sample to volume. This was homogenized again

and specific gravity (R_{40s}) was measured after 40s. A specific gravity (R_{7h}) reading was repeated after 7h.

A blank reading (R_{blank}) was performed with 200 ml of detergent solution and 800 ml of water. Calculations determining particle size were performed (Appendix 7).

Statistics

Student t-test was performed for all assays. The mean, standard deviation and variance were found for each site. To find whether the variance was equivalent between each site, the average was divided by the standard deviation and multiplied by 100 to give a percentage. The percentages were compared between the sites and if unequal the student t-distribution was determined by using each sites individual variance value. If the percentage was equal between the sites, the average variance between all sites was used to calculate the t-value. In all assays, unequal variance was determined.

The t-value was found by taking the difference of the means of two sites and dividing by the combined standard deviation between the two sites. The calculated t-value was compared to a t-table (Rohlf and Sokal, 1969), which lists t-values and their limits to indicate significant difference in data. Degrees of freedom were considered (n-1-1) for the determinations. If the t-value exceeded the limits imposed in the t-table, then the sites were considered significantly different (Bayless, 2001).

Correlation statistics were used to compare parameters. The equation used for the correlation is shown in appendix 8 (Schefler, 1969). The r value ranges from –1 to 1. If the sample value is positive it is considered positively correlated. If the sample value is negative, it is considered negatively correlated. A strong correlation is determined if the r value is in the 0.05 range closest to (+/-) 1.

Results and Discussion

Dehydrogenase Activity Method Development

A number of tests were performed to obtain a working method to measure dehydrogenase activity of the microbes in the sediment of the Lower Mahoning River. This method is a simple, effective, inexpensive and quick measure for dehydrogenase activity of microbes.

Efficacy was shown with the use of acetonitrile to extract the formazan from the sediments, also with no artificial reduction of INT. Alcohols did not cause chemical reduction but required high amounts for extraction. N,N-dimethylformamide (N,N-DMF), acetone, ethyl acetate and 10% formalin caused chemical reduction of INT in the presence of autoclaved sediments (Table 1).

Different INT concentrations tested show that 1.08 mM had the least variance and was in the range for the standard curve (Figure 6). Concentrations of 3.22 and 9.88 mM were tested but absorbency readings on the spectrophotometer were out of range (> 1.00 nm).

Sediments incubated with INT for 60 minutes had the highest formazan production, but the 2-minute incubations showed less deviation (Figure 7). Longer times were tested (120 minutes) but the results had a high variance and are not shown. Time trials performed show that the initial reduction of INT occurs quickly and a rate was caught at the 2-minute incubation. At 60-minutes, the majority of INT is reduced. For all assays 2 and 60-minute incubations were performed to show the rate of reduction and the total amount of reduction of INT.

Temperature trials performed showed an increase of enzyme activity as the temperatures increased up to 60°C. The 95°C incubation showed biotic termination of

activity. This is indicative of the assay being microbial mediated as most microbes were exterminated 95°C (Figure 8).

The standard curve was used to calculate μmol INTF produced from the absorbency readings. The r^2 coefficient value was 0.9973, the y-intercept was –0.0119, and the slope was 0.0035 (Figure 9).

Sodium azide does not appear effective as a respiratory inhibitor, there was high variability at different temperatures (Figures 10a and 10b). Perhaps the microbes found in the Lower Mahoning River sediment, having adapted to an extreme environment, has conferred tolerance to sodium azide. Also, previous research has shown that sodium azide is less effective in anaerobic conditions (Ning, 1996). Plus, azide will elevate the pH of samples at higher temperatures (Rozyecki and Bartha, 1981). Future studies are warranted to locate an effective respiratory inhibitor that will not cause discontinuance of the cell membrane.

A comparison of current data to previously published results is shown (Table 2). Sandstone from ancient monuments in the United Kingdom showed low values for INT reduction (Taylor and May, 2000). Beach sediment contaminated with crude oil and sandy loam soils had results similar to those of the Lower Mahoning River sediments (Mathew and Obbard, 2001; von Mersi and Schinner, 1991). Uncontaminated arable soils showed INT reduction at much greater rates (Trevors, 1983). Although there is a wide range of figures, data from the Lower Mahoning River is within the range of earlier studies.

Previously published methods using INT to measure dehydrogenase enzymatic activity were not successful in this study. Major modifications were necessary to

circumvent chemical reductions of INT. Substrates used in previous studies included non-contaminated soils, sandstone and sediments (Taylor and May, 2000; Brohon et al. 1999; Maurines-Carboneill et al. 1998; Wuertz et al. 1998; von Mersi and Schinner, 1991; Trevors, 1984). Two other studies performed INT reduction in organic contaminated substrates (Mathew and Obbard, 2001; Camina et al. 1997). A study using INT in metal contaminated environments showed that INT reduction was elevated in the presence of copper. Other metals did not effect the INTF production, nor were there any problems associated with solvent induced chemical reduction (Obbard, 2001). None of the above studies were conducted in substrates with a combination of organic and inorganic (heavy metal) contaminants. It can be concluded that interference in the assay was a combination of the nature of contaminants and their reactions with halogenated solvents.

Table 1: Results from solvent trials for microbial activity.

Solvent	ml required for extraction	Artificial Reduction
Ethanol	35	No
Methanol	40	No
n,n-DMF	NA	Yes
1:1 n,n-DMF/Ethanol	NA	Yes
Acetone	NA	Yes
Acetonitrile	10	No
Isopropanol	37	No
Ethyl Acetate	NA	Yes
10 % Formalin	NA	Yes

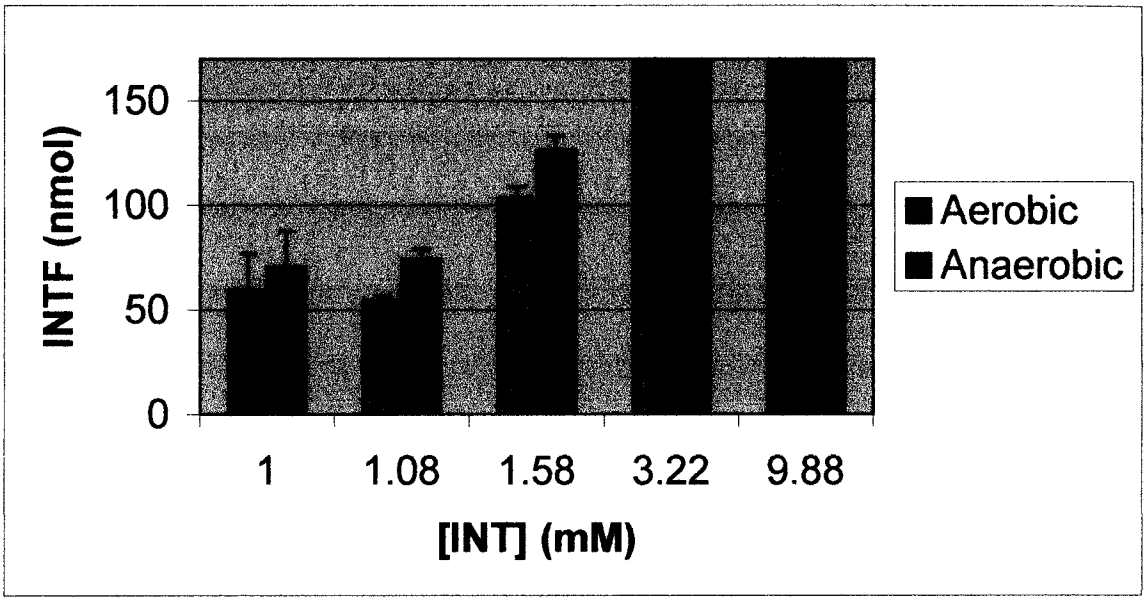

Figure 6: Incubation resulting in the reduction of INT (mM) to INTF (nmol/g) for selected concentrations in sediments of the Mahoning River at Youngstown (RM 20.5). The results from 3.22 and 9.88 mM were too dark to read in the spectrophotometer.

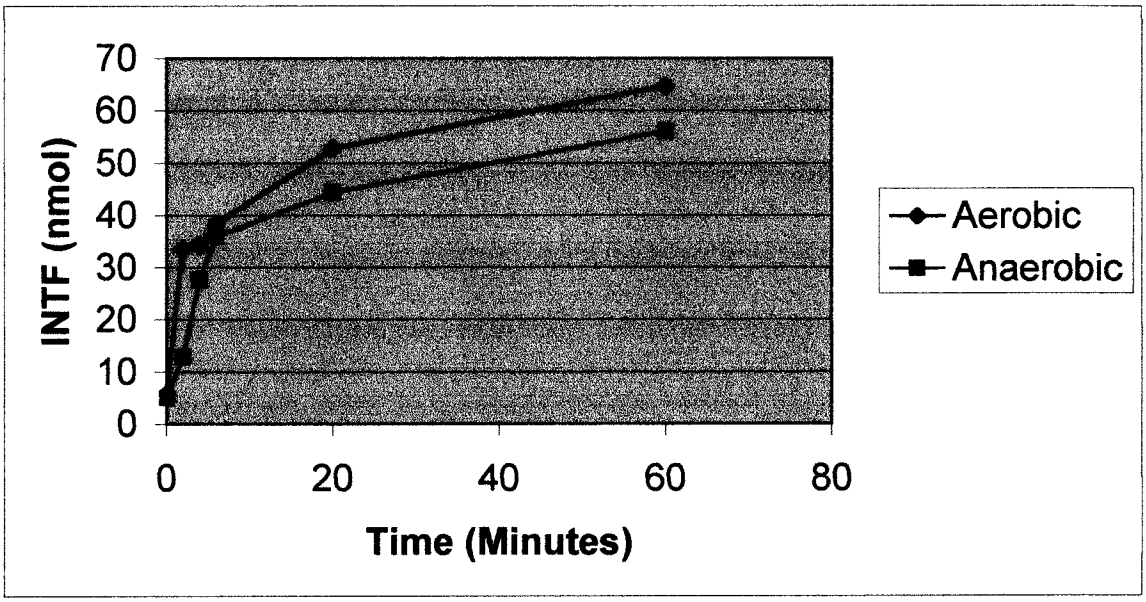

Figure 7: Time trial for iodonitrotetrazolium formazan (INTF) production aerobically vs. anaerobically in sediments from the Mahoning River in Youngstown (RM 20.5).

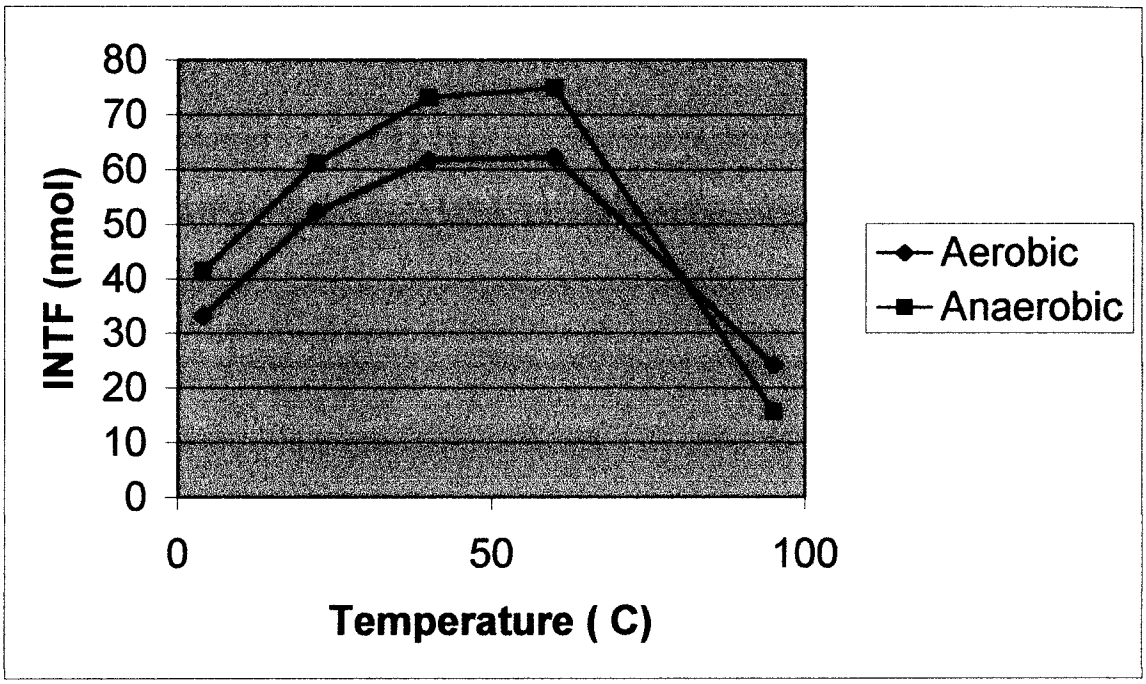

Figure 8: Reduction from iodonitrotetrazolium chloride (INT) to INTF for one hour incubations at different temperatures in sediments of the Mahoning River at Youngstown (RM 20.5). Abiotic controls using acetonitrile were subtracted from metabolic samples.

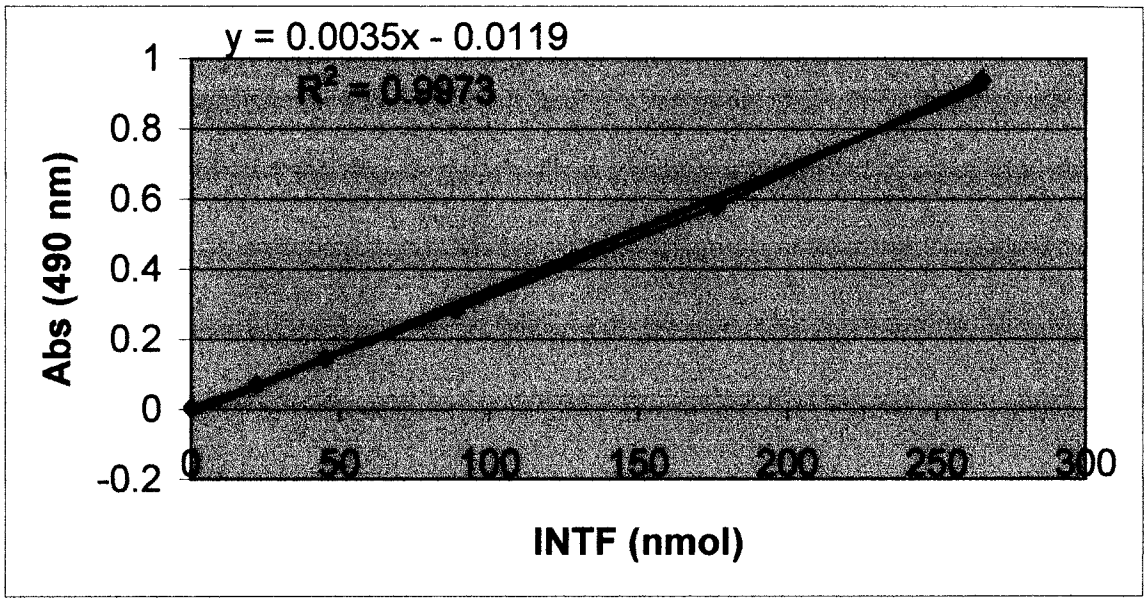

Figure 9: Standard curve of INTF in acetonitrile.

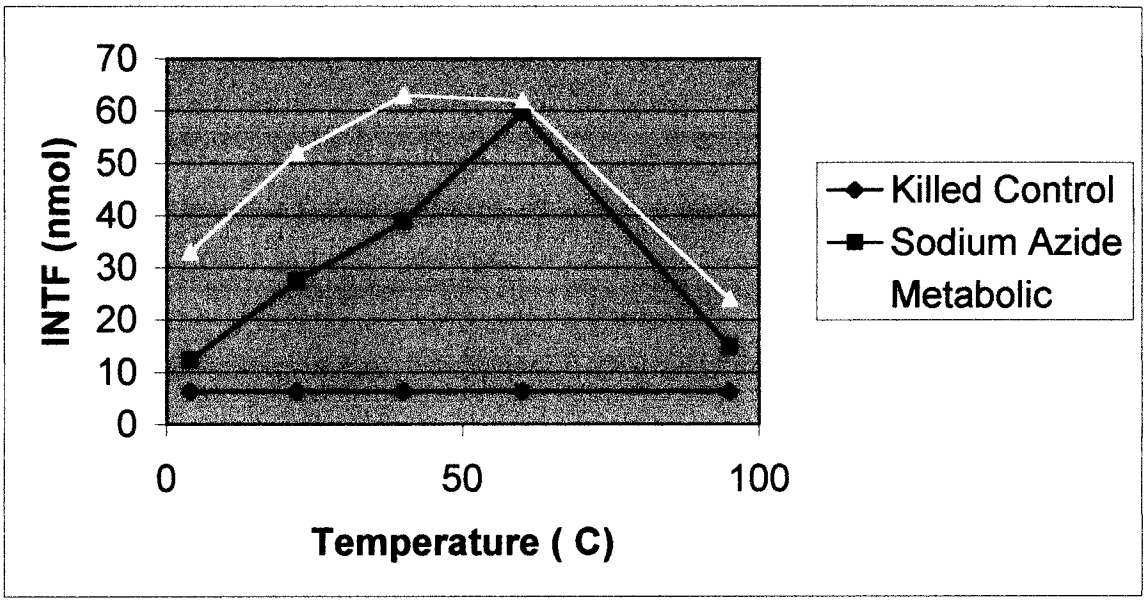

Figure 10a: Aerobic trials for sodium azide as abiotic control in microbial activity at different temperatures. Killed control represents samples pretreated with acetonitrile. Sodium azide samples were pretreated with sodium azide for 24 hours. Metabolic samples were not inhibited.

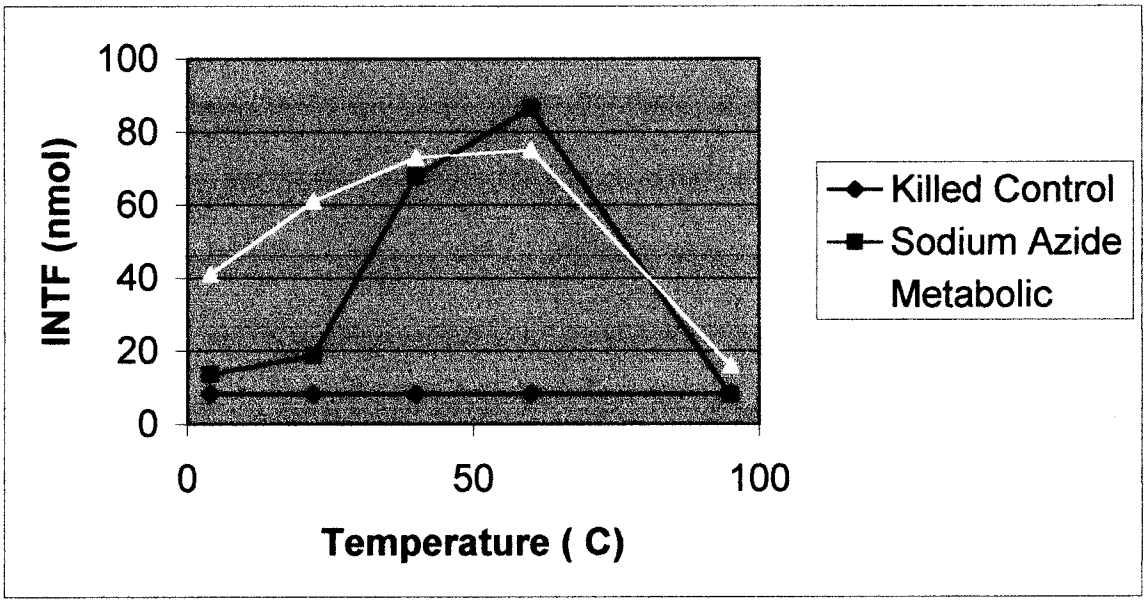

Figure 10b: Anaerobic trials for sodium azide as abiotic control in microbial activity at different temperatures. Killed control represents samples pretreated with acetonitrile. Sodium azide samples were pretreated with sodium azide for 24 hours. Metabolic samples were not inhibited.

Table 2: Comparison of microbial activity results incubated at 60 minutes to previously published methods. Results are shown as INTF produced $nmol^{-1}$ g^{-1} h^{-1}.

Substrate	**INTF (nmol/g)**
Leavittsburg Sediment [a]	36
Girard Sediment [a]	132
Youngstown Sediment [a]	93
Lowellville Sediment [a]	91
Lowellville Bank Sediment [a]	104
Sandstone from Portchester Castle, UK [b]	2.1-8.5
Beach Sediment contaminated with crude oil (Singapore) [c]	11.4
Arable Soil (Austria) [d]	200-480
Sandy Loam from Cambridge Research Station (Ontario) [e]	40-200

Key:
[a] Mahoning River Results
[b] Taylor and May, 2000
[c] Mathew and Obbard, 2001
[d] von Mersi and Schinner, 1991
[e] Trevors, 1983

River Survey

Lower Mahoning River sediments were sampled in triplicate at five locations. The main focus of this survey was to determine the quantity and respiration of microbes present in sediments throughout the Lower Mahoning River in comparison to the environmental contaminants. These parameters were examined in four sites for river bottom sediments and one site for riverbank sediments.

The physical composition of the sediments (i.e. particle size) did not differ between the five sites (Figure 11). Also, environmental conditions (i.e. water and sediment temperature) showed no significant differences except for Lowellville bank, where the sediments are covered by topsoil (Figures 12 & 13). The only parameters showing significant differences were pH (having Lowellville sediments higher than Leavittsburg and Youngstown) (Figure 14); water holding capacity (Lowellville bank was higher than Girard) (Figure 15); and percent moisture (Leavittsbsburg and Girard lower) (Figure 16). These data for physical and environmental conditions suggest that an accurate comparison can be made between the sites for the biological and chemical parameters, because there is little difference between physical characteristics between all sites.

Biological parameters for the sediments include assays determining microbial activity, microbial biomass and toxicity. Results for aerobic microbial activity two-minute incubation show Leavittsburg (3.5 nmol g^{-1}) significantly lower than Girard (17 nmol g^{-1}), Youngstown (14 nmol g^{-1}) and Lowellville (12 nmol g^{-1}) (Figure 17a). Anaerobic microbial activity incubated two-minutes showed Leavittsburg (5.6 nmol g^{-1}) significantly lower than Girard (22 nmol g^{-1}) and Lowellville bank (29 nmol g^{-1}) (Figure 17b).

Aerobic microbial activity with sixty-minute incubation showed Leavittsburg (13 nmol g^{-1} INTF) significantly lower than Girard (47 nmol g^{-1}), Youngstown (33 nmol g^{-1}), and Lowellville (32 nmol g^{-1}) and Lowellville bank (38 nmol g^{-1}). Also, Girard was significantly higher than the other four sites (Figure 18a). Anaerobic microbial activity incubated sixty minutes also showed Leavittsburg lower than the other four sites. Girard and Lowellville bank were also significantly higher than Youngstown (Figure 18b).

There was a direct positive correlation between metals and microbial activity (Table 3 and Figure 19). Metals data was taken from United States Army Corps of Engineers (1999). It is possible that the microbes are utilizing metals as a terminal electron acceptor. Microbes capable of reducing metals are also found to oxidize organic contaminants (Nevin and Lovley, 2000; Rooney-Varga et al. 1999; Lovley, 1993). A study, which examined metal contaminated soils, found that microbial biomass and microbial activity decreased when the levels of heavy metals increased (Kuperman and Carreiro, 1997). Although this finding is not consistent with the results of this study, the soil studied did not contain organic contaminants. Possibly the organic degrading microbes are dependent upon both organics and metals. More studies would be needed to investigate this phenomenon and identify possible metal reducing bacteria in the sediments.

Results for lipid phosphate biomass showed Leavittsburg (25.2 nmol phosphate g^{-1}) significantly lower than Girard (54.2 nmol g^{-1}) and Lowellville bank (90.8 nmol g^{-1}). Although Lowellville had a mean of 106 nmol phosphate g^{-1}, it was not significantly different from any site due to high variance (Figure 20).

Positive correlation was found between microbial biomass and total organic carbon (Figure 21). This finding is supported by previous research in organic contaminated

substrates. A study in an aquatic habitat has shown that microbial communities are capable of tolerating pollutant stressors (Barkay and Pritchard, 1988). This study has shown that there is a considerable lag time in activity and biomass where the community adjusts for survival and growth, however, it is uncertain if this adjustment is phenotypic or genotypic. After a tolerance to one pollutant has been achieved, the microbial community appears to be more tolerant to additional pollutants and stressors (Barkay and Pritchard, 1988). In this current study, anthropogenic pollutants have been present in the sediments over 80 years. It is expected that adaptation by the microbes would occur.

Microbes are capable responding to PAH contaminations at both genotypic and phenotypic levels. Phospholipid fatty acid profiles (PFLA) and nucleic acid analysis were used to measure biomass and identify genetic makeup of microbes in contaminated sediments of the Little Scioto River in central Ohio. It was concluded that PAH degrading microbes were found in higher amounts in sediments containing elevated PAH concentrations (Langworthy et al.1998). Another study showed microbes that have adapted to PAHs are capable of degrading them. Microbes utilize the organic pollutants as a carbon food source (Grosser et. al. 1991; Barkay and Pritchard, 1988).

Log EC_{50} values were significantly higher (less toxic) for Leavittsburg than Girard and Lowellville bank (Figure 22).. There was a high standard deviation for results for this assay, possibly due to high heterogeneity of the sediments. The results show that Girard, Youngstown and Lowellville bank have the most toxic to the marine bacteria *Vibrio fisheri*, while Leavittsburg and Lowellville are less toxic. The toxicity results from this study were compared to previous studies and from results obtained from Shite Creek (a non contaminated stream in southern Mahoning County) (Table 4). Shite Creek showed

low toxicity, while contaminated ground water leachates from sites in Lithuania displayed more toxicity (Sabaliunas et al. 2000). PAH contaminated leachates from a former coke oven site had a wide range of toxicity results (Bispo et al. 1999). The results from the Lower Mahoning River sediments are within the reaches of these prior reports. These results do not correlate with PAH contamination of the sediments (Figure 23), but are somewhat correlated with metal content (not significantly) (Figure 24).

A previous study in contaminated sediments, which include PAHs, PCBs and metals showed that metals were responsible for extirpation of *V. fisheri* more drastically than PAHs and PCBs (Salizzato et al. 1998). Other studies showed that PAHs and organic contaminants were responsible for toxicity in *V. fisheri*. The drawback of the Microtox® system is that *V. fisheri* is a marine bacteria. The use of an indigenous freshwater organism would give a better picture of toxicity. Studies have used *Daphnia magna* for toxicity assays (Weltons et al. 2000; Wernerson and Dave, 1997). *D. magna* is a filter feeding crustacean and found in most freshwater ecosystems. It is sensitive to organic and inorganic contaminants and is a reliable indicator of toxicity (Sabaliunas et al. 2000; Bispo et al. 1999).

Chemical characteristics analyzed in the sediments included: PAHs; hexane extractable hydrocarbons; and total organic carbon. PAH levels increased as the river progressed downstream through industrial regions. Leavittsburg showed only miniscule amounts of PAHs while Girard and Youngstown displayed moderately high amounts of PAHs. Further downstream, Lowellville and Lowellville bank recorded the highest amounts of organic contaminants (Figures 25-39). Hexane extractable hydrocarbon and total organic

carbon levels also displayed an increase during the progression downstream, with the exception of Youngstown and Girard (Figures 40 & 41).

The results indicate a possible adaptation of the microbes to contaminants in the sediments. The four contaminated sites had higher microbial activities than Leavittsburg. Also, the Leavittsburg site showed lower microbial biomass than the other sites, but only showed statistical difference from Girard and Lowellville bank. These sites also showed a trend with PAHs. Leavittsburg had miniscule amounts of PAHs present, whereas Girard and Youngstown had significantly higher levels and further downstream at Lowellville and Lowellville bank the numbers were the highest.

A previous study performed on Lower Mahoning River sediments showed differences in microbial assemblage between contaminated and noncontaminated sites (Xu, 1999). This study found that pollution possibly led to development of specific bacterial groups that utilize the anthropogenic contaminants as a food source.

In conclusion, the results of the feasibility study indicate a strong potential for bioremediation of the sediments for organic contaminants as indigenous microbes present seem to have adapted to the anthropogenic contaminants. Also, there is potential for metal reduction and dissimilation by the microbes since microbial biomass and activity were highest in sediments with the highest concentrations of metals.

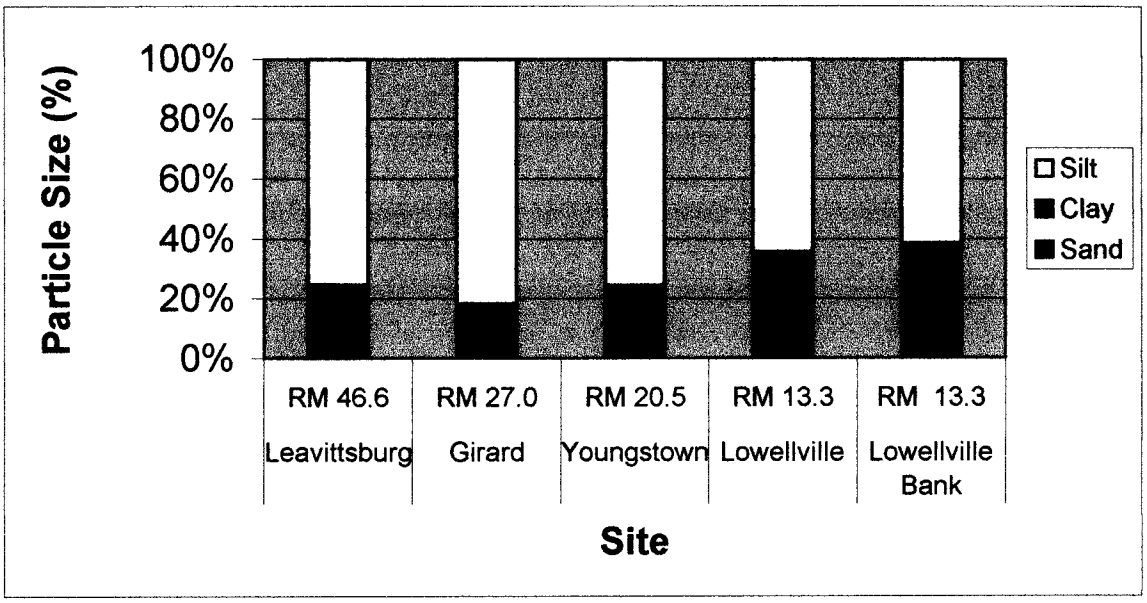

Figure 11: Particle Size Distribution (%) of sediments of the Lower Mahoning River.

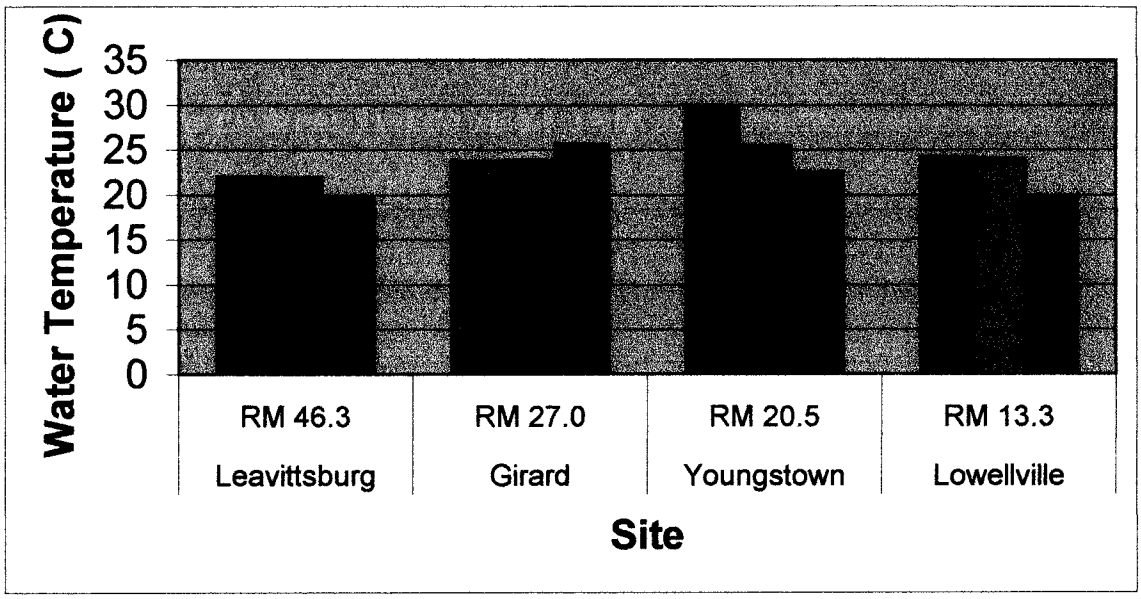

Figure 12: Water temperature (°C) of the Lower Mahoning River.

Figure Legend (core date):

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090801
092101

090101
090301
100601

080301
091401
091501

082001
090601
092201

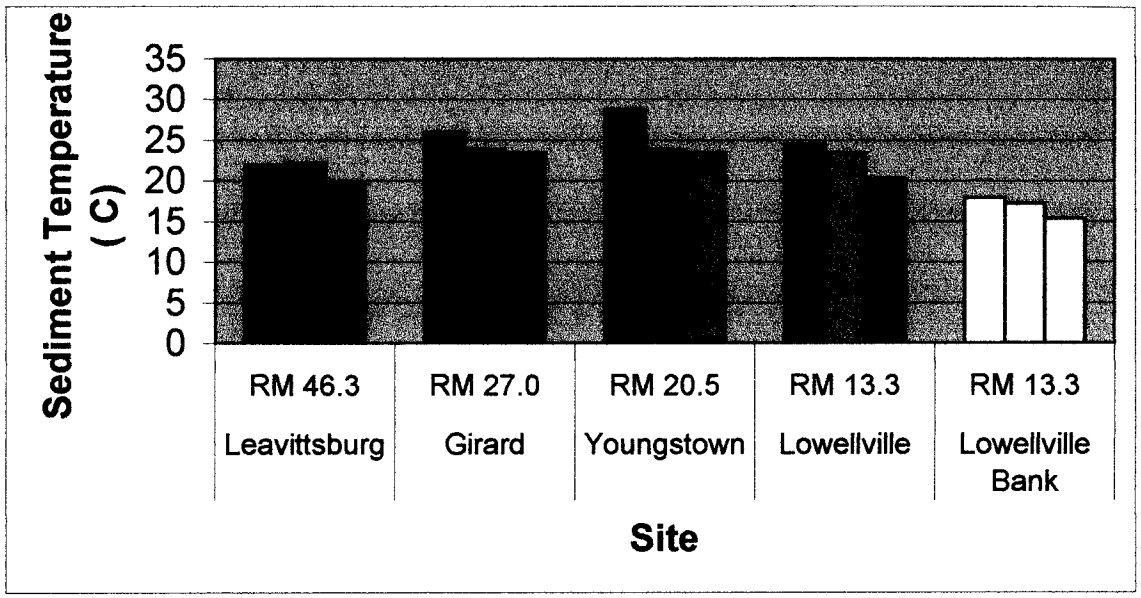

Figure 13: Sediment temperatures (°C) of the Lower Mahoning River.

Figure Legend (core date):

081601
090801
092101

090101
090301
100601

080301
091401
091501

082001
090601
092201

091401
092001
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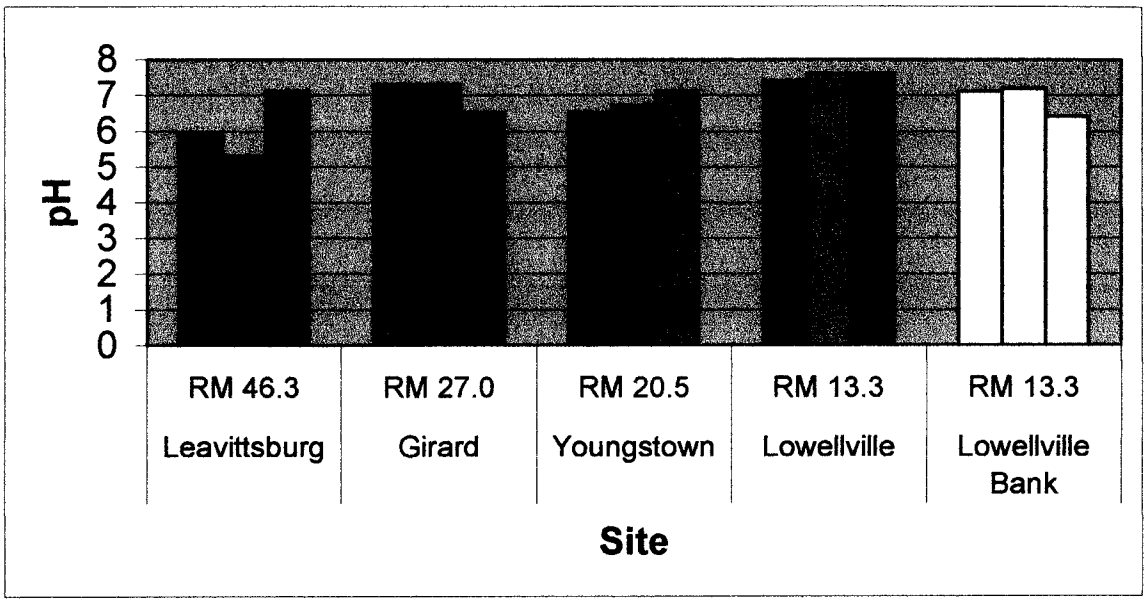

Figure 14: pH values of sediments in the Lower Mahoning River. See figure 13 for legend.

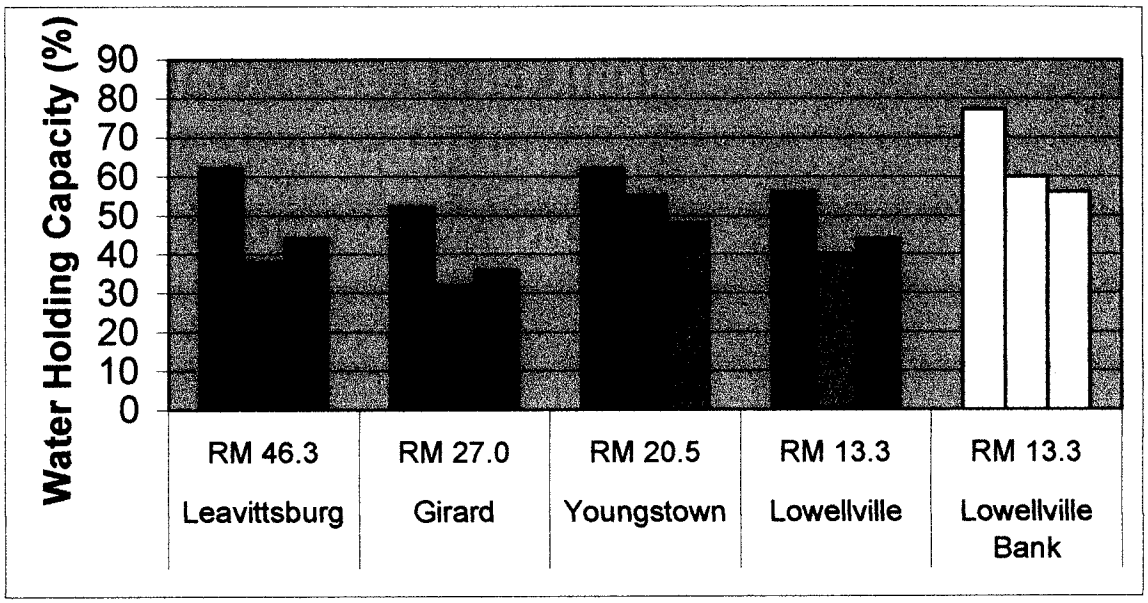

Figure 15: Water Holding Capacity (%) of sediments of the Lower Mahoning River. See figure 13 for legend.

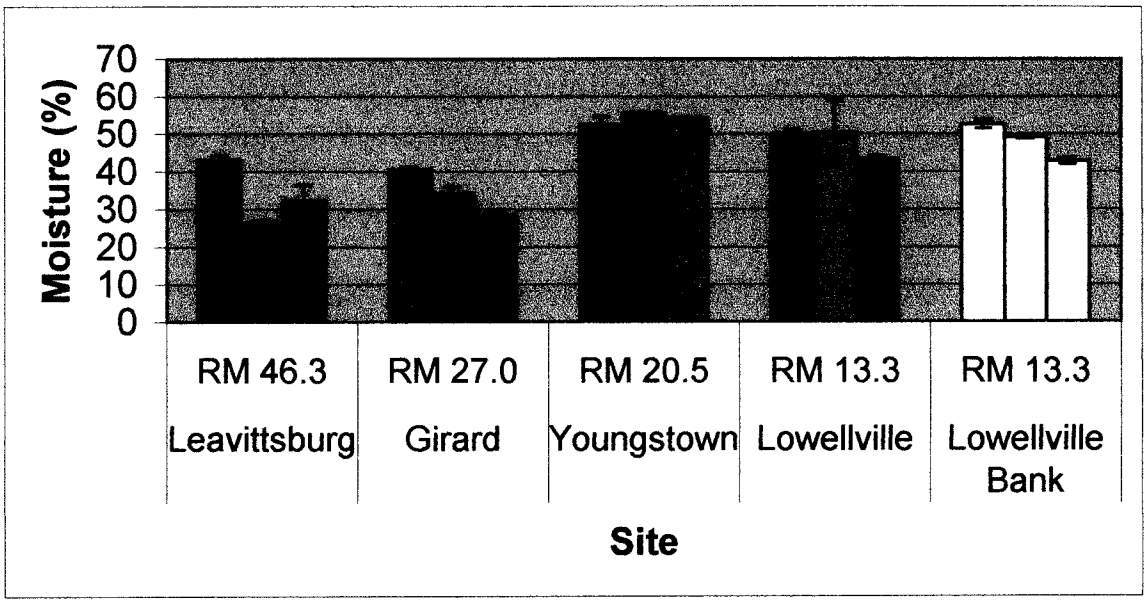

Figure 16: Percent Moisture (%) of sediments in the Lower Mahoning River. See figure 13 for legend.

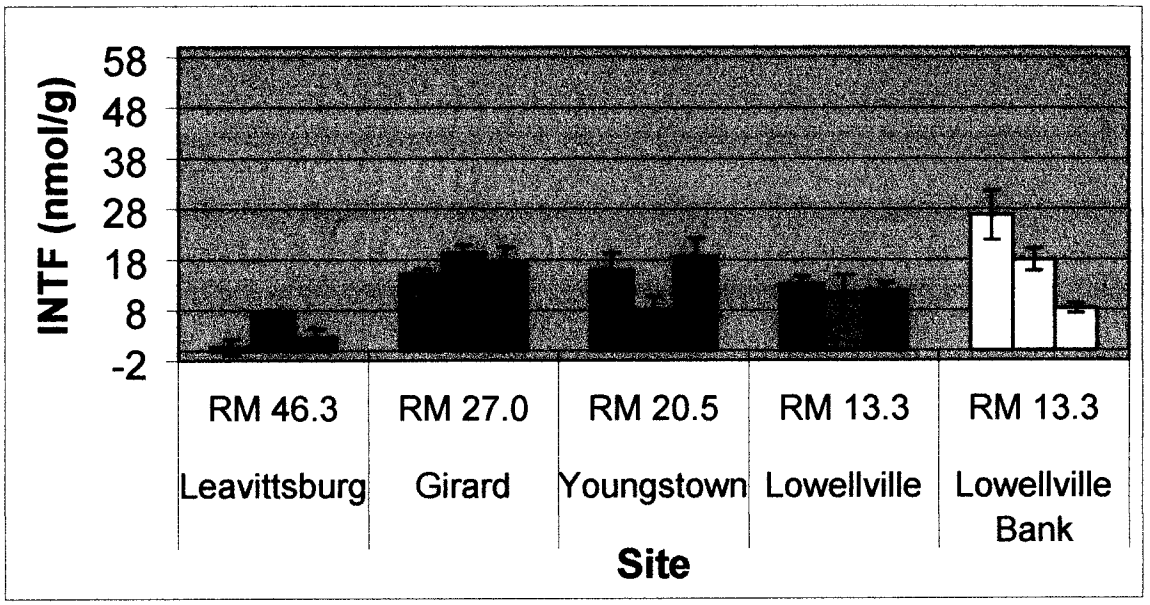

Figure 17a: Aerobic microbial activity for two-minute incubation for sediments in Lower Mahoning River. See figure 13 for legend.

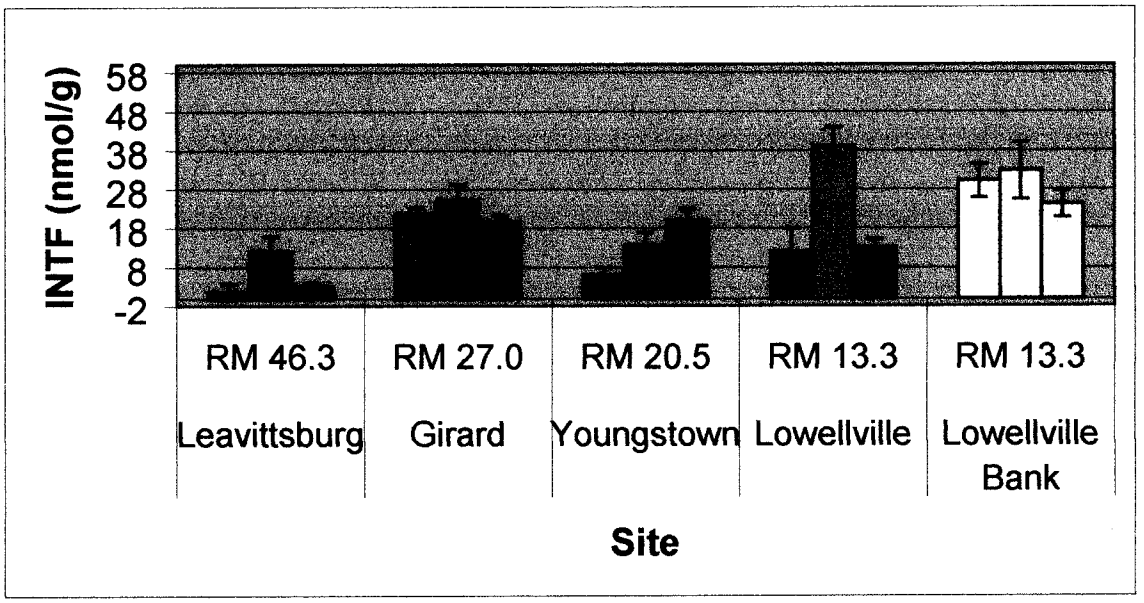

Figure 17b: Anaerobic microbial activity for 2-minute incubation for sediments of the Lower Mahoning River. See figure 13 for legend.

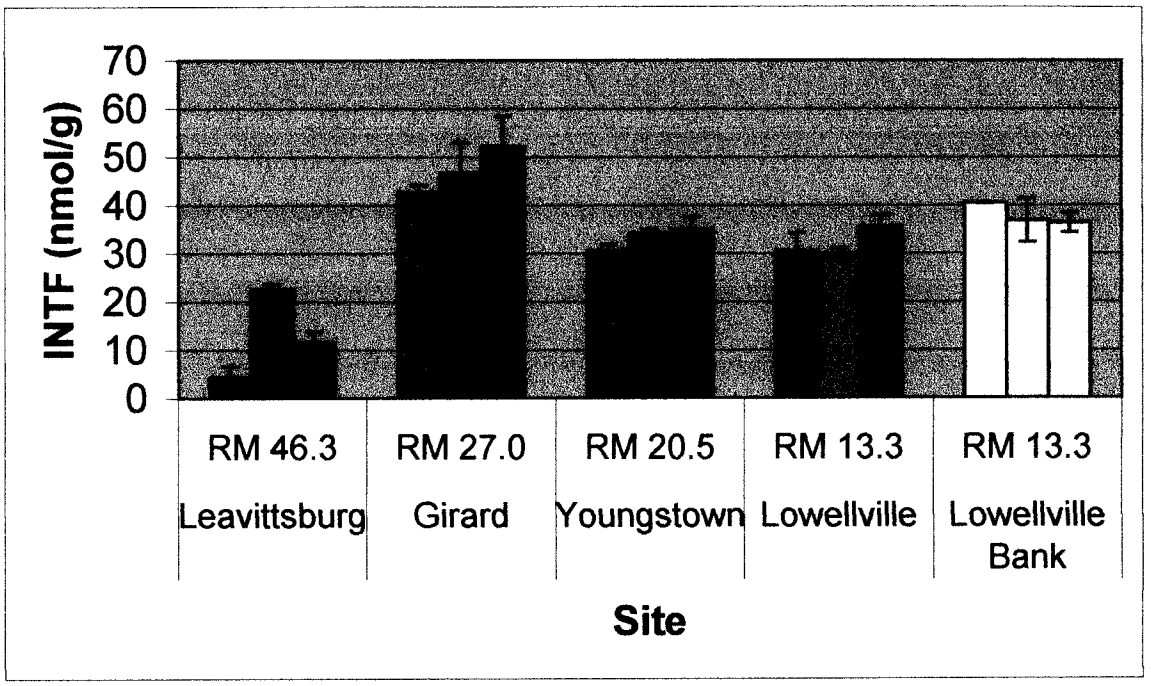

Figure 18a: Aerobic microbial activity for 60-minute incubation for sediments in the Lower Mahoning River. See figure 13 for legend.

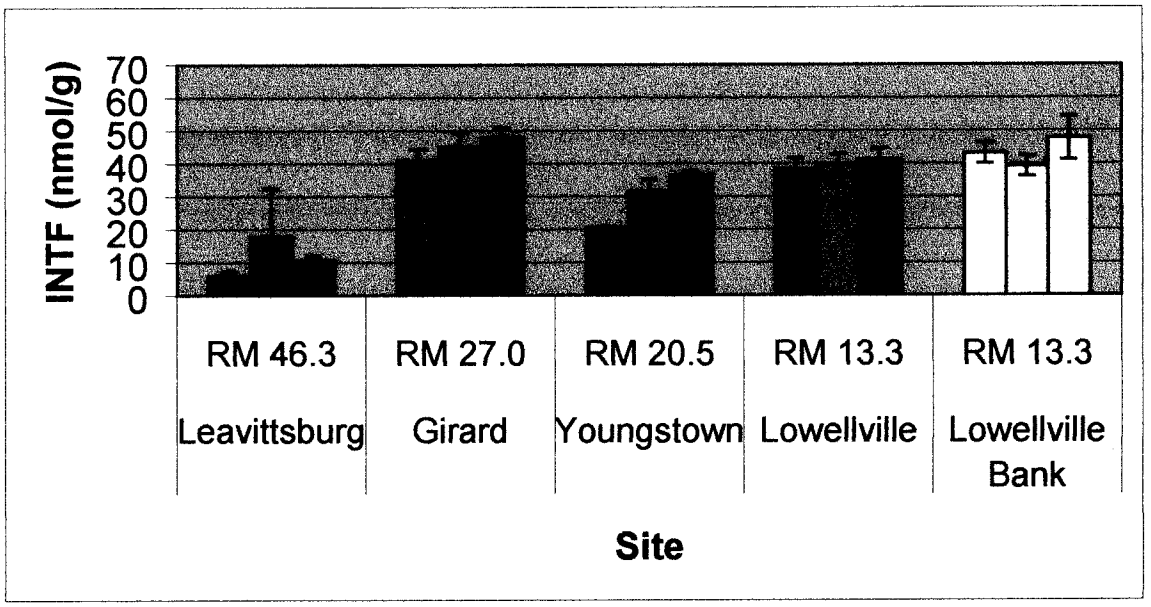

Figure 18b: Anaerobic microbial activity for 60-minutes for sediments of the Lower Mahoning River. See figure 13 for legend.

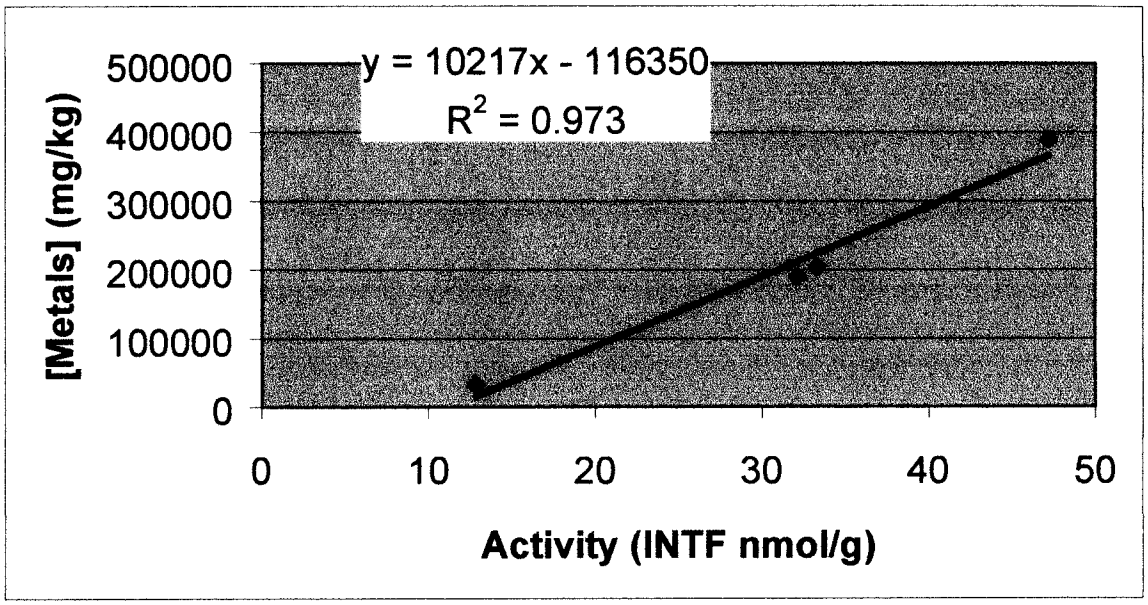

Figure 19: Comparison between microbial activity (nmol/g) and metal concentrations (mg/kg) of the sediments in the Lower Mahoning River. Metal data taken from U.S. Army Corps of Engineers (1999).

Table 3: Metal concentrations (mg/kg) in sediments of the Lower Mahoning River (US Army Corps, 1999).

	Leavittsburg	Girard	Youngstown	Lowellville
Silver	0	1	0.18	4.1
Aluminum	7170	11100	9360	9220
Arsenic	22.2	73.7	14.3	27.4
Barium	54.4	91.6	91.1	162
Beryllium	0.56	1.5	1	0.48
Cadmium	0.11	0	1	5
Cobalt	9.4	36.9	19.3	20.4
Chromium	16.2	56.8	42.1	276
Copper	16.4	114	61.2	216
Iron	20600	359000	153000	148000
Manganese	345	4260	1530	1590
Nickel	34.3	39	43.8	91.9
Lead	21.6	415	62.9	700
Antimony	0.24	6.7	3.8	2.9
Selenium	0.48	5.1	0.4	0
Thallium	1.1	0	2.6	4.1
Vanadium	12.8	41.1	11.2	22.6
Zinc	59	777	236	3710
Mercury	0.016	0.3	0.098	0.39
Sodium	94.4	242	217	138
Calcium	937	9450	34100	21600
Potassium	1400	1170	754	760
Magnesium	2730	2770	4170	3700
Total	**33525**	**389651**	**203722**	**190251**

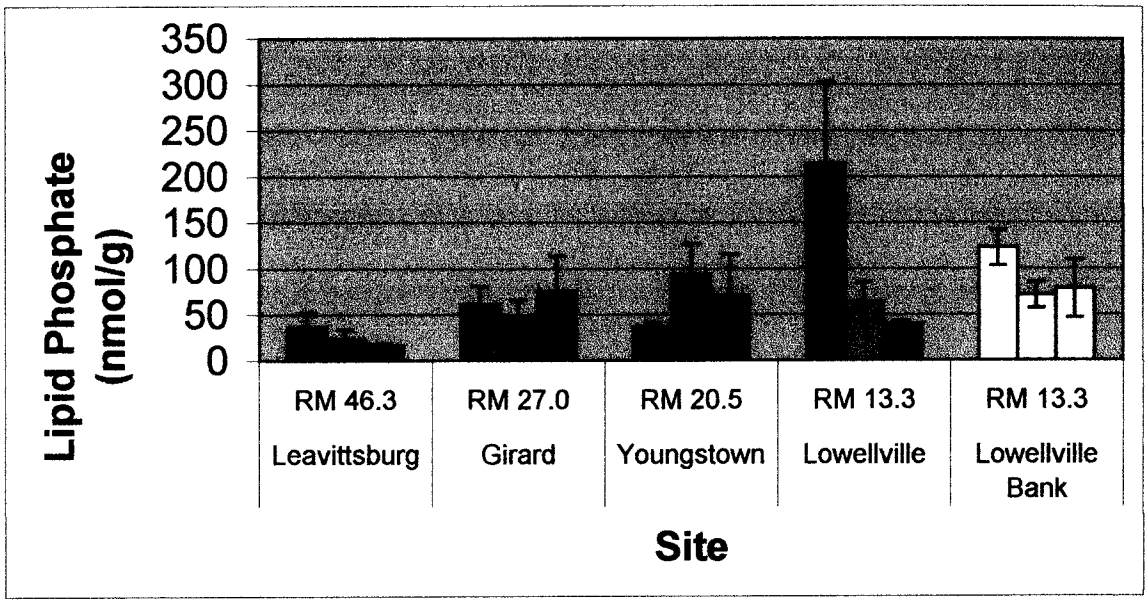

Figure 20: Microbial lipid phosphate biomass (nmol) per gram dry weight of the sediments of the Lower Mahoning River. See figure 13 for legend.

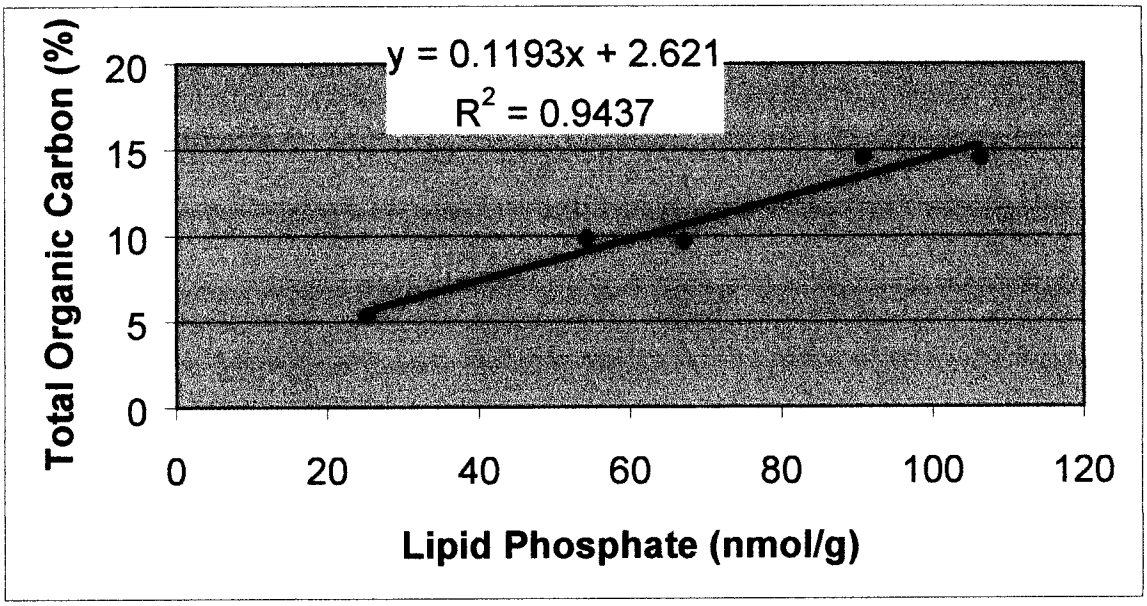

Figure 21: Comparison between lipid phosphate biomass (nmol/g) and total organic carbon (%) in the sediments of the Lower Mahoning River.

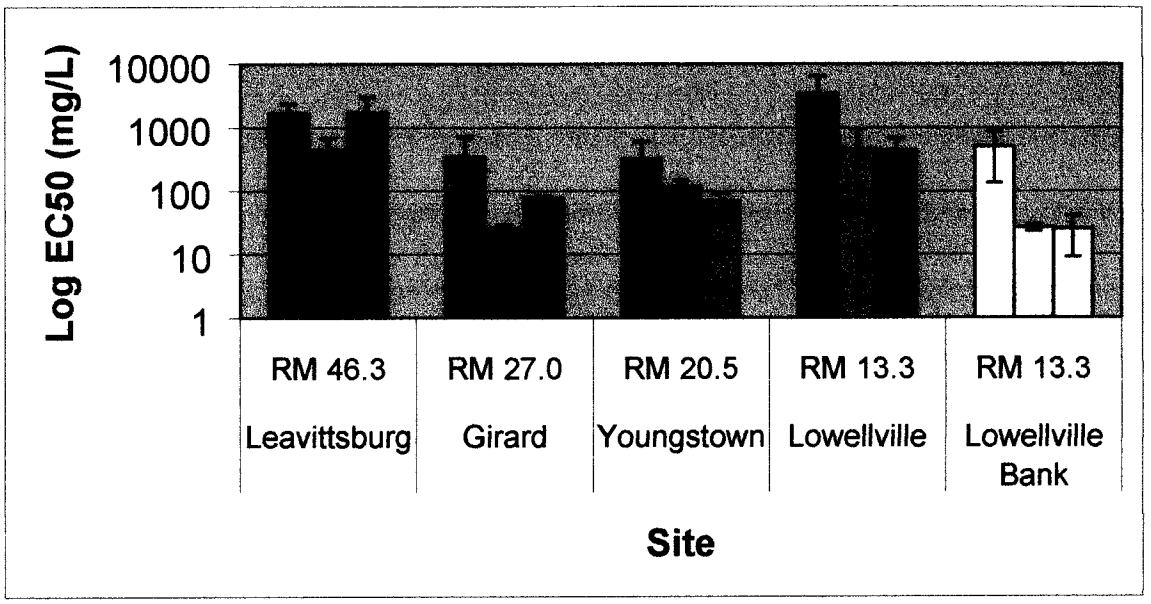

Figure 22: Toxicity values in Log EC_{50} concentration (mg/L) of Lower Mahoning River sediments. The lower the value, the higher the toxicity. See figure 13 for legend.

Table 4: Comparison of toxicity results to previously published studies. Results are shown as Toxicity EC_{50} (mg/L).

Substrate	Toxicity EC50 (mg/L)
Leavittsburg Sediment [a]	1275
Girard Sediment [a]	150
Youngstown Sediment [a]	170
Lowellville Sediment [a]	1440
Lowellville Bank Sediment [a]	185
Shite Creek Sediment [b]	32000
Contaminated Ground Water Samples (Lithuania) [c]	50-860
PAH Contaminated Soil Leachates (France) [d]	450-16000

Key:

[a] Mahoning River Results
[b] Shite Creek Results (Damascus, Ohio)
[c] Sabaliunas et al. 2000
[d] Bispo et al. 1999

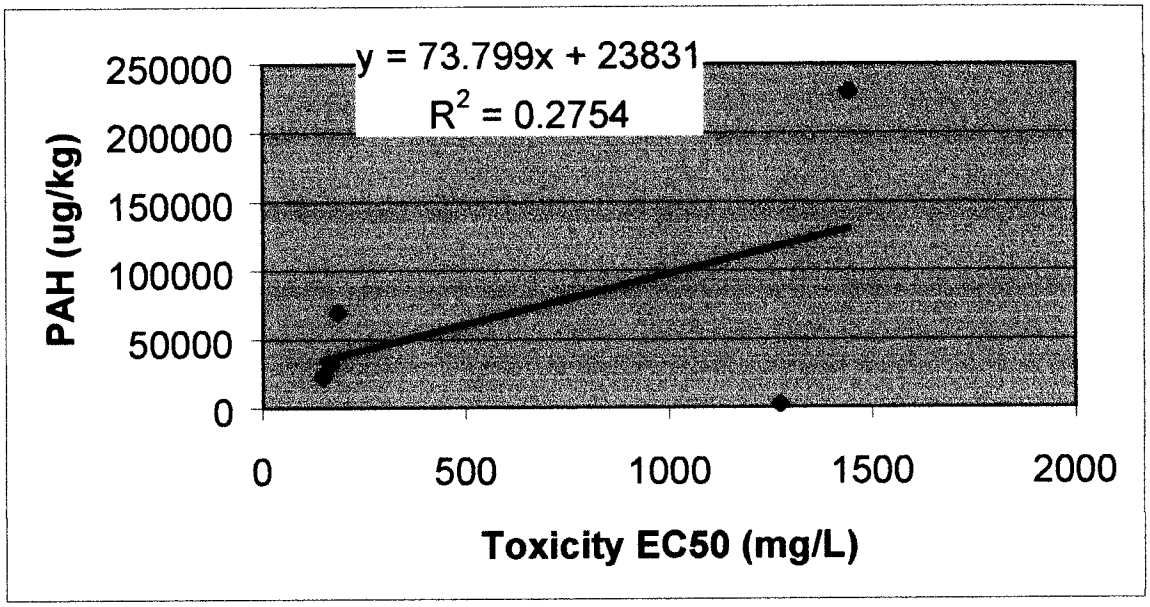

Figure 23: Comparison between toxicity EC_{50} (mg/L) and PAHs (µg/kg) in sediments from Lower Mahoning River.

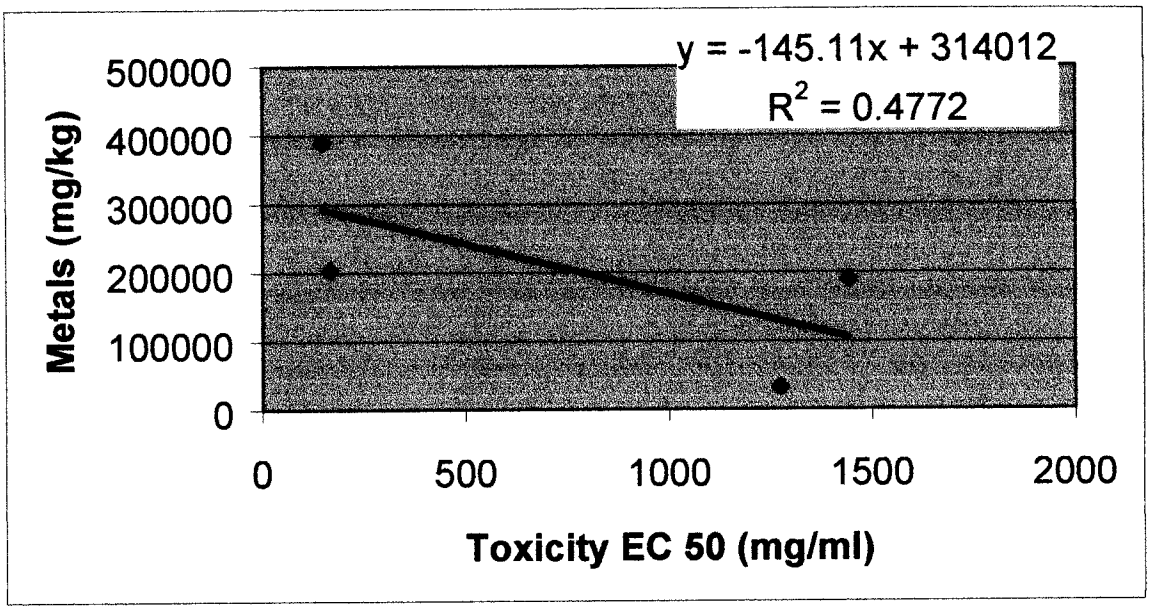

Figure 24: Comparison between metals (mg/kg) and toxicity EC_{50} (mg/L) of the sediments in the Lower Mahoning River.

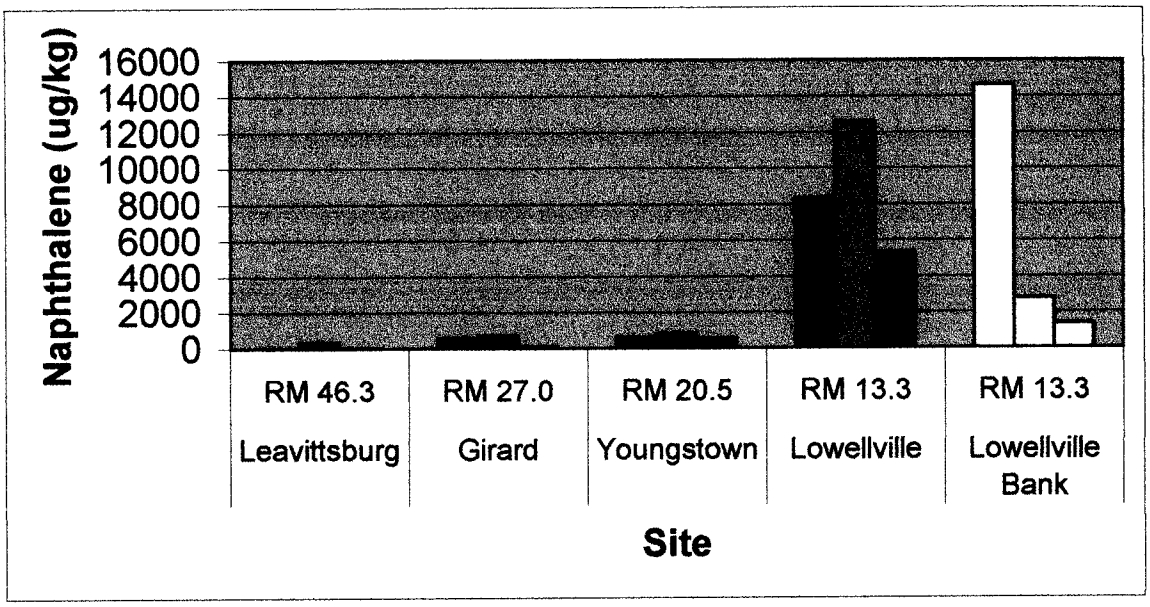

Figure 25: Naphthalene concentrations (µg/kg) in sediments of the Lower Mahoning River. See figure 13 for legend.

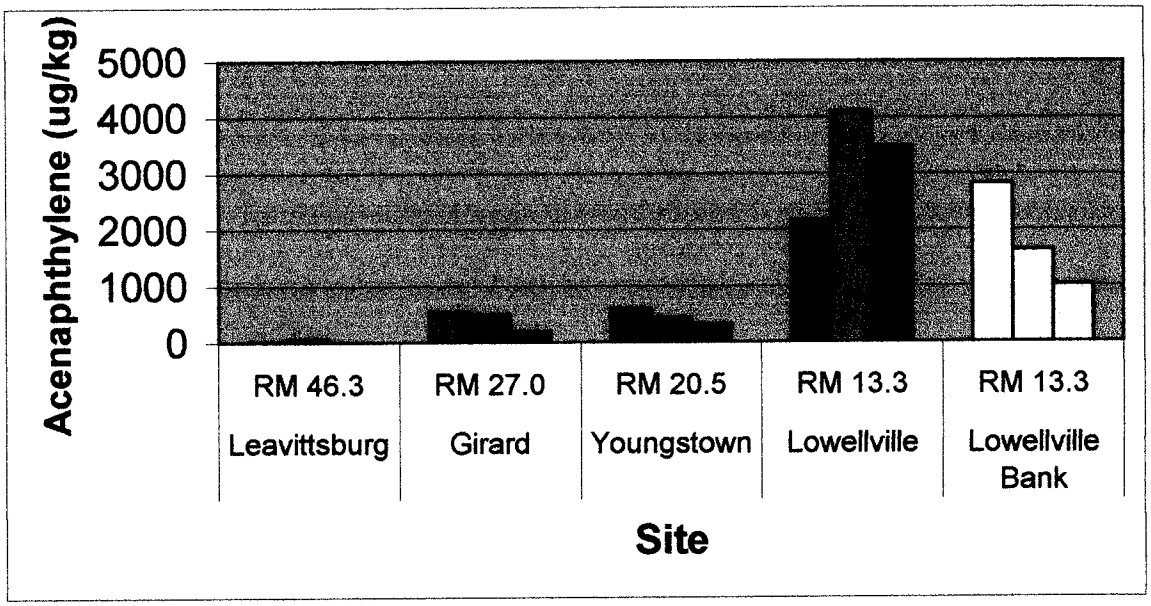

Figure 26: Acenaphthylene concentrations (μg/kg) in sediments of the Lower Mahoning River. See figure 13 for legend.

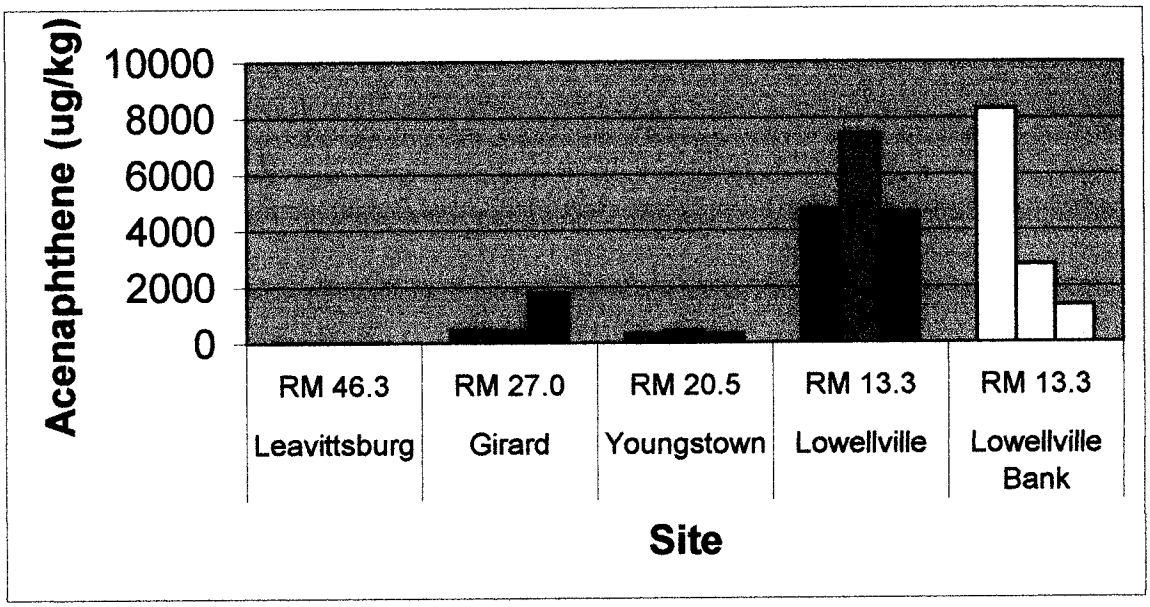

Figure 27: Acenaphthene concentrations (µg/kg) in sediments of the Lower Mahoning River. See figure 13 for legend.

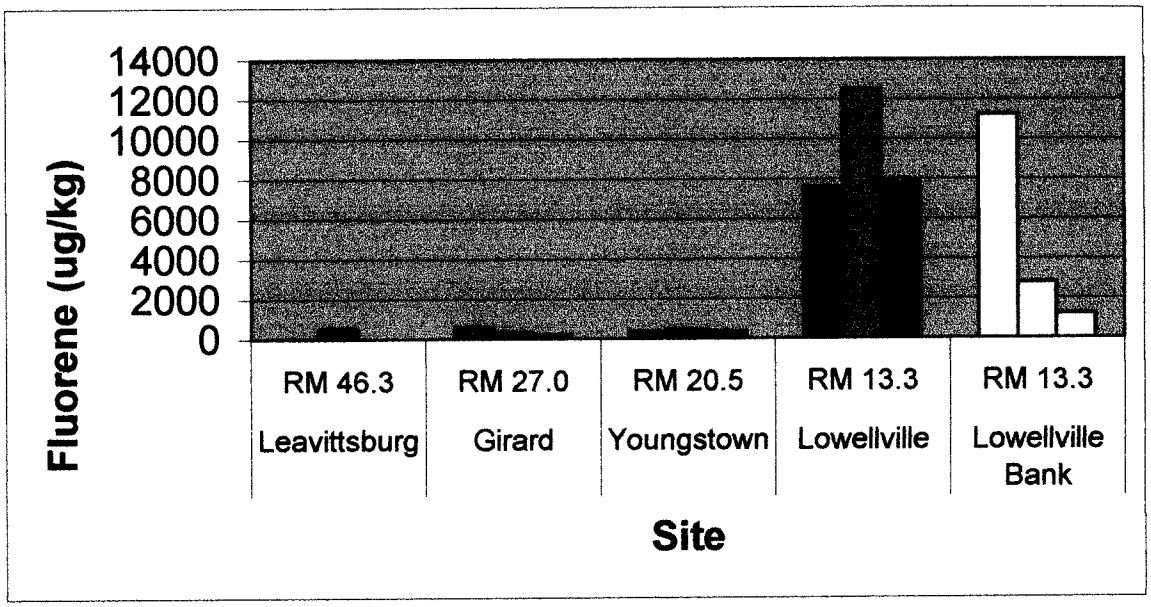

Figure 28: Fluorene concentrations (µg/kg) in sediments of the Lower Mahoning River. See figure 13 for legend.

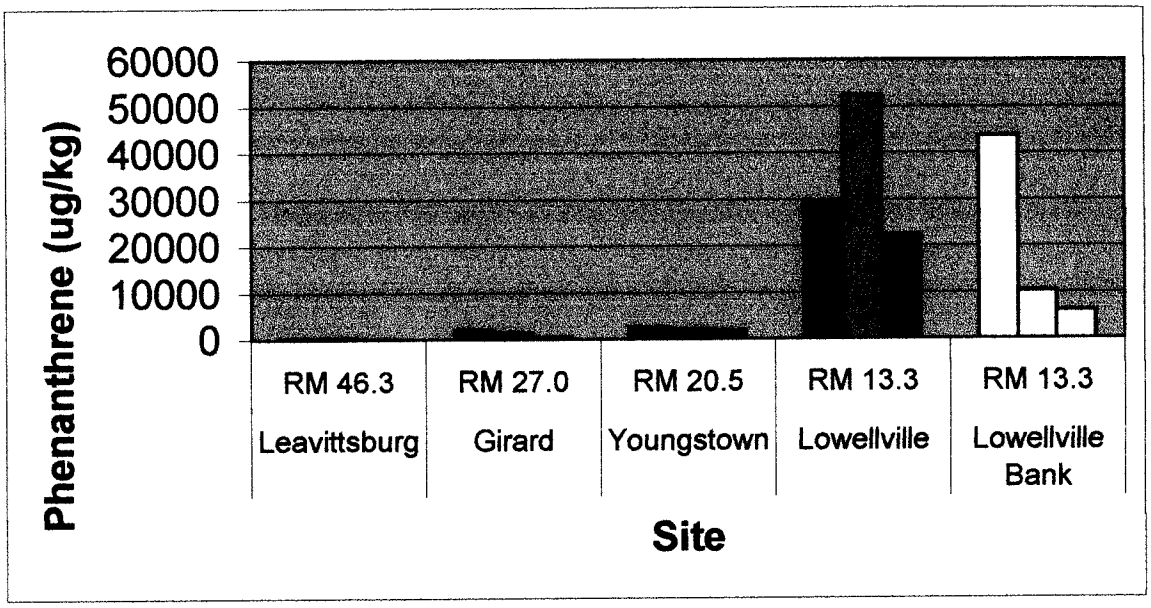

Figure 29: Phenanthrene concentration (μg/kg) of sediments of the Lower Mahoning River. See figure 13 for legend.

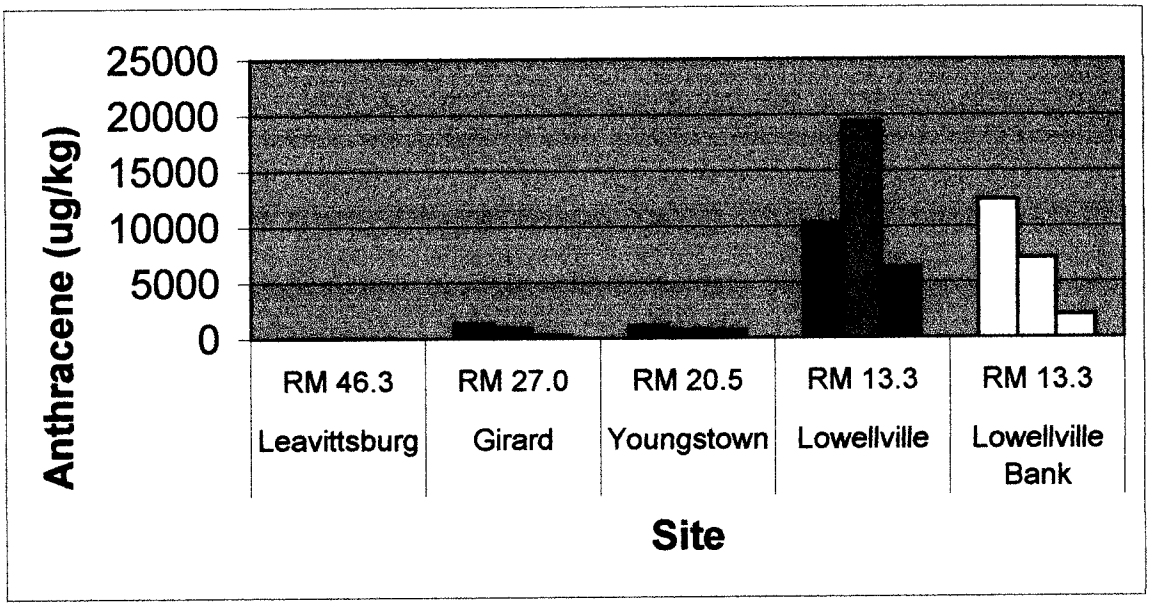

Figure 30: Anthracene concentrations (μg/kg) of sediments of the Lower Mahoning River. See figure 13 for legend.

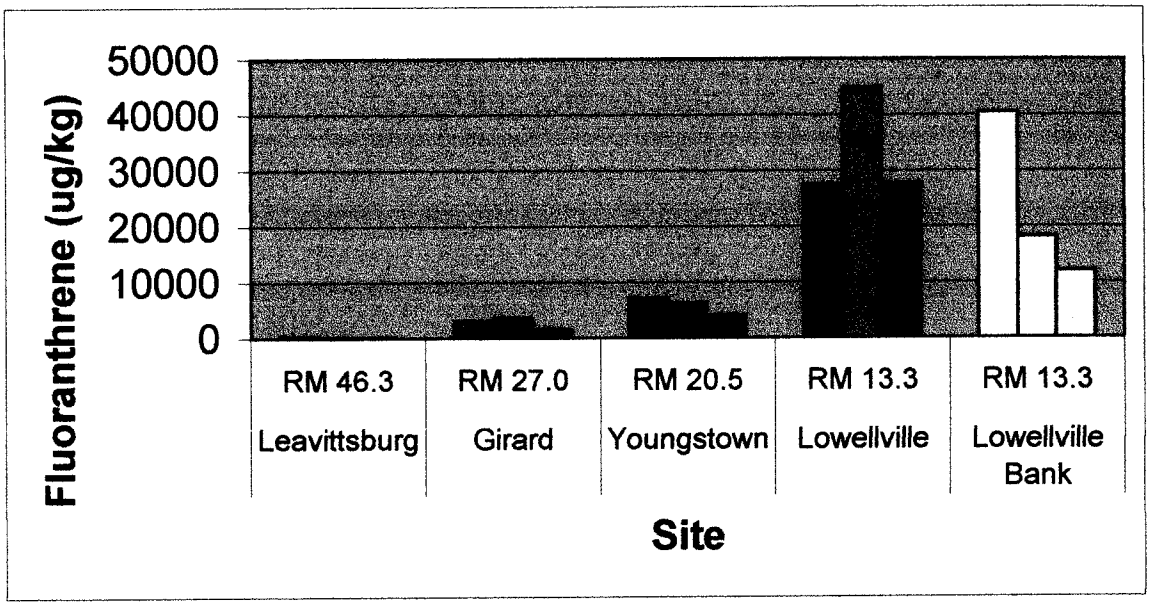

Figure 31: Fluoranthene concentrations (µg/kg) of sediments of the Lower Mahoning River. See figure 13 for legend.

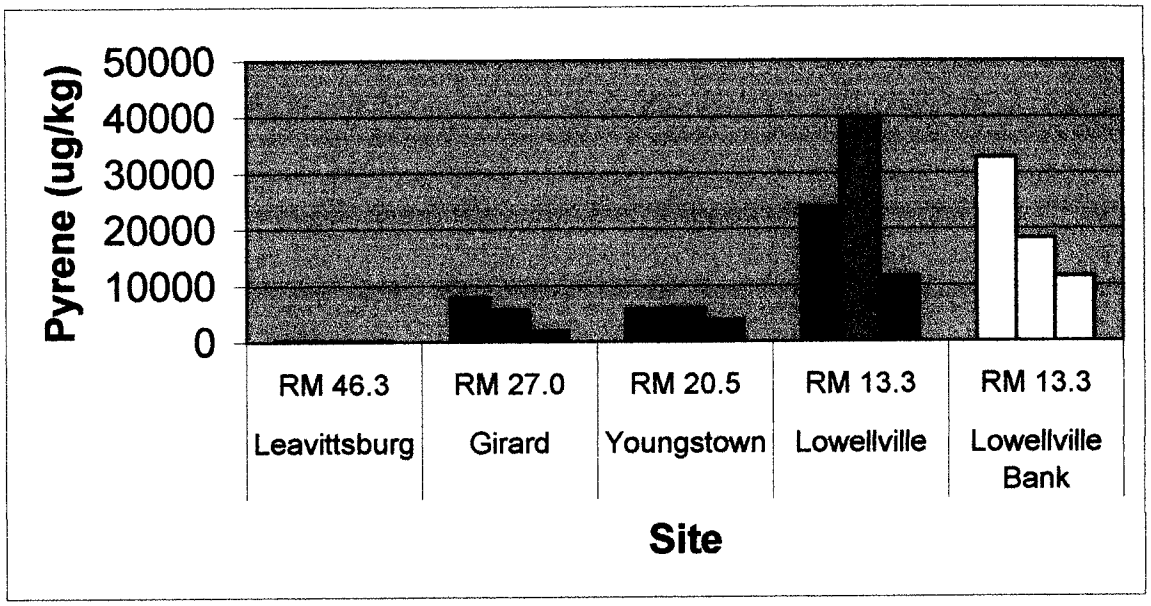

Figure 32: Pyrene concentrations (μg/kg) of sediment in the Lower Mahoning River. See figure 13 for legend.

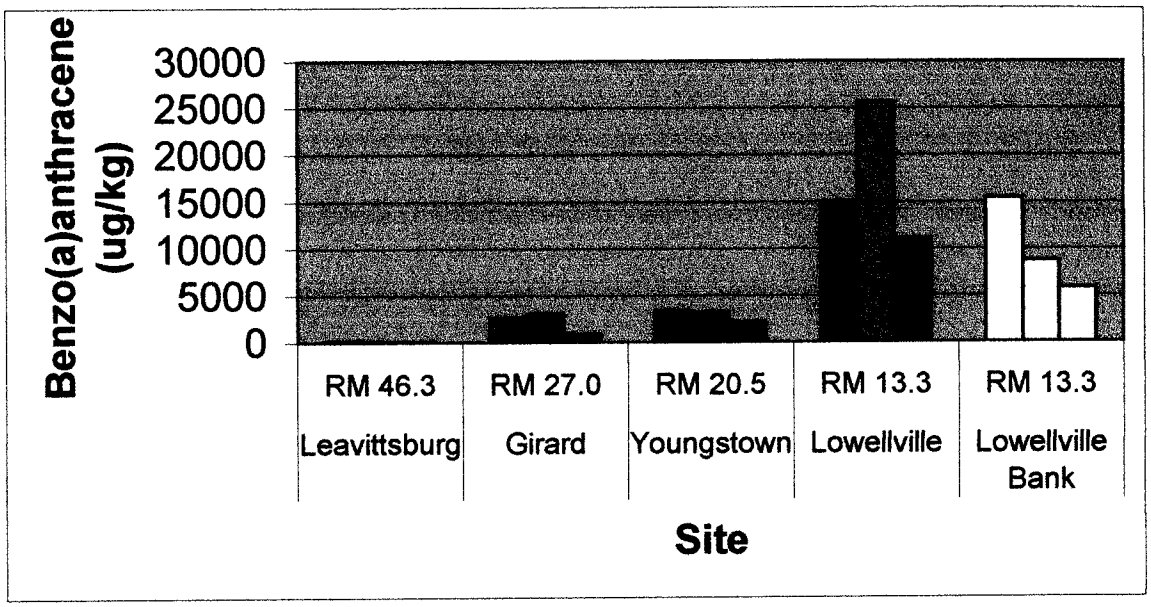

Figure 33: Benzo(a)anthracene concentrations (µg/kg) of sediments of the Lower Mahoning River. See figure 13 for legend.

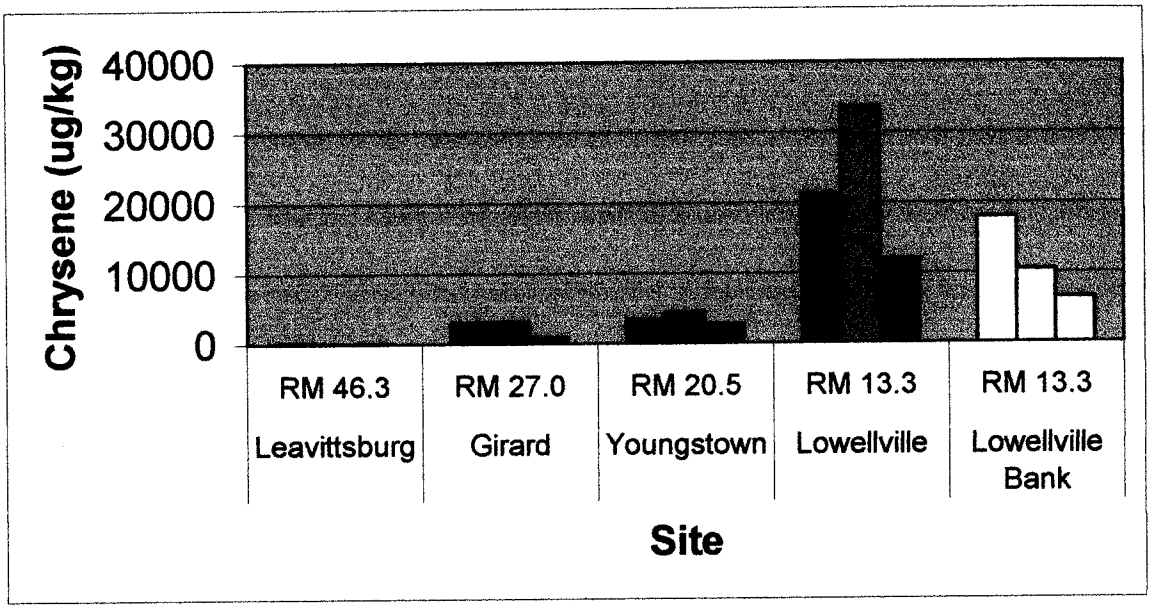

Figure 34: Chrysene concentrations (µg/kg) of sediments of the Lower Mahoning River. See figure 13 for legend.

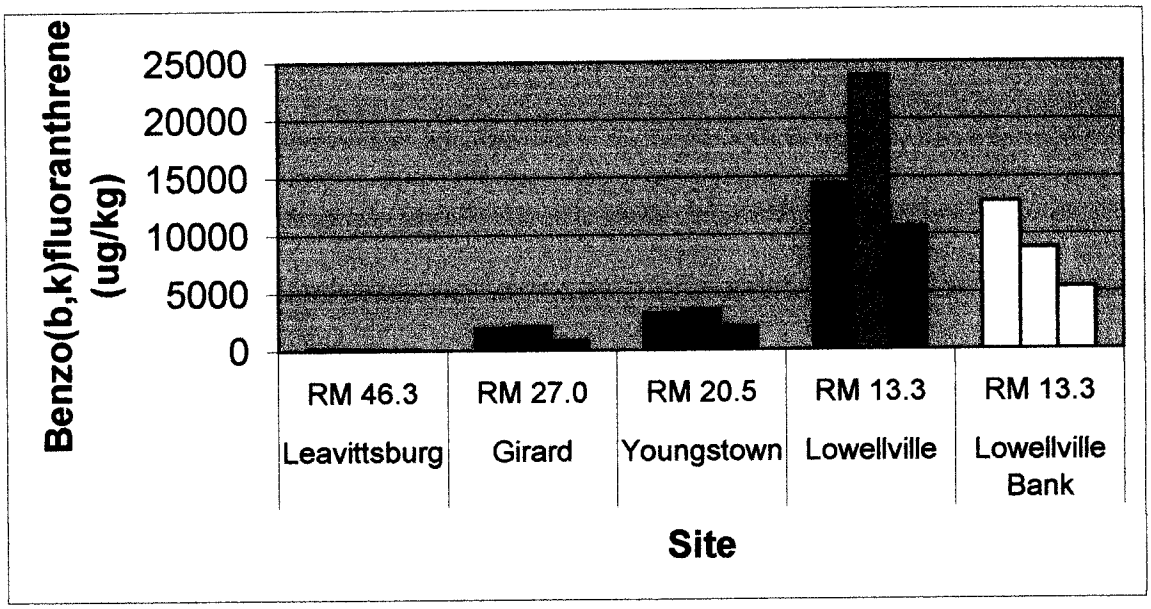

Figure 35: Benzo(b,k)fluoranthrene concentrations (µg/kg) of sediments of the Lower Mahoning River. See figure 13 for legend.

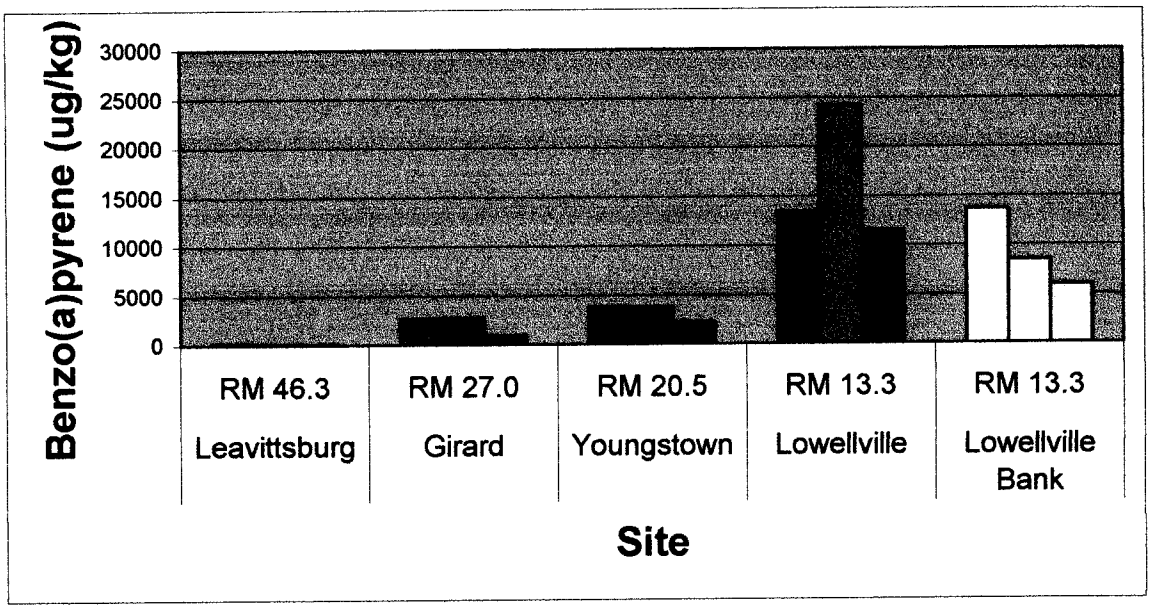

Figure 36: Benzo(a)pyrene concentrations (µg/kg) in sediments of the Lower Mahoning River. See figure 13 for legend.

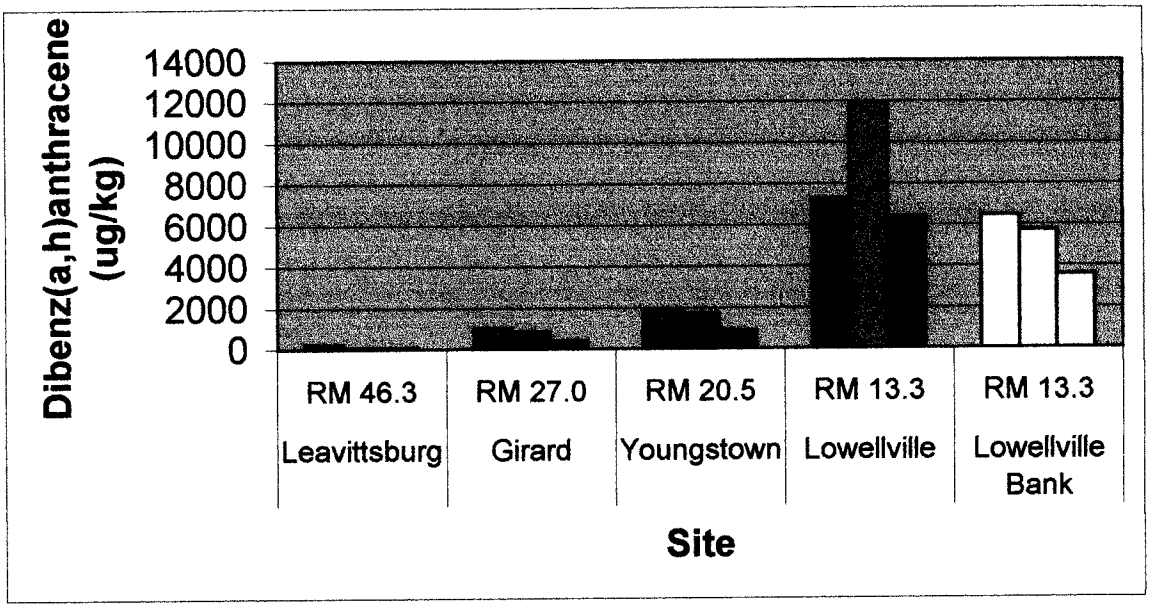

Figure 37: Dibenz(a,h)anthracene concentrations (µg/kg) in sediments of the Lower Mahoning River. See figure 13 for legend.

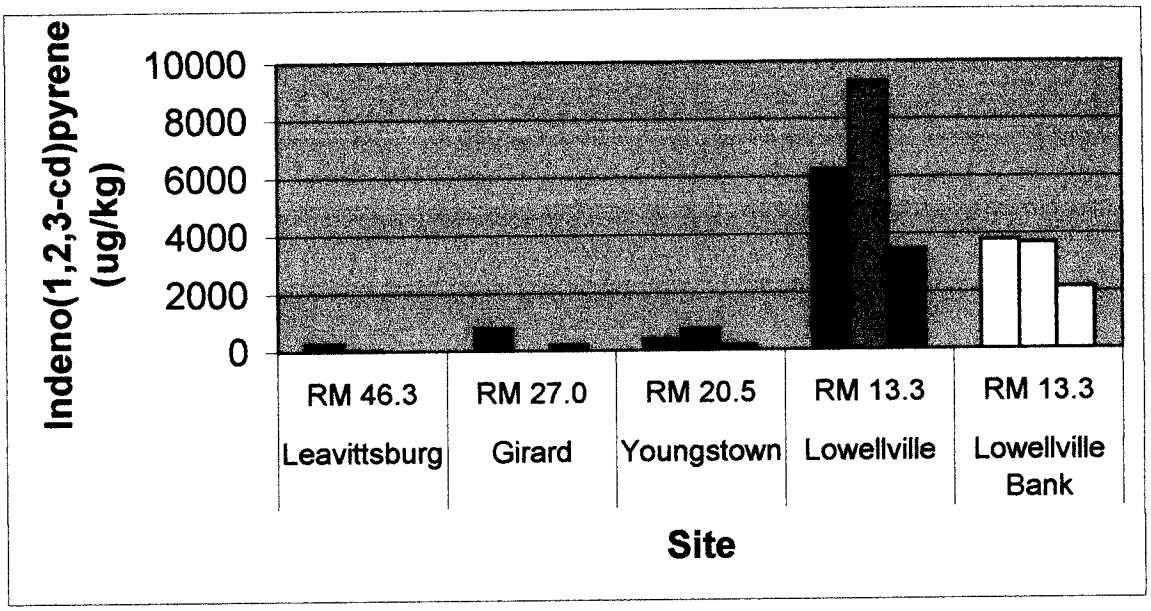

Figure 38: Indeno(1,2,3-cd)pyrene concentrations (µg/kg) in sediments of the Lower Mahoning River. See figure 13 for legend.

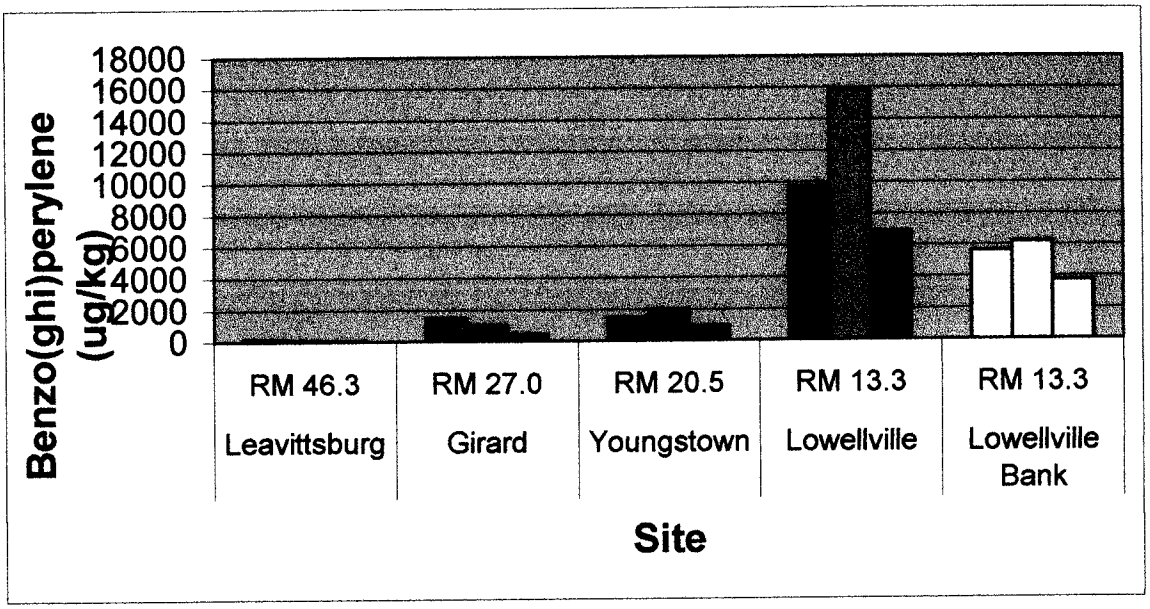

Figure 39: Benzo(ghi)perylene concentrations (µg/kg) in sediments of the Lower Mahoning River. See figure 13 for legend.

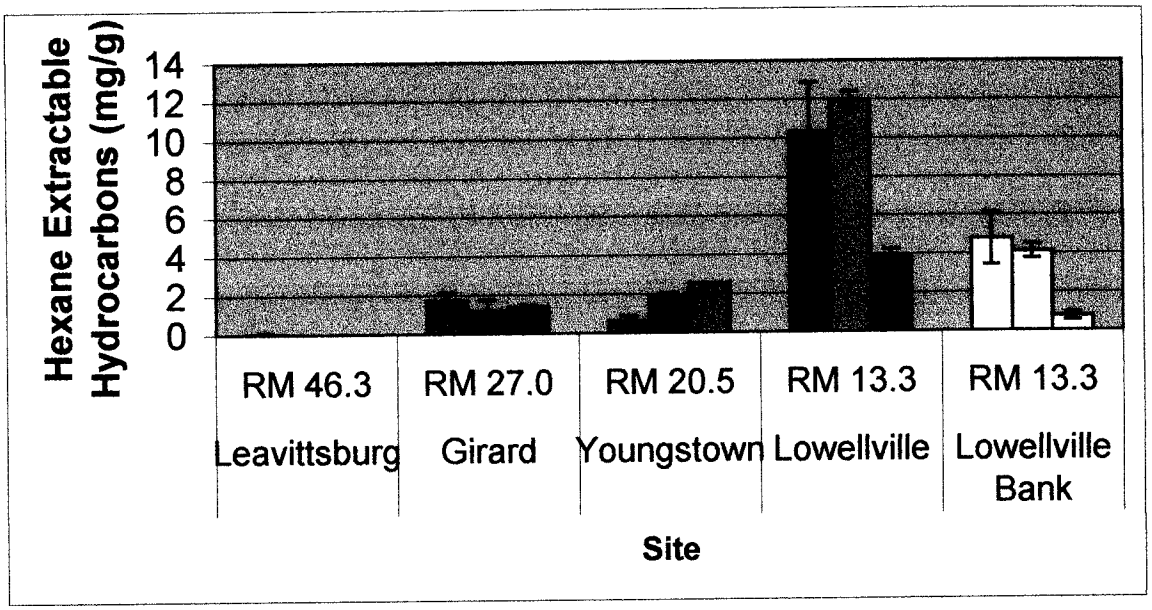

Figure 40: Hexane Extractable Hydrocarbons (mg/g) dry weight of sediments in the Lower Mahoning River. See figure 13 for legend.

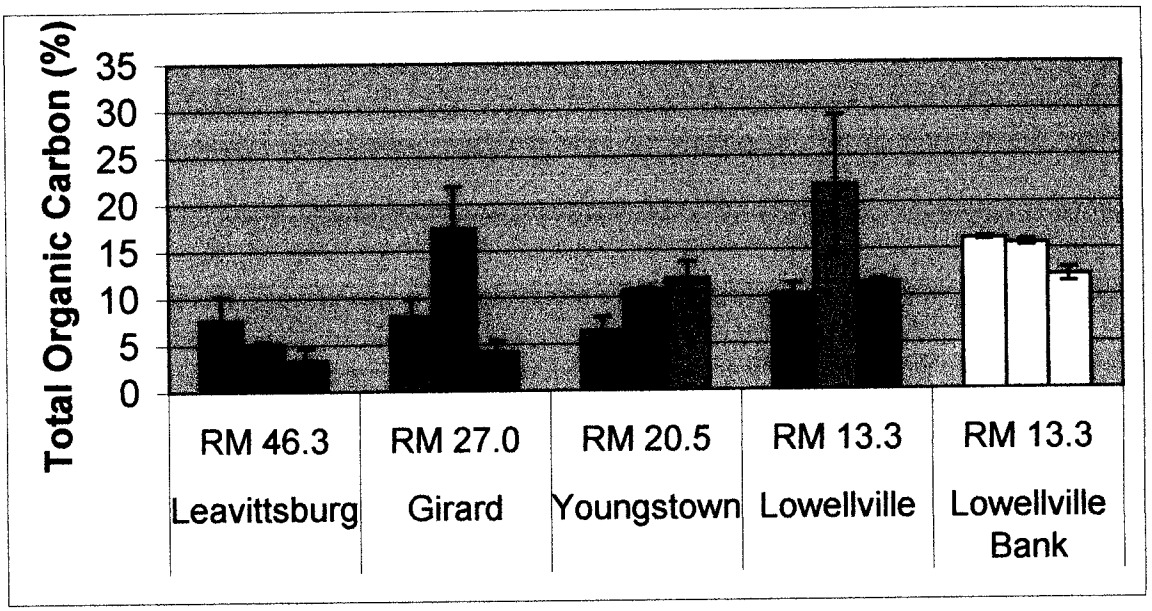

Figure 41: Total Organic Carbon (%) in sediments of the Lower Mahoning River. See figure 13 for legend.

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Youngstown State University Center for Urban Studies from U.S. Geological Survey, 2002. Map depicting sampling sites in the Lower Mahoning River.

Appendix 1: Solutions for Microbial Activities

1.08 mM iodonitrotetrazolium chloride solution: weighed 0.03 g of INT and placed into 100 ml amber glass bottle, added 0.25 ml of N,N-dimethylformadine and mixed well with a glass rod. Using 18 MΩ water, added 49.0 ml and sonicated with light heat for twenty minutes. This solution is light sensitive.
40 mM sodium azide: measured out 0.26 g sodium azide in a glass container and added 100 ml of MΩ water and shook well.
Iodonitrotetrazolium formazan (for standard curve): weighed out desired amount of INTF using the Cahn C-31 microscale balance:

0.0	nmol	0.00 mg INTF
22	nmol	0.105 mg INTF
45	nmol	0.210 mg INTF
89	nmol	0.420 mg INTF
176	nmol	0.830 mg INTF
265	nmol	1.250 mg INTF

N,N-dimethylformamide (10.0 µl) was added to the formazan, then slowly added 49.9 ml of acetonitrile while swirling to ensure dissipation of the crystals. The solution was sonicated for twenty minutes. These solutions were covered with aluminum foil, as they are light sensitive.

Appendix 2: Linear equation

$$y = mx + b$$

Where: y= absorbance at 490nm
m= slope from standard curve
x= unknown
b= Y intercept from standard curve

Appendix 3: Equation for combined standard deviations

$$\sigma_Q = (a^2\sigma_x^2 + b^2\sigma_y^2)\ ½$$

Where: σ_Q= combined standard deviation.
$a^2\sigma_x^2$= standard deviation of first site.
$b^2\sigma_y^2$= standard deviation of second site.

Appendix 4: Solutions for Lipid Phosphate Biomass

50 mM phosphate buffer: weighed 8.7 g of potassium phosphate and added to 1000 ml of MΩ water. Adjusted the pH to 7.4 with 1N hydrochloric acid.
Saturated potassium persulfate: dissolved 5.0 g of potassium sulfate in 100 ml of 0.36N sulfuric acid. This mixture is light sensitive. Stored in refrigerator until use.
2.5% ammonium molybdate: added 2.5 g of ammonium molybdate to 100 ml of 5.72N sulfuric acid.
Malachite green: added 1.11 g of polyvinyl alcohol to 1000 ml of 80°C MΩ water. Allowed cooling to room temperature and added 0.11 g of malachite green.
0.5 μM glycerol phosphate: added 1.08 g glycerol phosphate to 100 ml of MΩ water and blended well. Pipetted 1.0 ml of this solution to a 100 ml glass container and added 100 ml of MΩ water.

Appendix 5: Equation for lipid phosphate biomass

$$\text{Total Lipid Phosphate Biomass (nmol)} = \frac{(5/1^{st}\text{extraction}) \times (1/2^{nd}\text{ extraction}) \times (\text{unknown}(x))}{\text{sediment dry weight (g)}}$$

Where: 5= Amount of DCM used in assay (ml)
1st Extraction= Amount of sample pipetted from lower lipid layer (ml)
2nd Extraction= Amount of sample pipetted from resuspended sample (ml)
unknown (x)= Value derived from linear equation
sediment weight (dry)= sediment weight x % solid

Appendix 6: Equation for converting PAHs from µg/ml to µg/kg

$$\frac{\mu g}{ml} \times \frac{\text{extract vol.(ml)}}{kg} \times \frac{1}{\%\text{ solid}} = \frac{\mu g}{kg}$$

Appendix 7: Equation for determining particle size distribution

$$\text{Sand \%} = (R_{40s} - R_{blank}) \times \frac{100}{\text{dry sediment (g)}}$$

$$\text{Clay \%} = (R_{7h} - R_{blank}) \times \frac{100}{\text{dry sediment (g)}}$$

Silt % = 100 - (Sand % - Clay %)

Where R= specific gravity

Appendix 8: Equation for Pearson correlation statistics

$$r = \frac{\Sigma XY - \Sigma X \Sigma Y/N}{(\Sigma X^2 - (\Sigma X)^2/N)(\Sigma Y^2 - (\Sigma Y)^2/N)^{1/2}}$$

Where: X= means of first array
Y= means of second array
N= sample size