

Lake Erie Protection Fund Small Grant Final Report  
Project: Investigation of Microbial Activity in Seeps along Williams Ditch

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The Lake Erie Commission issued a Lake Erie Protection Fund Small Grant to the University of Toledo for \$7500 to fund an investigation of microbial activity in Williams Ditch. The majority of the funds were used for supplies and a summer research assistantship for Christine Foreman, the contributions by other personnel, Robert Sinsabaugh and Rachel Ammonette, were supported by the University of Toledo.

The study site for the project was Williams Ditch. Williams Ditch is located in the Arco Industrial Park in Toledo, Ohio (see Figure 1). From 1923 through 1962 the Federal Creosoting Corporation and the American Creosoting company operated a creosoting facility for railroad ties on this site. The site is now more commonly referred to as the Toledo Tie Site. Historically wooden railroad ties were treated with creosote as a preserving agent. Wood preservation was carried out on site from 1923 to 1962, and during the 1950's it was one of Ohio's largest wood preserving facilities (Lesniak 1995). Due to its past history the site has been known to harbor creosote materials, including polycyclic aromatic hydrocarbons

(PAHs), which pose environmental, health, and safety concerns. Creosote is considered a potential human carcinogen by the National Institute of Occupational Safety and Health. From 1987 to the present, the Toledo Tie Site has been the focus of several studies by the Ohio Environmental Protection Agency (EPA), private companies (Bowser-Morner 1987, Environmental Consultants Inc. 1989, Midwest Environmental Consultants 1990, Geraghty & Miller Inc. 1990, and Hull and Associates Incorporate 1997) as well as investigators at the University of Toledo (Lesniak 1995, Blakely 2000, Foreman and Sinsabaugh this study). Williams Ditch, which is believed to be hydrologically linked to the Toledo Tie Site, empties into the Ottawa River and ultimately into Lake Erie. The Toledo Tie site has been declared a CERCLA Superfund site and is in the process of being cleaned.

Creosote is a complex mixture of over 200 compounds, of which approximately 85% are polycyclic aromatic hydrocarabons (PAH's), 10% phenolic compounds, and 5% N-, S-, and O- heterocyclic compounds (Lesniak 1995). Organic chemicals such as PAH's do not break down easily and tend to get bio-magnified in the food chain. The pollution of aquatic environments with toxic organic compounds has become a major issue in water quality and environmental management (Rav-Acha and Rebuhn 1992).

Our study was aimed at investigating the microbial consortia in the segment of Williams Ditch that flows past the Toledo Tie site. Beginning in July of 1998 seven surface water samples were collected. Bacterial productivity levels, extracellular enzyme activities, substrate utilization parameters and water quality were measured. Samples were to have been analyzed by Dr. Allison Spongberg, UT Geology Department, for the presence of PAHs and other creosote signature materials. Unfortunately, Dr. Sponberg was unable to complete the analysis on the samples collected so we will refer to past studies which have documented the presence of creosote and creosote breakdown products in this water.

### Study Site

We selected two sample sites in Williams Ditch (Fe site-Fig. 2 and the Clear site Fig. 3). These sites were located near the western most portion of the Toledo Tie site, in an area that corresponds to sampling sites (see Fig. 4) ETL39, MESY 39, ETL40, MESY40,ETL44, MESY44, ETL45, and MESY45 from a 1993 site inspection conducted by OEPA (Wylie 1993). These sites were shown to have elevated concentrations of PAHs (3J ppm benzo(a)pyrene and 10J ppm fluoranthene) and evidence of creosote contamination. In addition to this creosote influence the site receives runoff from the City of Toledo's leaf storage area. Our upstream site was

presumed to be a groundwater seep because of the presence of oxidized iron deposits on the sediment surface. The second, downstream site showed no visible signs of iron deposition.

## Materials and Methods

### *Water quality analysis*

At each site water quality parameters were monitored. Water temperature was measured with a thermometer, pH with a Hanna Piccolo pH pen, conductivity and total dissolved solids (TDS) with a Hach model 44600 meter. Nitrate-N, nitrite-N, ammonium-N, chloride and sulfate concentrations were measured using Hach test kits. Water samples were collected in acid-washed Nalgene bottles and returned to the laboratory.

In the laboratory, soluble reactive phosphorus (SRP) concentration was measured using the ascorbic acid method (Greenberg et al 1985). In addition to the estimates of total dissolved solids (TDS) provided by the conductivity meter, we also estimated TDS gravimetrically. Water samples were filtered through 0.45  $\mu\text{m}$  membrane filters; the filtrates were evaporated at 103°C; and the residue was weighed. Total solids (TS) were estimated by evaporating unfiltered water samples at 103°C and weighing the residue.

### *Bacterial Productivity*

Bacterial productivity was estimated from the rate of incorporation of  $^3\text{H}$ -(methyl)-thymidine into DNA (Findlay et al. 1984, Sinsabaugh et al. 1997). Samples consisted of 10 mL of river water in polypropylene tubes. Negative controls were prepared by boiling 10 mL of sample water for 30 min. Each assay consisted of three analytical and two negative control replicates. 40  $\mu\text{Ci}$   $^3\text{H}$ -(methyl)-thymidine (specific activity 70Ci/mmol, final thymidine concentration 57 nM) was added to each tube, which was vortexed and incubated for 1 h at 20°C. Results were calculated as cells  $\text{h}^{-1} \text{mL}^{-1}$ , using a conversion factor of  $2 \times 10^9$  bacteria produced per nmole of  $^3\text{H}$ -thymidine incorporated (Riemann et al. 1990). Counting efficiencies were determined by external standardization. Isotopic dilution trials were conducted and corrections applied as needed (Findlay et al. 1984).

### *Enzyme assays*

Enzyme activities are useful for providing functional comparisons between microbial communities. In this study enzyme assays followed the protocol presented by Sinsabaugh *et al.* (1997). Briefly, stock solutions of 4-methylumbelliferyl (MUF) linked substrates were prepared in 5 mM, pH 8, bicarbonate buffer, which approximated the conditions

found in William's Ditch. The substrates for the  $\alpha$ -1,4-glucosidase (EC 3.2.1.20),  $\beta$ -1,4-glucosidase (EC 3.2.1.21), leucine aminopeptidase (EC 3.4.11.1), and alkaline phosphatase (EC 3.1.3.1) assays were 4-MUF- $\alpha$ -D-glucoside, 4-MUF- $\beta$ -D-glucoside, L-Leucine 7-amido-4-methyl-coumarin, and 4-MUF-phosphate, respectively. Final concentrations for the substrates ranged from 10-200  $\mu\text{M}$  for 4-MUF- $\alpha$ -D-glucoside and 4-MUF- $\beta$ -D-glucoside, and from 1-120  $\mu\text{M}$  for L-Leucine 7-amido-4-methyl-coumarin and 4-MUF-phosphate. Enzyme activities were expressed as  $\mu\text{moles}$  of substrate hydrolyzed per liter of water per day.

Enzyme kinetics were assumed to follow the Michaelis-Menton model. For each enzyme the reaction rate ( $V$ ) was calculated for eight substrate concentrations with three analytical replicates at each concentration per reactor. Using the Eadie-Hofstee linear rearrangement of the model the kinetic parameters  $V_{\text{max}}$  (maximum velocity of the reaction under substrate saturation) and  $K_m$  (the half saturation constant) were calculated .

A special class of enzymes capable of oxidizing phenols are referred to as oxidative enzymes. These enzymes are involved in the breakdown of

lignin and other aromatic compounds and the formation of humic complexes. Phenol oxidase activity was measured colorimetrically on a Gilford spectrophotometer 260 using 5 mM L-3,4-dihydroxyphenylalanine, L-DOPA, prepared in 5 mM NaHCO<sub>3</sub> buffer (pH 8.1), as a substrate (Harkin & Obst 1973; Pind et al., 1994). There were three analytical replicates for each sample. Activity was calculated as  $\mu\text{mol}$  substrate converted  $\text{h}^{-1} \text{L}^{-1}$ , using an empirically derived extinction coefficient of  $1.66 \mu\text{mol}^{-1}$ .

#### *Substrate Utilization*

Substrate utilization assays provide information on the functional diversity of microbial communities. The BIOLOG® GN (BIOLOG, Inc. Hayward, CA) system consists of a 96-well microtiter plate containing 95 different carbon substrates and one non-carbon blank. Each well contains a redox dye, tetrazolium violet, that is reduced to formazan by the respiratory activity of the microbes. The degree to which each substrate is utilized is determined by measuring the color intensity of each well. To assay the samples, 150  $\mu\text{l}$  of water was added to each well and the plates were then incubated in the dark at 25°C. Readings of optical density were made at 24 h intervals using a microplate spectrophotometer (Molecular Devices, eMax) set to a wavelength of 590 nm.

ECO plates are also made by the BIOLOG company but contain 31 substrates plus a water blank; each substrate is replicated three times on a plate (Insam 1997).

#### *Collection of samples for DNA analysis*

Samples were collected by filtering 200 ml of water from each site onto five separate 0.2  $\mu\text{m}$  membrane filters (Gelman Supor 200). The filters were immediately placed into pre-labeled whirlpak bags and dropped into liquid nitrogen. The filters were stored at  $-70^{\circ}\text{C}$  until DNA extraction.

#### *Extraction and Quantification of DNA*

Each filter was aseptically cut up and divided between two 2 ml micro-centrifuge tubes containing a proprietary bead mixture, columns, and reagents (Fast Spin DNA Soil kit, Bio 101). Samples were homogenized using a mini bead-beater/8 (Biospec, Tulsa, OK) for 2 minutes, 30 sec on 30 sec off. The bead-beater was kept in a  $4^{\circ}\text{C}$  cold room in order to minimize frictional heating. After extraction the DNA was subjected to an additional clean-up step using the Wizard Mini-Prep kit (Promega). The DNA was quantified fluorometrically using Pico-Green dsDNA reagent (Molecular Probes, Eugene, OR) on a Molecular Devices Model fMax fluorometer set to an excitation wavelength of 480 nm and an emission

wavelength of 520 nm.

### *Community Structure Analysis using RAPDS*

Community similarity between the two sites was followed using randomly amplified polymorphic DNA (RAPD) analysis (Williams *et al.* 1990, Welsh and McClelland 1990). The RAPD analysis is a PCR based technique using a single arbitrary primer to scan for small inverted repeats and then amplify the intervening DNA segments (Hadrys *et al.* 1993). RAPD analysis primers were obtained from Amersham/Pharmacia Biotech and included:

Primer 1 (5'-d[GGTGCGGGAA]-3')

Primer 2 (5'-d[GTTTCGCTCC]-3')

Primer 3 (5'-d[GTAGACCCGT]-3')

Primer 4 (5'-d[AAGAGCCCGT]-3')

Primer 5 (5'-d(AACGCGCAAC)-3')

Primer 6 (5'-d[CCCGTCAGCA]-3')

Amplification was conducted on a Thermolyne Amplitron II thermal cycler. The thermal cycling profile was as follows: 1 cycle at 95°C for 5 min, followed by 45 cycles of denaturation for 1 min at 95°C, primer annealing at 38°C 1 min, and elongation at 72°C 2 min, followed by a 4°C chill hold. 50  $\mu$ l reactions were run using 25 pmol of a single rapid

primer, 50 ng of template DNA, 200  $\mu$ M of each dNTP (dATP, dCTP, dGTP, and dTTP), 1.5 mM MgCl<sub>2</sub>, 2.5 units of Master Taq DNA polymerase and Taqmaster PCR enhancer (Eppendorf, Westbury, NY).

Amplicons were separated by gel electrophoresis on a long 2% 1X TAE agarose gel containing 0.5  $\mu$ g/ml of ethidium bromide. The gels were photographed using Polaroid 57 film on a UV light transilluminator and the photographs scanned on a pdi 420oe scanning densitometer. Banding patterns were analyzed using Quantity One image analysis software (Bio-Rad, Hercules, CA). Similarity values based on the molecular weight of the bands produced in the analysis were used to generate a data matrix for use in cluster analysis.

## Results

The microbial community in the vicinity of the groundwater seep (Iron site) was substantially different from that found downstream at the clear site. For most of the parameters measured the iron site showed greater activity. Microorganisms from the iron site had higher levels of substrate utilization, and higher phenol oxidase (mean= 0.260  $\mu$ mol/h/ml, range 0.108-0.508) and peroxidase (mean= 0.263  $\mu$ mol/h/ml, range 0.093-0.495) activities. The levels of oxidative activity at the iron site are the highest we have recorded in this region. The activities of the

five hydrolytic enzymes we measured ( $\alpha$ -glucosidase,  $\beta$ -glucosidase, arginine, leucine aminopeptidase, and alkaline phosphatase) were also greater at the iron site. Despite large differences in activity, bacterial productivity was similar at both sites throughout the summer months. Levels decreased in December (Fig. 5).

Conductivity at the iron site (mean= 6.45 mS/cm, range 2.44 - too high to meas.) was higher than at the clear site (mean= 1.75 mS/cm, range 1.09 - 2.16). Total dissolved solids concentration was higher at the iron site (mean= 5.19 g/L versus 1.04 g/L for the clear site). The pH at both sites was circumneutral. Concentrations of nitrate, nitrite, chloride, and sulfate were similar at both sites and both showed high levels of total iron. Spectrophotometric scans of the surface water showed that water entering the channel from the iron seep was more highly colored than the water from the clear site.

A molecular profile of the microbial community was generated through the use of RAPD analysis. Six RAPD primers were used on DNA extracted from samples collected on August 17, and December 2, 1998. Different primers yielded diverse banding patterns. The use of multiple primers reduced individual bias. Each fragment on the gel was treated as an individual unit and scored as either present or absent. These data,

based on the estimated molecular weight of the bands, were used to generate a data matrix for use in cluster analysis (based on Ward's amalgamation scheme and euclidean distances). Cluster analysis indicated that the microbial communities associated with the two sites were 100% dissimilar, on both sampling dates (Fig. 6). In comparisons across seasons the clear samples from August and December were about 8% similar while those from the iron site were approximately 15% similar.

## Discussion

Other research has documented that creosote and creosote related compounds are found as contaminants in Williams Ditch. We studied two sites in William's Ditch in order to get a better understanding of microbial community dynamics. The iron site received local groundwater inputs. These inputs are diluted by upstream flow. The result is that the clear site (located approximately 10m downstream) is chemically and microbiologically distinct from the iron site. The magnitude of the impact and the size of the area affected is related to the volume of groundwater leachate and the flow of water in the ditch. The microbiological differences indicate that the substrate entering the seeps is clearly different in kind from that supporting microbial activity in the

main channel. Structurally the two sites appear to be completely different as well. For comparison, the structural divergence provided by the presence of iron and elevated levels of humic compounds was larger than that associated with seasonal succession.

We originally became involved in this project after a sheen was reported on the water surface of the ditch. The city of Toledo's leaf collection facility was located next to William's Ditch and it was suspected of leaching water with a high level of coloring or staining. Rather than being problematic the presence of these humic leachates may promote the degradation of organic pollutants from the Toledo Tie site.

It has been found that humic acids are able to stimulate the degradation of benzene when iron is present as the terminal electron acceptor (Lovely et al 1996). Humic substances in aquatic systems also strongly affect adsorption-desorption and biodegradation of organic compounds (Rav-Acha and Rebuhn 1992). It is not clear what the relative contributions of compost and creosote leachates are to water quality; however, it is clear that the oxidation of aromatic substrates is a major microbial activity. The contribution of leaf leachates enriches the community with microbes that can degrade aromatic compounds. For this reason it is conceivable that a composting operation could stimulate the

breakdown of recalcitrant creosote compounds that enter Williams Ditch.

## References

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Figure 1. Location of Williams Ditch study site. (from Lesniak 1995).

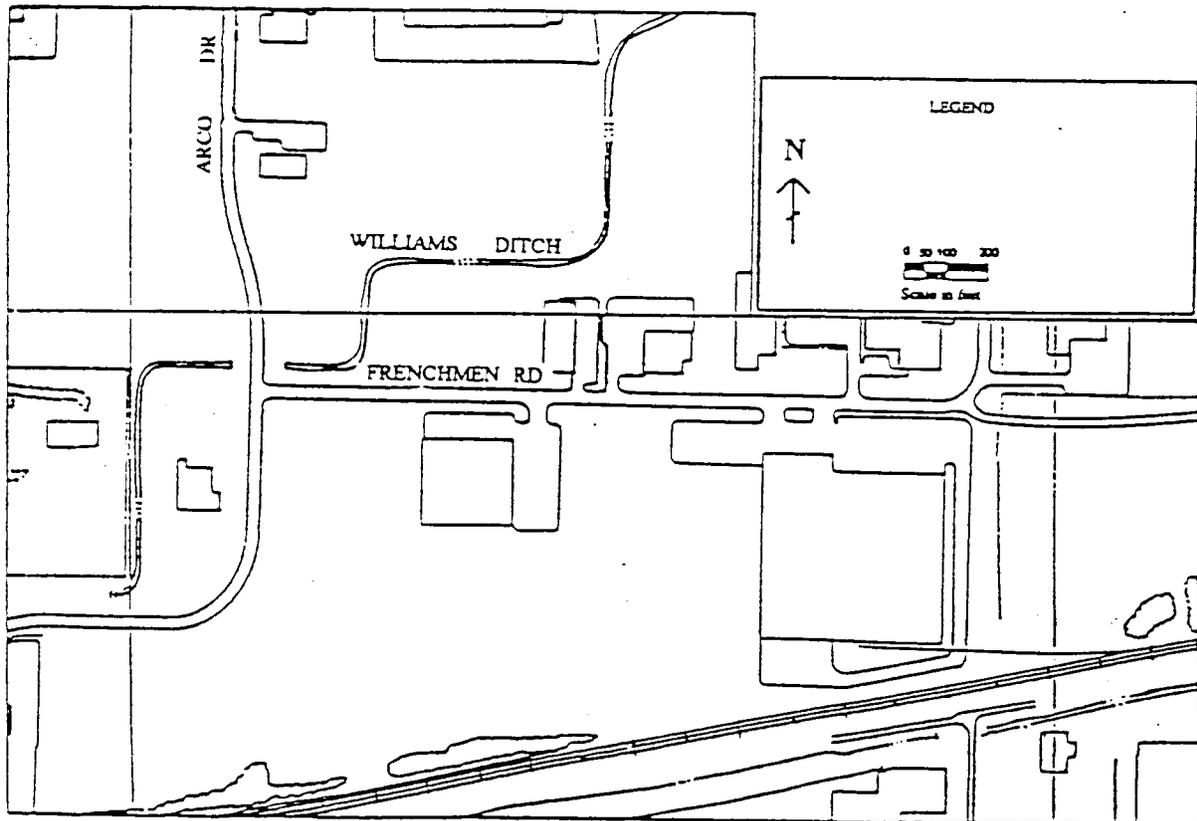
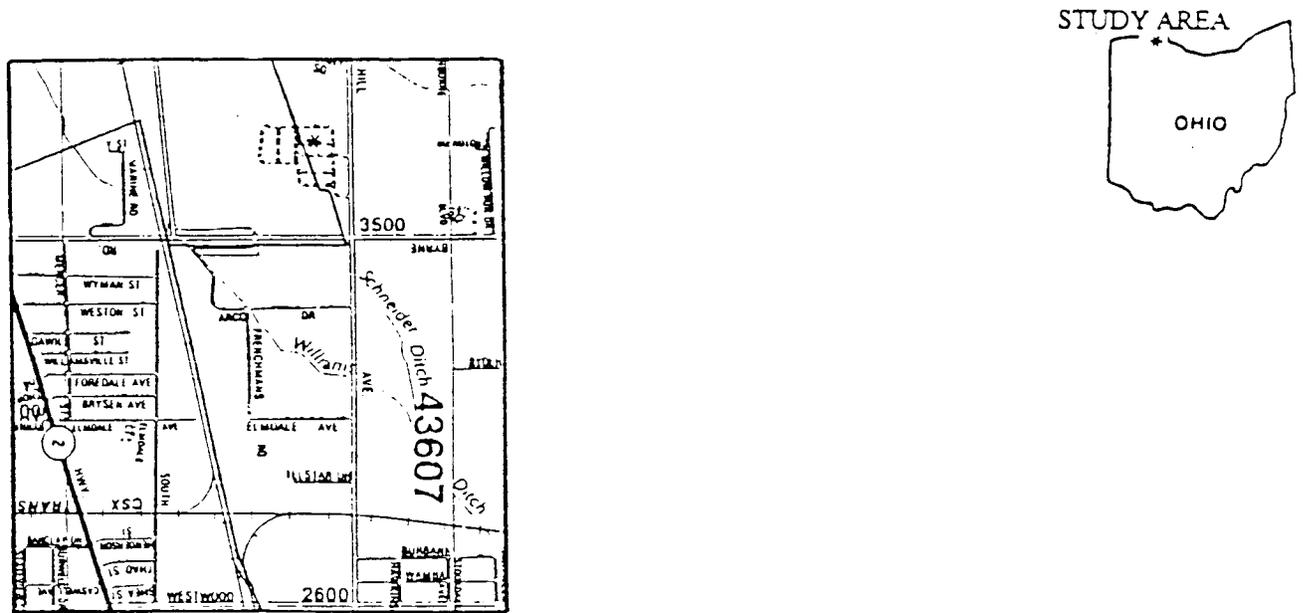




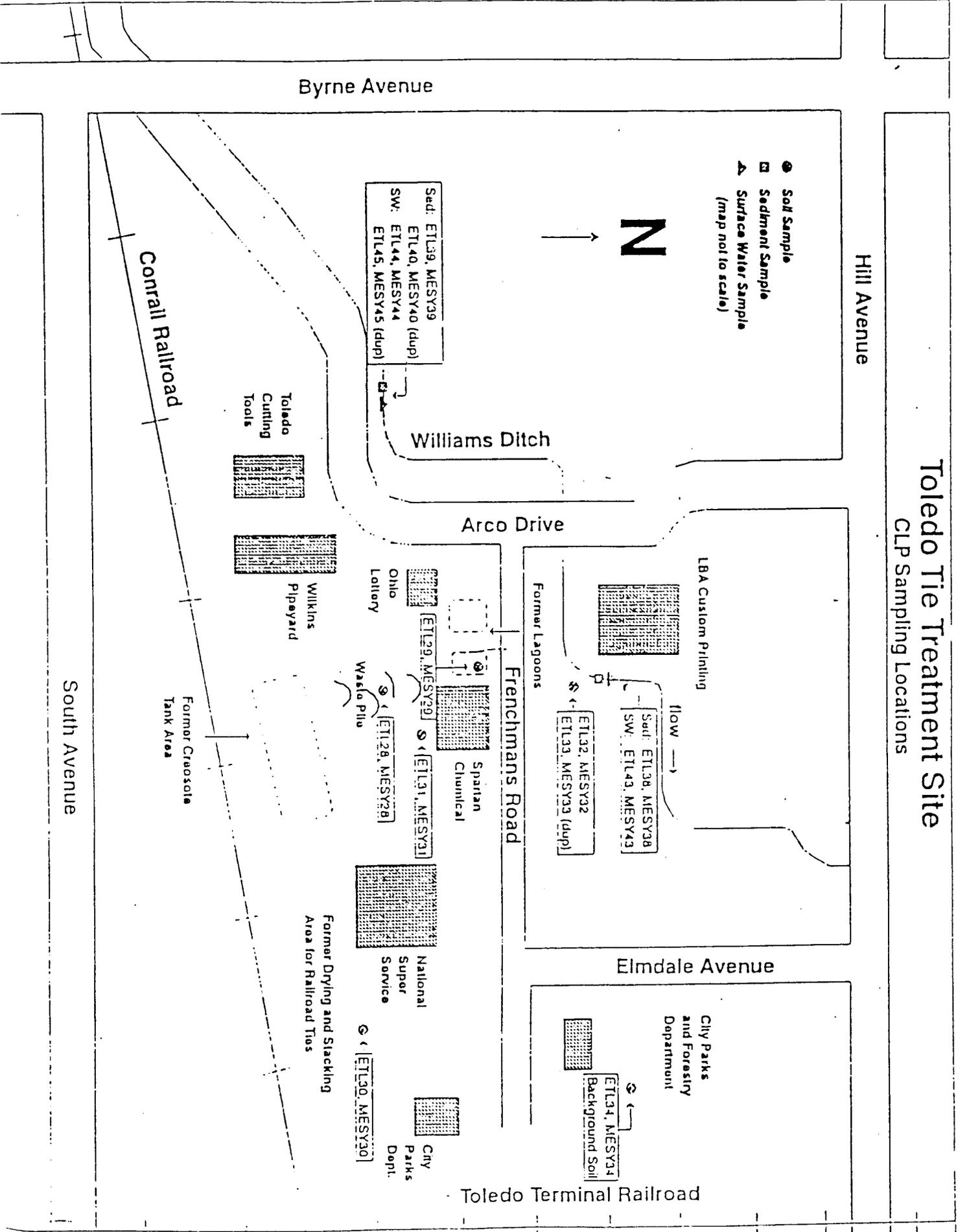
Figure 2 Williams Ditch Iron Site



Figure 3. Williams Ditch Clear sampling site

# Toledo Tie Treatment Site

## CLP Sampling Locations



- Soil Sample
- ▣ Sediment Sample
- ▲ Surface Water Sample  
(map not to scale)



Sed: ETL39, MESY39  
ETL40, MESY40 (dup)  
SW: ETL44, MESY44  
ETL45, MESY45 (dup)

Sed: ETL38, MESY38  
SW: ETL43, MESY43  
ETL32, MESY32  
ETL33, MESY33 (dup)

ETL29, MESY29  
ETL31, MESY31  
ETL129, MESY28

ETL34, MESY34  
Background Soil

ETL30, MESY30

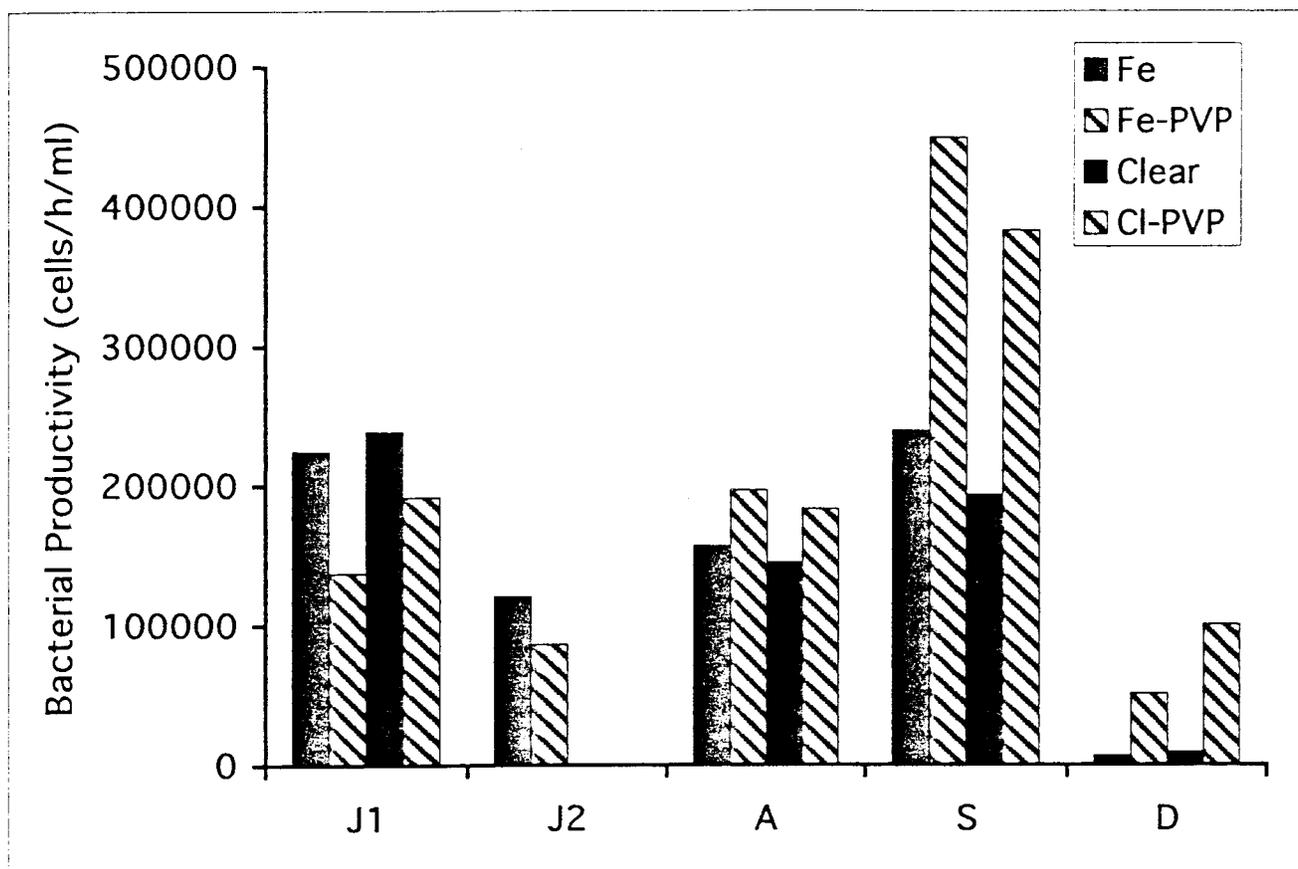


Figure 5  
 Bacterial Productivity in Williams Ditch. J1 represents samples collected on July 13, J2 on July 27, A on August 17, S on September 9, and D on December 2, 1998.

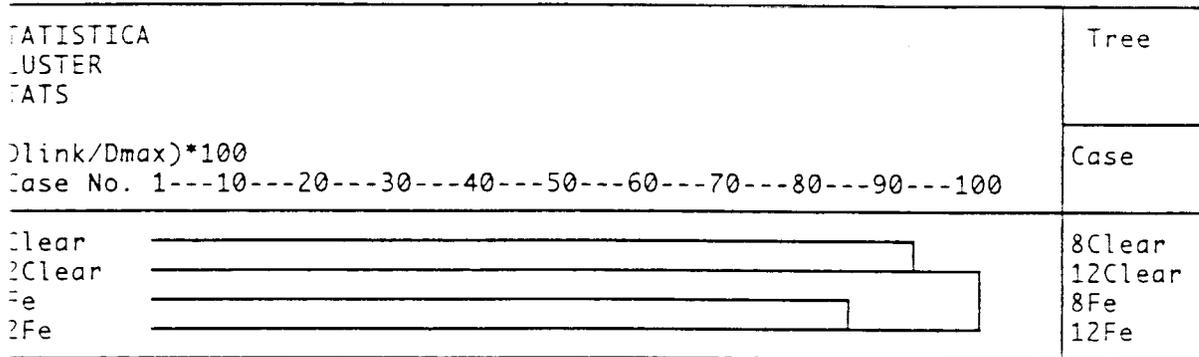


Figure 6

Cluster analysis based on Wards amalgamation scheme and Euclidean distances. 8 Clear and 8 Fe represent samples collected on August 17, 1998 from the Clear and the Iron sites respectively. 12 Clear and 12 Fe represent samples collected on December 2, 1998 from the Clear and Iron sites.