

**THE EFFECTS OF TOLUENE CONTAMINATION ON EPILITHIC
BACTERIAL COMMUNITIES IN A STREAM**

by

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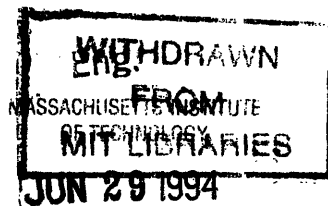
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ABSTRACT

The effects of toluene contamination on the epilithic bacterial communities in a stream were studied by comparing samples from contaminated and uncontaminated portions of the same stream. This comparison was based on the relative abilities of both contaminant exposed and unexposed communities to degrade toluene, on the relative abundance of toluene degrading organisms, and on the respective proportion of toluene degrading bacteria within the heterotrophic population. Samples from the contaminated sites were found to be able to degrade toluene approximately ten times faster than those from the pristine site. Toluene degrading bacteria make up between 4 and 7 % of the heterotrophic population at contaminated sites, whereas they correspond to only a 0.12 to 0.14 % of the population at uncontaminated sites. However, total bacterial numbers and total heterotrophic numbers, with respect to biomass weight, have not been significantly affected by toluene. Our result indicate that the epilithic bacterial community has adapted to the presence of toluene in the stream.

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CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1.- INTRODUCTION

Contamination of the natural environment with aromatic hydrocarbons and other petroleum derivatives is a widespread phenomenon. Degradation of these contaminants by naturally occurring microorganisms is a very common occurrence. Biodegradation has the potential for cleaning up the contaminants, and in some instances may be the only process capable of completely removing the pollutants from a contaminated site (Armstrong et al., 1991). Therefore the importance of a thorough understanding of the biodegradation processes. Such understanding will eventually allow us to have a greater control of these mechanisms, so that they may be effectively used for clean up purposes.

An important part of that understanding is the assessment of the effects of a given contaminant on the bacterial community at a contaminated site. That is the object of our study. The study site is the East Drainage Ditch, a small freshwater stream in northeastern Massachusetts, part of the Aberjona watershed, which is contaminated with micromolar quantities of toluene. The bacterial community of interest is that attached to the surface of the rocks lining the stream bottom.

This first chapter provides background information on the study site, its history and characteristics. It will also cover general information about the contaminant of interest: toluene. Literature concerning the biodegradation of toluene and its fate at the study site has been reviewed. Finally, a brief review of the characteristics of epilithic communities, and of the effects that hydrocarbon contamination has been reported to have on indigenous bacterial communities, is presented. The following chapter describes

the experiments performed to assess the influence of toluene contamination on the epilithic bacterial communities in the East Drainage Ditch.

1.2.- THE SITE

1.2.1.- THE ABERJONA WATERSHED

The site of our study is part of a watershed draining to the Aberjona river, which discharges into the Upper Mystic Lake. The Aberjona watershed is located in northeastern Massachusetts (see Figure 1.1), and it has been a heavily industrialized area since the mid 1800's. Hazardous chemicals of industrial origin have been discharged into the watershed for over 150 years. Many dump sites have been discovered in recent years and the extent of both groundwater and surface water contamination has not been completely assessed. There is a growing concern for the possible effects of this contamination on the local inhabitants (DEP, 1989).

The Massachusetts Department of Environmental Protection (DEP) and the US Environmental Protection Agency (EPA) have proof, or strong suspicion, of contamination at over 50 sites (DEP, 1989. USEPA, 1986). For the location of those sites within the watershed see Figure 1.2. Two of those sites (Industriplex and Wells G & H) have been designated Superfund sites. At Wells G & H an epidemiological study conducted by Lagakos *et al.* (1986) found positive correlation between consumption of the well's water and the incidence of childhood leukemia, perinatal deaths, congenital defects, and other childhood disorders. Researchers have also found that the chemicals contaminating the Aberjona watershed are present in areas far from the points of their original environmental discharge, this indicates that the chemicals have been transported through the watershed (Durant, 1991).

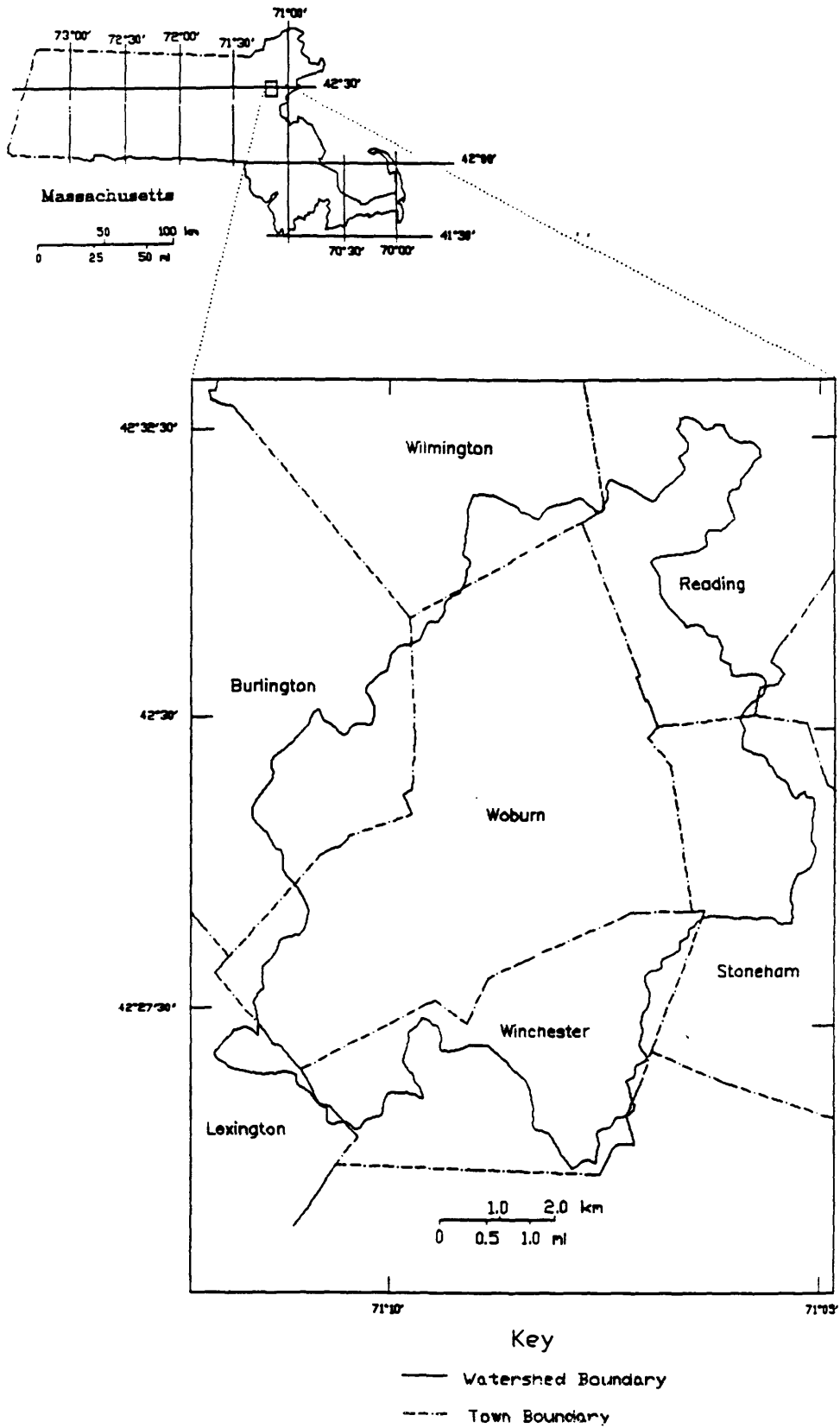


Figure 1.1.- Location of the Aberjona Watershed. (Durant, 1991).

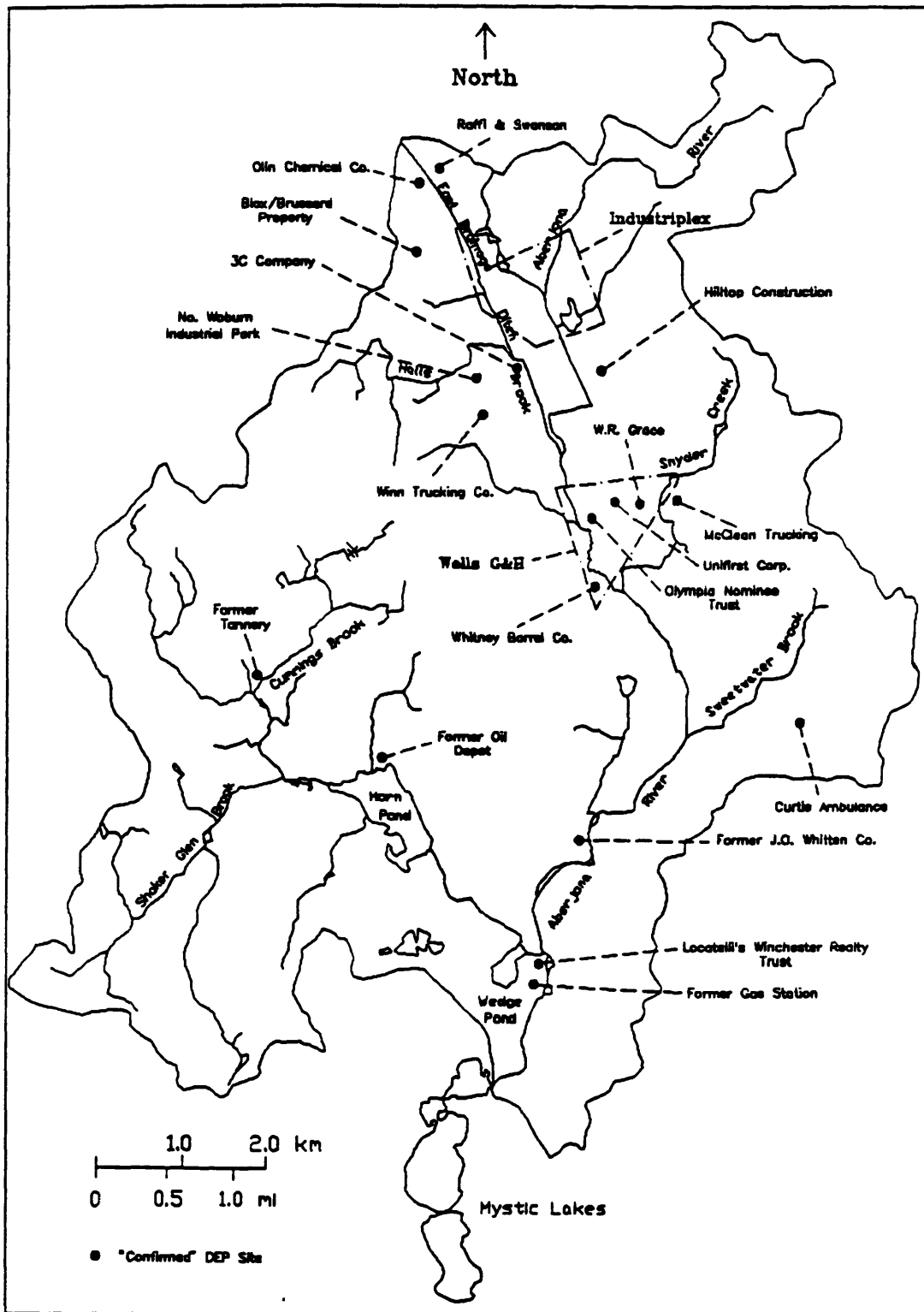


Figure 1.2.- Location of the East Drainage Ditch in the Aberjona watershed and with respect to confirmed DEP and EPA Superfund sites. (Durant, 1991).

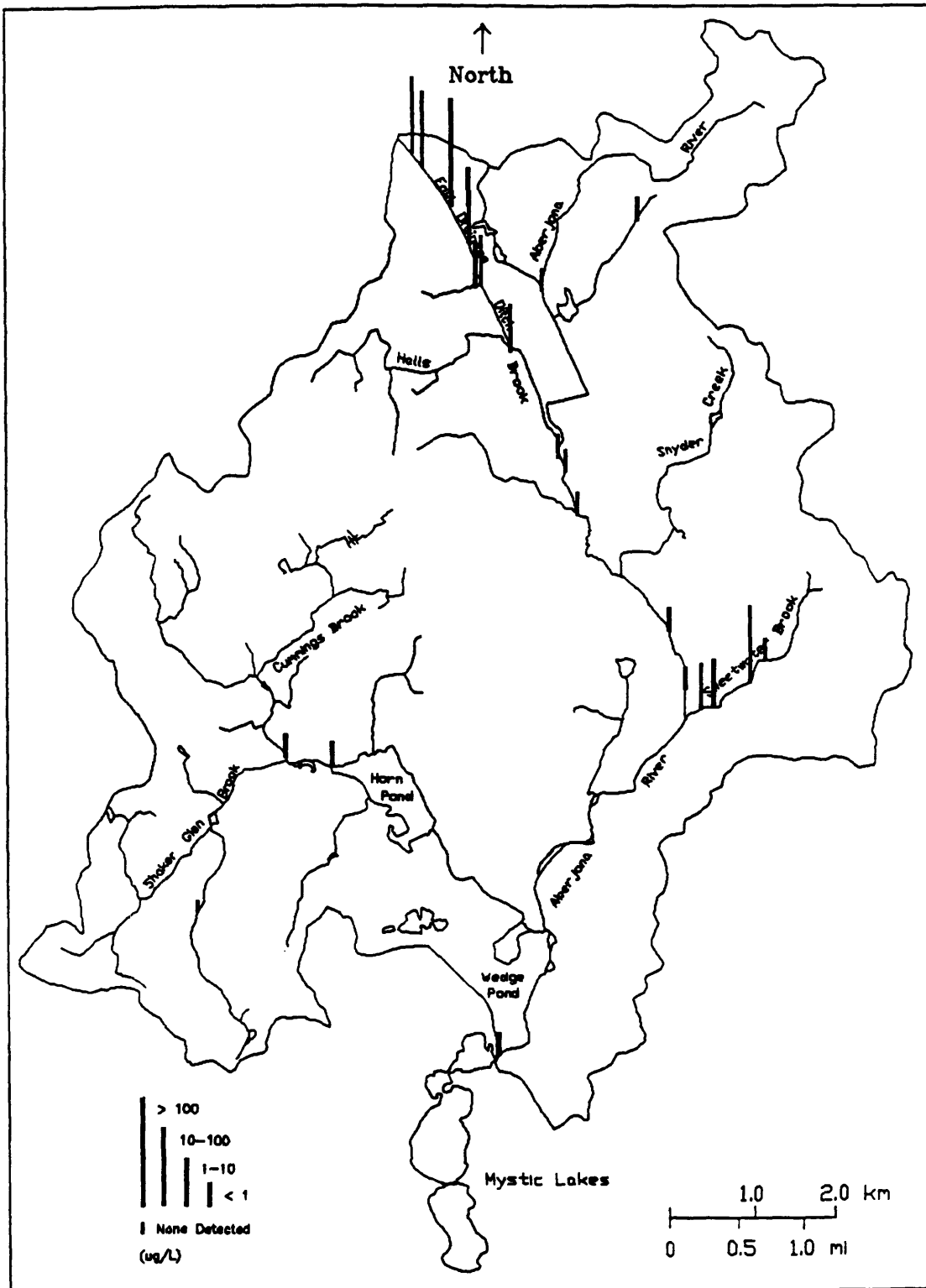


Figure 1.3. - Distribution of total volatile organic compounds in surface waters in the Aberjona watershed. (Durant, 1991).

Historically, one of the most important industrial activities in the watershed has been that of leather tanning, finishing and rendering (Durant, 1991). The leather industry has been established in the area since the late 1600's. Processes related to the leather industry, as well as other chemical manufacturing activities, have left the watershed polluted with a variety of metals, among which chromium, arsenic and lead predominate, there is also indication of the presence of various volatile organic compounds (Figure 1.3) and oils (Durant, 1991). These metals can be found in surface sediments throughout the watershed. The highest arsenic and chromium concentrations (1,000-1,500 mg/kg of dry weight) appear to be within the Industriplex site and points near downstream. Lead levels in the watershed sediments are more evenly distributed.

1.2.2.- THE EAST DRAINAGE DITCH

The East Drainage Ditch (EDD) flows from north to south and discharges into Halls Brook which flows into the Aberjona river (Figure 1.2). The Ditch is contaminated with a variety of organic and inorganic compounds. Chromium, cadmium, lead and arsenic have been found in the stream's sediments, both within and upstream from the Industriplex site. Chromium quantities in the EDD have been found to range between 1 and 500 mg/kg, dry weight of sediment. Lead concentration in sediments from the EDD ranges from 1 to 400 mg/kg. Most interesting for us are the high concentration of volatile organic compounds that can be found in the Ditch as compared with the rest of the watershed (Figure 1.3).

Like the rest of the watershed, the area surrounding the East Drainage Ditch has been an industrial site for over a century. Among other historical sites, the East Drainage Ditch flows through the Industriplex site, which is one of the oldest industrial sites in the

watershed (Durant, 1991), and which was added to the EPA National Priorities List in 1982 as a Superfund site (USEPA, 1986). Figure 1.2. This industrial site was originally developed by Chemical Works Co., a manufacturer of acids and other chemicals for the leather, textiles and paper industries. Ten years after it was first established, in 1863, Chemical Works was acquired by Merrimac Chemical, which manufactured arsenic and lead based insecticides and explosives such as trinitrotoluene (TNT). By the beginning of the 20th century Merrimac had become one of the largest chemical producers of New England. The site had three more owners between 1927 and 1960, the Monsanto Co. (1927-1934), the New England Chemical Co.(1934-1936), and Consolidated Chemical Industries (1936-1960). Under these owners the site functioned as a rendering factory, producing glue and grease from tanning industry refuse. Between 1960 and 1968 the site belonged to Stauffer Chemical Co., a glue manufacturer.

While trying to transform the site into an industrial park, construction workers began to uncover several piles of materials that previous owners had disposed of on the site. These discoveries eventually led to a DEP and EPA investigation on the site. The results of these investigations indicated, among other things, the existence of sludge lagoons contaminated with high levels of chromium, pits filled with lead and arsenic contaminated soil, and plumes of benzene and toluene in the ground water.(USEPA, 1986)

1.3.- THE CONTAMINANT

1.3.1.- TOLUENE

Among the numerous contaminants present in the East Drainage Ditch, the one of interest for our study is toluene. Also known as methylbenzene, methylbenzol, and

phenylmethane, toluene is an aromatic compound with a molecular weight of 92.14, and molecular formula C_7H_8 . Poorly soluble in water [0.53 g/l at 25°C, (Lide (ed.), 1991)], toluene is a volatile organic compound which is readily absorbed through the lungs, less so through the skin or the gastrointestinal tract (Antti-Poika, 1987). Toluene main toxicological effect is on the nervous system, it has not yet been found to be carcinogenic or mutagenic (Antti-Poika, 1987). Because of its effects on the central nervous system toluene has been designated as a priority pollutant and the US Environmental Protection Agency has set a drinking water standard of no more than 2 mg of toluene per liter of water for lifetime exposure (USPHS, 1989)

Toluene is a component of gasoline and its presence in the environment is often related with gasoline, or other fossil fuels, spills, and with leakage of gasoline from underground storage tanks. Toluene is commercially obtained from petroleum, by dehydrogenation of cycloparaffin fractions, or by aromatization of saturated aliphatic hydrocarbons (Antti-Poika, 1987). It is among the industrial chemicals with the largest production volumes, which are in the order of millions of tons per year (Smith, 1990). The industrial uses of toluene include the manufacture of benzene, benzoic acid, and benzyl chloride; it is also used as a solvent for paints, coatings, adhesives and inks; and in the pharmaceutical industry.

1.3.2.- THE MICROBIAL DEGRADATION OF TOLUENE

Aromatic hydrocarbons are among the most widely distributed substances on this planet. Although their origin in the environment is still a debatable issue, it is commonly accepted that they are mostly derived from the natural pyrolysis of organic compounds, and not directly from biosynthesis (Gibson and Subramanian, 1984). Thus, it is not surprising that microorganisms with the ability to degrade aromatic hydrocarbons are also

commonplace in nature. Nowadays there is a growing concern about the effects that the release into the environment of aromatic compounds of anthropogenic origin may have. From this perspective, the microbial degradation of aromatic compounds has become more than a natural part of the carbon cycle and now it is also viewed as a remediation tool. (Atlas, 1984)

This is not intended to be an exhaustive review of the literature that have been published on the biodegradation of toluene in recent years, since such a task would constitute a completely different project on itself. Rather, we will try to highlight the most relevant aspects of what constitutes today's knowledge of the process. Recently, evidence has been presented for the anaerobic biodegradation of aromatic hydrocarbons. The anaerobic degradation of toluene by a *Pseudomonas* sp. which utilizes NO_3 or N_2O as a terminal electron acceptor has been reported by Zeyer *et al.* (1990); also, toluene degradation by an iron reducing organism has been reported by Lovey and Lonergan (1990); and Grbic-Galic and Vogel (1987) have reported the anaerobic biotransformation of toluene and benzene by mixed methanogenic cultures. Because the experiments that constitute Chapter 2 of this paper dealt only with aerobic organisms and reactions, those are the pathways for toluene degradation that will be studied with greater detail.

Two main pathways have been established for the aerobic degradation of toluene by bacteria (Gibson, 1971). In the first pathway, which is present in *Pseudomonas aeruginosa* and which was the first ever to be studied, toluene is oxidized through benzyl alcohol, benzaldehyde, benzoic acid and catechol. Catechol can then be further oxidized either via the *ortho* pathway, which involves cleavage of the carbon-carbon bond between the two hydroxyl groups of catechol yielding *cis*, *cis*-muconic acid; or via the *meta*

pathway, which involves cleavage of the bond between a carbon atom without a hydroxyl group and the adjacent atom with a hydroxyl group, resulting in 2-hydroxymuconic semialdehyde (Cerniglia, 1984). The second pathway for aerobic degradation of toluene that has been known to researchers for some time, involves initial oxidation of the aromatic ring (instead of initial attack on the methyl substituent) yielding cis-2,3-dihydroxy-2,3-dihydrotoluene. This compound is then transformed to 3-methylcatechol, which is then further oxidized to acetate, acetaldehyde and pyruvate (Gibson, 1971).

In the past five years evidence has been presented of three other aerobic degradation pathways for toluene. These pathways involve initial attack on the aromatic ring by a monooxygenase, resulting in the formation of *meta*, *ortho* or *para* cresol (Shields, 1989; Kukor, 1990; Whited, 1991; respectively). The degradation process continues through either the formation of methylcatechol or *p*-hydroxybenzylalcohol.

Evidence has also been presented in recent years of the plasmid encoded degradation of toluene. This degradation would generally follow the benzylalcohol pathway (ie. oxidation of the methyl group as the first step). Studies have shown that the enzymes for the degradation of toluene are encoded on plasmids, designated as TOL (Bayly and Barbour, 1984). Different studies indicate that these plasmids contain two operons, one that encodes enzymes for the oxidation of toluene to the corresponding alcohol, aldehyde, and carboxylic acid derivatives, the other encodes for the conversion of the carboxylic acids to catechol (Smith, 1990).

1.3.3.- THE FATE OF TOLUENE IN THE EAST DRAINAGE DITCH

The water of the East Drainage Ditch is contaminated with toluene in concentrations that go up to 350 μg per liter. The contaminant is suspected to be entering the stream from a contaminated groundwater plume. In two studies conducted by Kim et al. (1994), and Cohen et al. (1994) at the Massachusetts Institute of Technology, biodegradation was shown to be one of the most important sinks for toluene in the East Drainage Ditch.

Kim's study used a mass balance approach for the determination of the fate of toluene in the stream. Dilution and volatilization were considered to be the only important abiotic processes for toluene loss in the stream. Propane was added to the stream as a volatile tracer and sodium chloride as a conservative tracer (to account for volatilization and dilution, respectively). All three concentrations (toluene, propane, and sodium chloride) were measured at different points downstream. Propane volatilization rate was used to estimate that of toluene, the difference between total toluene loss rate and its estimated volatilization and dilution rate was taken as toluene's biodegradation rate in the field. This study's results indicate that biodegradation is responsible for $66\pm 7\%$ of the toluene loss during the summer, while volatilization is responsible for $26\pm 6\%$ of the loss during the same period. Volatilization and biodegradation rates were found to be very similar during winter months. Dilution was found to account for 25 % of the decrease in toluene during the spring (high flow season), and ca. 8 % during the summer (low flow season). This study concludes that the site conditions, specially high flow velocities, high stream bottom surface area to water volume ratio, and long microbial adaptation period, are responsible for biodegradation being the most important sink for toluene throughout the year.

Concurrently, a series of laboratory experiments conducted by Cohen et al. (1994), supported the findings described above and determined that stream surfaces were responsible for more than 95 % of the toluene degradation. It was found that sediments and rocks from the stream bottom were the most important compartments. This study used shake flasks and columns to determine biodegradation rates under batch and continuous flow conditions, respectively. Rates determined using shake-flasks were better indicators of those in the field. Biodegradation rates of the environmental samples were also found to be strongly dependent on temperature, with results obtained under winter conditions being 11 to 14% of those obtained under summer conditions.

1.4.- THE BACTERIAL COMMUNITY

1.4.1.- EPILITHIC BACTERIAL COMMUNITIES

With the term epilithic bacterial communities we refer to a microbial assemblage growing attached to the surface of a rock. Epilithic communities have been found to be a mixture of biotic forms containing algae, bacteria, cyanobacteria, and, to a lesser extent, fungi. The proportion of photosynthetic forms would be governed by light availability. A structural model proposed by Lock *et al.* suggested that the biotic elements were held together and attached to the rock's surface by extracellular polysaccharides of microbial origin. This same model also suggested that the polysaccharide matrix serves as a site for entrapment of soluble and particulate matter; a possible deposit for extracellular enzymes; and a trap for any products of extracellular enzymatic hydrolyses. Other studies have demonstrated the existence of a rather loosely attached assemblage of organisms surrounding a more firmly attached component. (Lock, 1993)

As has been already discussed, Cohen et al.(1994) found that in the East Drainage Ditch most of the toluene degradation is carried out by microorganisms associated with the sediments and attached to the rocks lining the stream bottom. A similar phenomenon has been observed by other researchers for a variety of organic contaminants(Atlas, 1984; Leahy and Colwell, 1990). In another example, Pignatello et al. (1983) reported that most of the bacteria responsible for the degradation of pentachlorophenol in artificial freshwater streams were attached to surfaces. In a related study, the authors point out that their results indicate the importance of environmental surfaces to the degradation of xenobiotic compounds in aquatic systems (Pignatello et al., 1985). They conclude that the high concentration of microorganisms on these surfaces allows for a tremendous substrate utilization rate. Also, studies by Spain et al.(1980, 1983), and Van Veld and Spain (1983) point to the possibility that attachment plays a role in bacterial adaptation to contaminants, since it would facilitate transferring of genetic information between different members of the bacterial assemblage.

A study by Shreve and Vogel (1992) looks into the effects of immobilization on bacterial activity, particularly cell growth and substrate utilization kinetics. There are several factors that could affect the activity of attached versus free living bacteria, namely local water activity, pH, temperature, ion concentration, nutrient and waste products concentration, oxygen and nutrient diffusion, and fluid shear effects (Lock, 1993). Shreve's study concludes that changes in the activities of attached microbes are probably due to changes in the physical or chemical environment of the cell, and not to a generalized effect of immobilization on the growth and metabolism of the microorganisms. Particularly, they did not find that immobilization affected the substrate utilization rate for the *Pseudomonas* sp. they were working with.

1.4.2.- EFFECTS OF HYDROCARBON CONTAMINATION ON BACTERIAL COMMUNITIES.

In general, prior exposure of a community to hydrocarbons have been found to result in an increase in the community's ability to degrade the contaminants. This phenomenon is known as adaptation. This adaptation is said to occur through three major mechanisms: enzyme induction/repression; mutations that result in new metabolic capabilities; and/or, preferential growth of those organisms capable of degrading the contaminant or contaminants of interest.(Spain et al.,1980; Spain et al. 1983). Several studies have reported increases in the numbers of hydrocarbon degrading organisms, and in their proportion with respect to the heterotrophic population upon exposure to hydrocarbon contamination (Bossert and Bartha, 1984). Sometimes this phenomenon is associated with the predominance of a few hydrocarbon degrading species, but other studies indicate that the diversity of the population has not changed, and in others it has been shown to increase (Pfaender and Buckley, 1984). Such different results seem to indicate that the response of the community is highly specific to a given situation.

A study on pentachlorophenol degradation in artificial freshwater streams by Pignatello et al. (1983, 1985, 1986) is a good example of the effect of adaptation on microbial communities. Pignatello et al. (1983) studied the abiotic and biotic degradation of pentachlorophenol at a research station in Minnesota. The facility consisted of a several 520 m channel that are continuously fed with Mississippi river water. Pentachlorophenol was added to some of the channels while others were used as controls. Their results indicate that initially photolysis is the major sink for the contaminant. But after about three weeks biodegradation becomes the major removal process, presumably

because the microflora in the channels had adapted to the presence pentachlorophenol. They also found that total bacterial numbers were not significantly affected by the contaminant. The authors suggest that the community adaptation was achieved through an increase in the population of degrading bacteria from an originally low background population of pentachlorophenol degraders, and not through enzyme induction mechanisms (Pignatello et al., 1986). They base their conclusion on, among other reasons, the relatively long lag period, which is indicative of growth ; and the fact that the mineralization potential for pentachlorophenol persisted in the channel bacterial population for months after the addition of the contaminant ceased. Similar results were obtained in a study on p-nitrophenol (PNP) biodegradation conducted by Spain et al. (1984). They also concluded that bacterial adaptation to PNP was a result of the selection of organisms capable of growing in this compound. Similarities between different test systems were taken to indicate that adaptation could not have been, in this case, the result of a random event such as a gene mutation. In this study total heterotrophic population was found to remain unchanged, while PNP degrading bacteria numbers increased.

Madsen et al. (1991) examined the microbial patterns in an aquifer contaminated with polycyclic aromatic hydrocarbons (PAH's). Samples were taken from boreholes within the contaminant plume (upgradient, which was the closest to the contamination source, and downgradient), and from a pristine borehole. They found the following patterns: PAH mineralizing activity was found only in samples from within the contaminant's plume, but microorganisms from all three site were capable of mineralizing p-hydroxybenzoate (PBH); lag periods before mineralization were observed in the downgradient and pristine samples only; viable count of aerobic heterotrophic bacteria

showed that samples from the borehole closest to the contamination source had the largest heterotrophic population and the pristine site the lowest; no significant differences were observed in total bacterial numbers between the three sites. These results indicate how proximity to the contaminant source influenced the composition of the bacterial communities at this site.

In a study regarding the effects of several factors (ie. concentration, exposure time, inoculum and chemical structure) on the adaptation of microbial communities to degrade xenobiotic compounds, Spain et al. (1983) found that the extent of the adaptation of a community was not directly related to the concentration of the contaminant to which it was previously exposed. This is an interesting result since it points to the possibility of preparing the communities near a potential release site by feeding it with small amounts of the contaminants of interest. Other results from this same study indicate that adaptation can last for several weeks, and that biodegradation rates are not necessarily dependent on the total bacteria biomass. It is also suggested that there may be a threshold concentration, for certain compounds, below which no adaptation occurs.

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CHAPTER 2

EFFECTS OF TOLUENE CONTAMINATION ON THE EPILITHIC BACTERIAL COMMUNITY IN A MASSACHUSETTS STREAM.

2.1.-INTRODUCTION

Contamination of the natural environment with hydrocarbons, both from natural and synthetic origins, has been a cause of growing concern during the past two decades. Since microorganisms with the ability to degrade hydrocarbons have been found to be ubiquitous in natural environments (Atlas, 1981), a considerable amount of research has been devoted to study the potential use of the indigenous microflora as a clean up tool at contaminated sites (Atlas, 1984; Leahy and Colwell, 1990). As a part of this research effort, several studies have been concentrated in determining whether a prior exposure to hydrocarbons would make a bacterial community more adept at dealing with subsequent inputs, ie. whether its hydrocarbon degrading potential would increase (Leahy and Colwell, 1990).

Researchers have found that, in general, bacterial communities tend to adapt to their contaminated environment (Spain et al., 1980; Spain et al. 1983). This adaptation is said to occur through three major mechanisms: enzyme induction/repression; mutations that result in new metabolic capabilities; and/or, preferential growth of those organisms capable of degrading the contaminant or contaminants of interest. So it can be inferred that adaptation of the community is likely to cause some changes in its structure (Bossert & Bartha, 1984). These changes may prove to be difficult to predict, mainly because of the different effects of the contaminant on different members of the bacterial assemblage. It has been observed that while hydrocarbon degrading bacteria can grow on these

compounds, they may be toxic for other species within the community, and have no effect on several others (Pfaender and Buckley, 1984).

This paper will study the effects of toluene contamination on the epilithic bacterial community in a freshwater stream. It will focus on changes in the community of toluene degrading bacteria as influenced by a plume of toluene and other contaminants into the stream. In this study, the emphasis is on the relative abundance of toluene degrading bacteria and on the relative capabilities of the communities to degrade our compound of interest. Comparisons have also been made on the basis of total bacterial counts and cultivatable heterotrophic bacterial counts, that allow us to arrive at some preliminary conclusions as to the overall effects of the contaminant's presence on the bacterial assemblage.

Our study site, the East Drainage Ditch, is a fresh water stream in northeastern Massachusetts, and it is part of the Aberjona watershed. This stream is contaminated with toluene, among other volatile organic compounds, which is suspected to be coming into the stream from a ground water plume. Downstream from the study site, the East Drainage Ditch runs through an old industrial site, known as the Industriplex site, which became a superfund site in 1982 (Durant, 1991). Toluene concentrations of up to 350 ppb have been measured in the stream's waters (Cohen et al., 1994). In a related study by Kim et al. (1994), biodegradation was found to be the most significant sink for toluene in this site. It was also determined that the microbial community responsible for most of the degradation was that attached to the rocks and sediments lining the bottom of the stream. We have chosen to study epilithic bacterial communities, mainly because these organisms

are exposed directly to soluble materials in the water and are known to have significant biodegradative potential (Cohen et al., 1993).

Measurements of toluene concentration in the stream water show that very small quantities of toluene (0-6 ppb) are present 50 meters upstream from the suspected source, and no toluene can be found 100 meters upstream from the source. This situation presented us with the opportunity to observe how the presence of toluene has influenced the microbial community. A comparison has been made of the relative abilities of both contaminant exposed and unexposed communities to degrade toluene, and on the relative abundance of toluene degrading bacteria within those communities.

2.2.-MATERIALS AND METHODS

2.2.1.-SAMPLING SITE AND CONDITIONS

All of the samples for this study were collected from the East Drainage Ditch (Figure 2.10), a fresh water stream that is part of the Aberjona watershed, in northeastern Massachusetts. Sampling started in June and continued until September of 1993. Average temperature in the area for this time of the year ranges from 20° to 25°C. Flow rates have been found to range between 25 and 40 m³/hr for the summer months (Kim et al., 1994).

The Aberjona watershed has been the site of numerous industrial activities since the turn of the century. Industries located in the watershed have included leather tanning and chemical manufacture. Parts of the East Drainage Ditch are contaminated with metals, such as Arsenic and Chromium, and with a variety of organic compounds, such as toluene, which seem to be introduced to the stream by a contaminated ground water

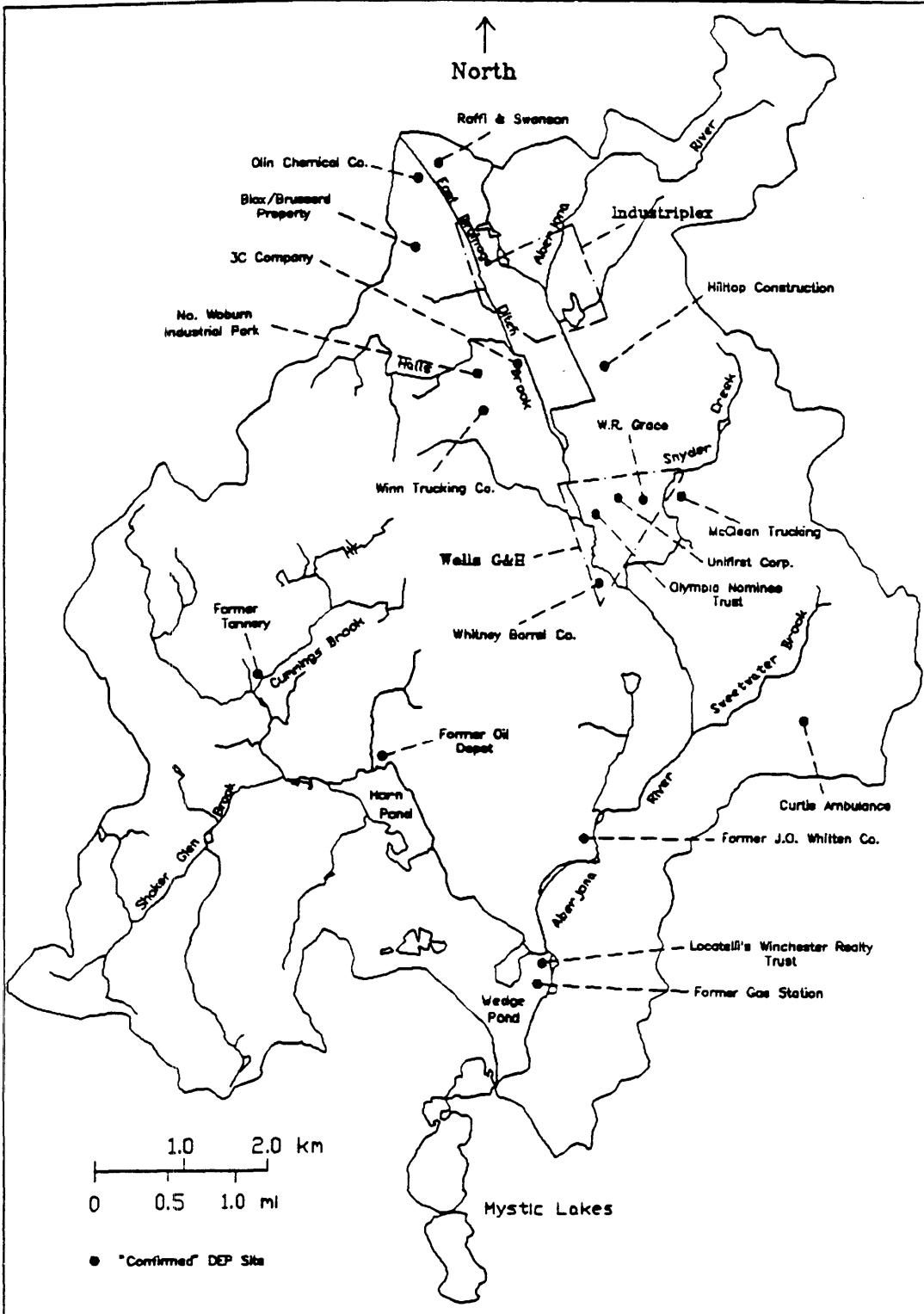


Figure 2.1.- Location of the East Drainage Ditch within the Aberjona watershed and with respect to confirmed DEP sites and EPA Superfund sites. (Source: Durant, 1991).

plume (Durant, 1991). The introduction into the stream of the contaminated groundwater appears to be occurring underneath a culvert which is approximately 80 m long. This culvert is located approximately 1,600 m upstream from the confluence of the East Drainage Ditch with Halls Brook. Because of this, the location of the sampling points refer to distances either upstream from the culvert entrance or downstream from the culvert end.

2.2.2.-DETERMINATION OF TOLUENE LEVELS IN THE STREAM WATER

In order to determine toluene concentrations in the waters of the East Drainage Ditch, water samples were collected at 100, 50 and 1 m upstream, and at 5, 50 and 400 m downstream from the culvert. Duplicate 25 ml samples were collected in 40 ml EPA vials provided with hollow screw caps and Teflon coated silicone septa. Mercuric chloride was added to the samples to a final concentration of 15 mg/l. Aqueous solutions of toluene were prepared as standards in concentrations of 0, 0.22, 0.54, 1.08, and 5.43 μM (0, 20, 50, 100, and 500 ppb). The appropriate amount of toluene was added to each standard in one milliliter of methanol. One milliliter of methanol was added to the samples so all condition were the same. All of the samples, as well as the standards, were shaken for two hours to achieve equilibrium. One ml sample was removed from the head space of both the samples and the standards, and toluene was quantitated using a Carlo Erba HRGC 5300 Mega Series Gas Chromatograph (Fison Instruments, Valencia California), equipped with a Restek RTX-5 capillary column and a flame ionization detector. Oven temperature was held constant at 40°C for two minutes, and then raised to 120°C, with an increase rate of 10°C/min.

2.2.3.-BIODEGRADATION RATE DETERMINATIONS

Batch reactors were set up to determine degradation rates. Toluene loss from the reactors was assayed by gas chromatography, using the same instrument described above. Our samples consisted of rocks collected from the bottom of the East Drainage Ditch, the sampling sites were 100 and 50 meters upstream, and 5 and 50 meters downstream from the culvert. The samples were kept at 4°C and used within twenty four hours of collection. Care was taken while handling the samples to avoid excessive damage to the biomass attached to the rocks.

These experiments were performed using 500 ml round bottom flasks fitted with glass stopcocks. All assays were run in duplicate. 250 ml of stream water were placed in each flask. The flasks were then shaken for one hour to achieve equilibrium, and the original amount of toluene in the water was determined. Toluene was then added to a final concentration between 2.17-2.71 μM (200-250 ppb). The water was allowed to equilibrate again, and then 80 g (± 2.8 g) of rock were placed in each flask. The initial toluene concentration was determined at this point. The flasks were shaken at 22°C for 8.5 hours. Every 1.5 hours a 1 ml headspace sample was withdrawn from each flask in a gas tight syringe and injected into the gas chromatograph for analysis. Controls killed with mercuric chloride were also run in the same manner, to account for abiotic toluene losses.

The data points obtained from the GC analysis were plotted against time, and the equation for the best fit line was calculated using the least squares method. The degradation rate is equal to the slope of this line. The data was adjusted using the weight of the rocks instead of their surface area primarily because of the characteristics of the streambed. The sediments in the East Drainage Ditch become rapidly anaerobic with

depth so that only a portion of any one rock would be supporting an aerobic community at the time of collection. All of the rocks used for the experiment were very similar in size and shape.

2.2.4.-ENUMERATION OF EPILITHIC MICROORGANISMS. TOTAL DIRECT COUNTS

Rock samples were taken from 100 and 50 m upstream, and 5 and 50 m downstream from the culvert. Biomass was scraped from the rocks using an autoclaved small spatula. 0.2 g of the biomass from each site were suspended in 10 ml of Minimal Salts (MS) dilution medium (MS medium contained, per liter of distilled water, 10.55 g Na_2HPO_4 ; 3.50 g KH_2PO_4 ; 1.50 g NH_4Cl ; 0.50 g MgSO_4 ; 0.05 g CaCl_2 ; and, 5.00 g NaNO_3 . One ml of a trace elements solution was added after sterilization.), and shaken for about five minutes to ensure proper separation (Hobbie et al., 1977). The samples were then fixed with a glutaraldehyde-cacodylate solution (final concentration 2% glutaraldehyde, and 0.1 M cacodylate). Successive dilutions in glutaraldehyde were performed as needed.

One ml subsamples were then stained with 10 μ l of 10 mg/ml DAPI solution (4',6-diamidino-2-phenylindole) for 10 minutes. The 1 ml samples were then filtered through a 0.2 μ m pore size, black polycarbonate membrane filter (Millipore Co.). Bacterial cells retained on the filter were then counted under an epifluorescence microscope. At least 20 grids and 300 cells were counted. All samples were done in duplicate. Cells that were observed to be attached to a particle were counted twice, assuming that the same number would be attached to the particle on the underside.

2.2.5.-TOTAL HETEROTROPHIC BACTERIAL COUNTS

Rocks samples were collected from the same sites and under the same conditions as described above for the biodegradation rates determination. Biomass was scraped from the rock surface. 0.2 grams of biomass from each site were then suspended in ten milliliters of Minimal Salts medium, and shaken for at least five minutes on a wrist-action shaker. The resulting cell suspension was then successively diluted to obtain 10^{-2} to 10^{-7} dilutions. These dilutions (0.10 ml) were spread, using a glass rod, onto petri dishes containing 35 mls. of 1% PTYG agar (1% PTYG agar contained, per liter of distilled water, 0.05 g peptone; 0.05 g tryptose; 0.1 g yeast extract; 0.1 g glucose; 0.6 g $MgSO_4$; 0.07 g $CaCl_2$; and, 15 g of agar.). The plates were incubated in the dark, at 20°C, for three weeks. Colonies were counted every three days, until no new colonies were observed.

2.2.6.-PLATE COUNTS OF TOLUENE UTILIZING BACTERIA

Epilithic bacterial samples were collected as described above. Dilutions were prepared , and petri dishes containing 35 ml of Minimal Salts agar (MS medium plus 15 g of agar per liter of solution) were inoculated. After inoculation the plates were placed in glass dessicators. A beaker containing toluene saturated water was also located inside the dessicators. The amount of toluene saturated water within the container was adjusted so that the final toluene concentration in the petri dishes, after equilibrium, was 0.11 mM (10 ppm).

The plates were incubated in the dark for three weeks, at 20°C. To maintain the toluene concentration constant, the containers were opened and the toluene saturated water changed every third day. Colonies were also routinely counted, until no new colonies could be detected. Control MS agar plates were inoculated and incubated under

the same conditions described above but without any toluene. The difference between the toluene plates counts and the control counts would give an initial estimation of the number of toluene degrading bacteria. At the end of the three weeks incubation period the colonies growing on the highest dilution (ie. 10^{-5} , 10^{-6} , 10^{-7}) toluene plates were isolated and transferred to new plates for a second incubation period. These isolates were again grown with and without toluene. Those isolates that grew only on toluene, and not (or not to a significant extent) on the controls, were then counted as toluene degrading bacteria.

2.2.7.-ESTIMATION OF THE MOST PROBABLE NUMBER OF TOLUENE MINERALIZING BACTERIA

For the determination of the most probable number of toluene degrading bacteria an experiment was set up to quantitate bacteria capable of converting ^{14}C labeled toluene (u-ring label, specific activity 9.7 mCi/mmol; Sigma Chemical Co.) to $^{14}\text{CO}_2$ in dilutions of epilithic bacteria. The technique used is an adaptation of that presented by Lehmicke et al., (1979). Rock samples were collected and dilutions prepared as explained above. One ml of MS medium was placed in a 1/2 dram glass vial, and was inoculated with 100 μl of the corresponding dilution. Six dilutions were made (10^{-2} to 10^{-7}), and five vials were inoculated per dilution. The inoculated vial was then placed inside an inverted 40 ml serum bottle, which was then capped with a Teflon coated serum stopper, and sealed. Keeping the serum bottle in this upside down position, one milliliter of 0.5 M NaOH, for trapping evolved $^{14}\text{CO}_2$, was injected to the bottle. Toluene saturated water had been added to the sodium hydroxide in such an amount as to obtain a final concentration in the inner vial's medium of 1.08 μM (100 ppb). Approximately 0.01 μCi (ca. 10,000 counts per minute), as radiolabeled toluene, were placed in each bottled. This set up allowed us

to work with the volatile substrate without need for a carrier solvent, and without losses of the substrate either by volatilization or by adsorption to the vial walls.

After one month of incubation (in the dark, at 20°C), the bottles were opened and the inner vials were taken out. The bottles were left open under a ventilation hood to allow any labeled toluene that had not been mineralized to escape. The next day 10 ml of scintillation cocktail were added to each serum bottle, mixed well with the sodium hydroxide, and then transferred to a scintillation vial. The amount of $^{14}\text{CO}_2$ that had been trapped in the NaOH was then determined by liquid scintillation counting. Each bottle was counted for ten minutes and all were counted twice.

Positive controls that were inoculated with a loop full of a pure culture of toluene degrading organisms were also run. Negative controls consisting of uninoculated vials were also prepared. Those vials evolving at least twice as many counts as the negative controls were scored as positive (or ca. 20% of the counts originally added). This activity level was considerably larger than background levels. $^{14}\text{CO}_2$ trapping efficiency was determined to be 100% using $\text{NaH}^{14}\text{CO}_3$. MPN values were obtained from the tables in Greenberg et al. (Eds.), (1993).

2.3.-RESULTS

2.3.1.-TOLUENE CONCENTRATION IN THE EAST DRAINAGE DITCH

Samples for the determination of the toluene concentration in the stream's water were collected at the beginning and at the end of the summer. Although flow rates were not measured at the time of collection, it was observed that the water level gets considerably lower as summer advances. It is also worth noting that water levels are

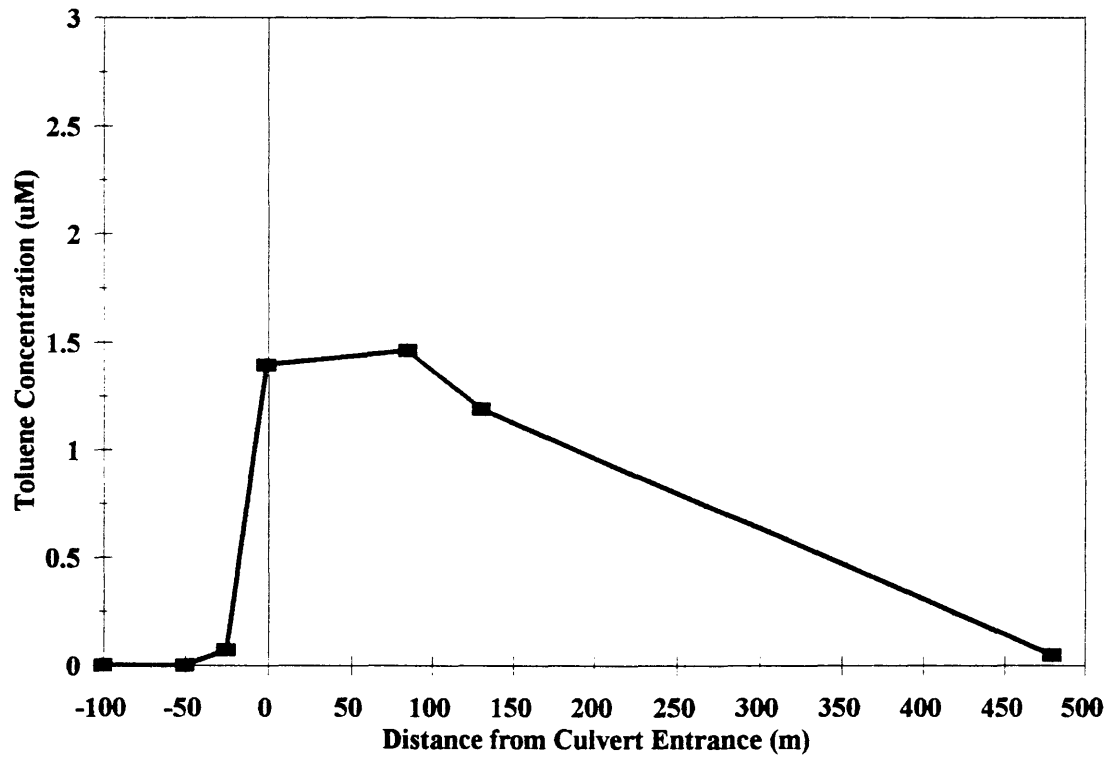


Figure 2.2.- Toluene concentrations in the water of the East Drainage Ditch. Data from samples collected on June 25, 1993.

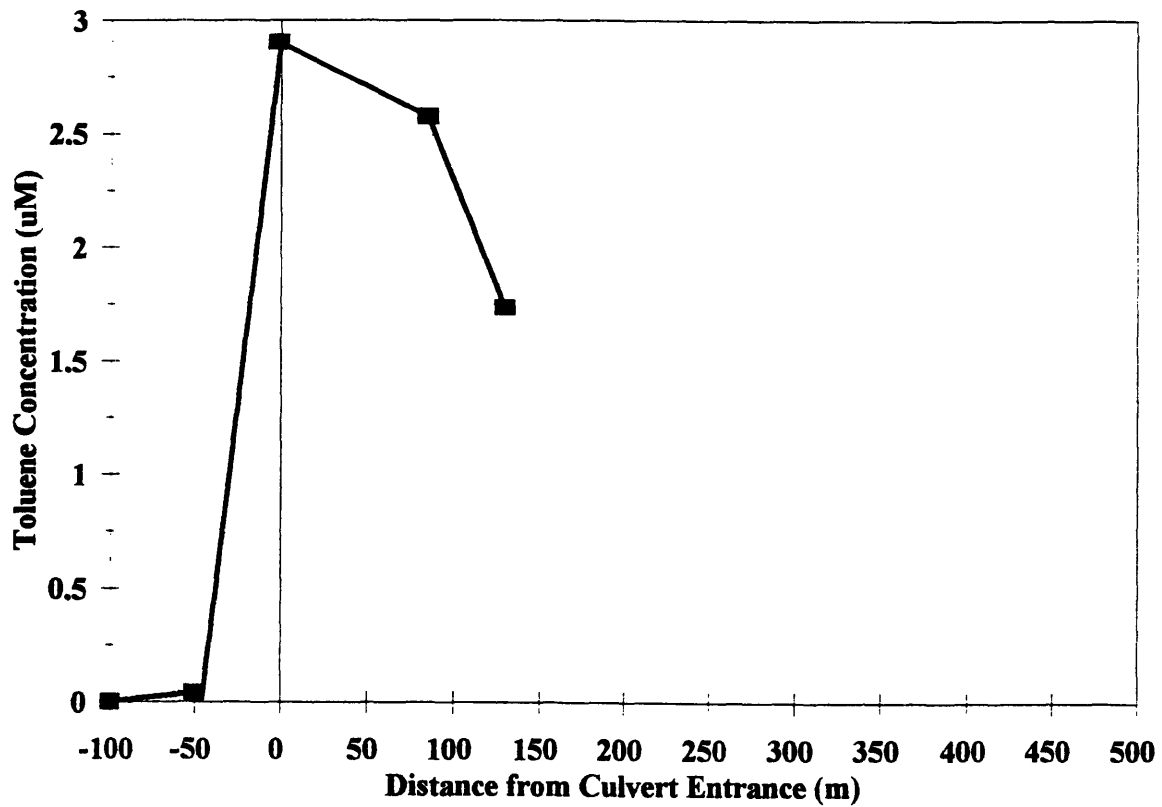


Figure 2.3.- Toluene concentrations in the water of the East Drainage Ditch. Data from samples collected September 19, 1993.

much lower, and flow rates are much smaller, upstream from the point where toluene first appears in the stream.

Results obtained from water samples taken on June 25, 1993 (Figure 2.2, also see Table A.1, Appendix 1) showed that no toluene was present at the sampling points located 100 m and 50 m upstream from the toluene source. On September 19, 1993 only five points were sampled. Again, 100 m upstream from the source no toluene was found; but the sample from 50 m upstream showed a concentration of $0.041 \pm 0.022 \mu\text{M}$ (3.75 ± 2.07 ppb) (Figure 2.3, also see Appendix A, Table A.1). These results indicate that bacterial communities from points 100 m upstream from the culvert are not likely to be exposed to any toluene, whereas communities from 50 m upstream are sometimes exposed to very low concentrations of toluene. On the other hand, bacterial assemblages from points downstream from the culvert are regularly exposed to toluene, and they may have adapted to degrade the contaminant.

2.3.2.-BIODEGRADATION RATE DETERMINATIONS

For the toluene degradation rate determinations, rocks samples were collected from points 100 and 50 m upstream from source, and points located 5 and 50 m downstream from the culvert. Our intent was to see if any correlation could be established between the sampling points aqueous toluene levels and the ability of the local epilithic bacterial community to degrade toluene. Rock samples were collected from the points located 50 m upstream and 50 m downstream from the source, on August 10 and on August 30, 1993. From 100 m upstream and 5 m downstream samples were collected on August 30 and on September 19, 1993. The average biodegradation rate for each site is indicated in Table A.2, Appendix A. The individual results for each particular sampling

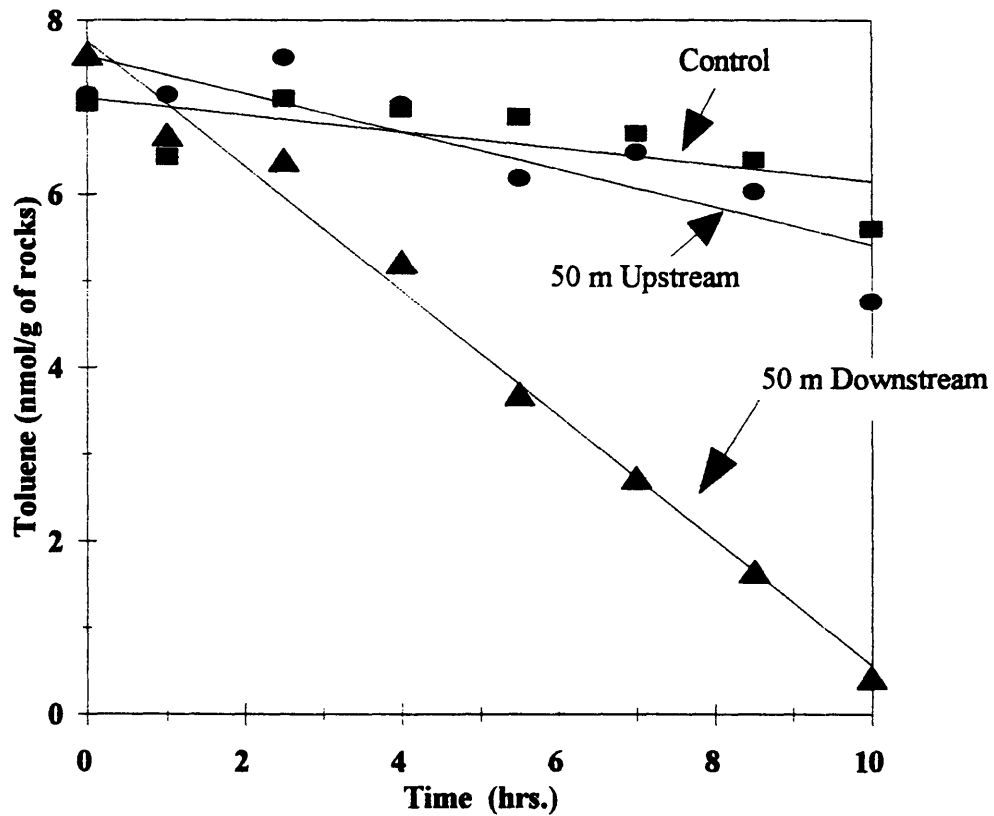


Figure 2.4.- Toluene loss vs. time plot for the determination of biodegradation rates. Data from samples collected August 10, 1993. For the control the best fit line equation is $Y = -0.096 X + 7.1$, $r^2 = 0.47$. For samples collected from 50 m upstream from the culvert's entrance (50 m "upstream from the source") the equation is $Y = -0.22 X + 7.6$, $r^2 = 0.76$. And, for samples collected from 50 m downstream from the culvert's end (50 m "downstream from the source") the equation is $Y = -0.72 X + 7.7$, $r^2 = 0.99$

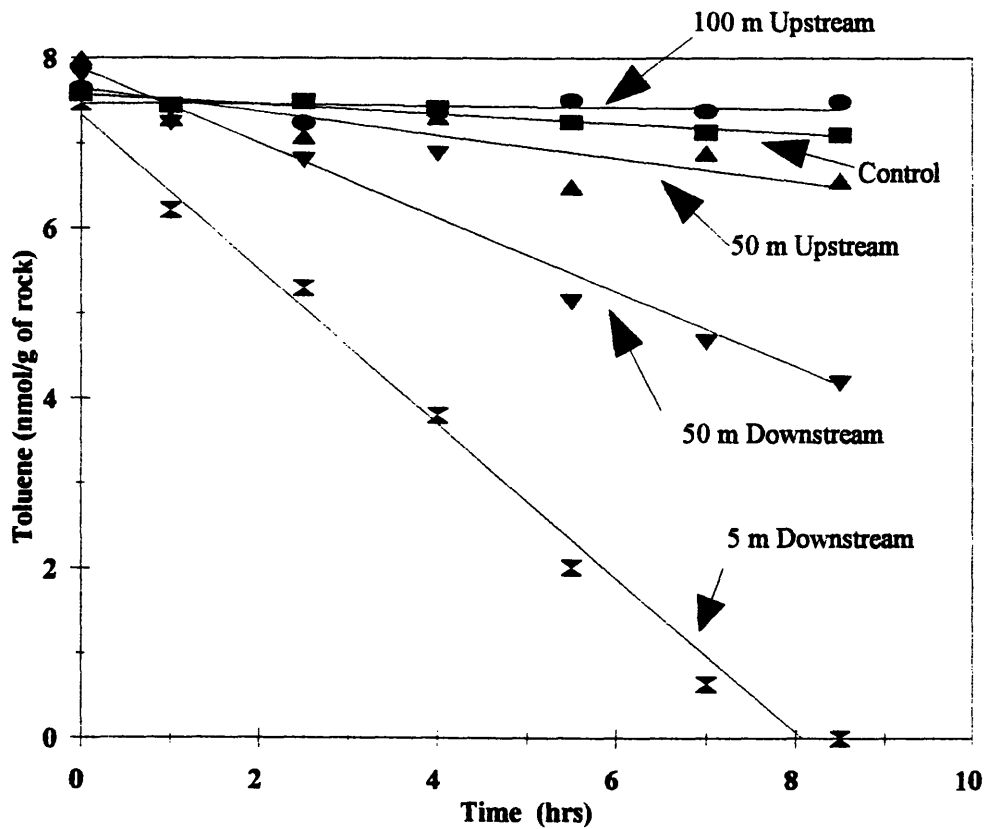


Figure 2.5.- Toluene loss vs. time plot for the determination of biodegradation rates. Data from samples collected on August 30, 1993. For the control the best fit line equation is $Y = -0.060 X + 7.6$, $r^2=0.92$. For samples collected from the point 100 m upstream from the culvert entrance, the equation is $Y = -0.017 X + 7.6$, $r^2= 0.72$. For samples collected 50 m upstream from the culvert's entrance, the equation is $Y = -0.138 X + 7.6$, $r^2= 0.70$. For samples collected 5 m downstream from the culvert's end, the equation is $Y = -0.909 X + 7.4$, $r^2= 0.99$. And, for samples collected from 50 m downstream from the culvert's end, the equation is $Y= -0.437 X + 7.6$, $r^2= 0.94$.

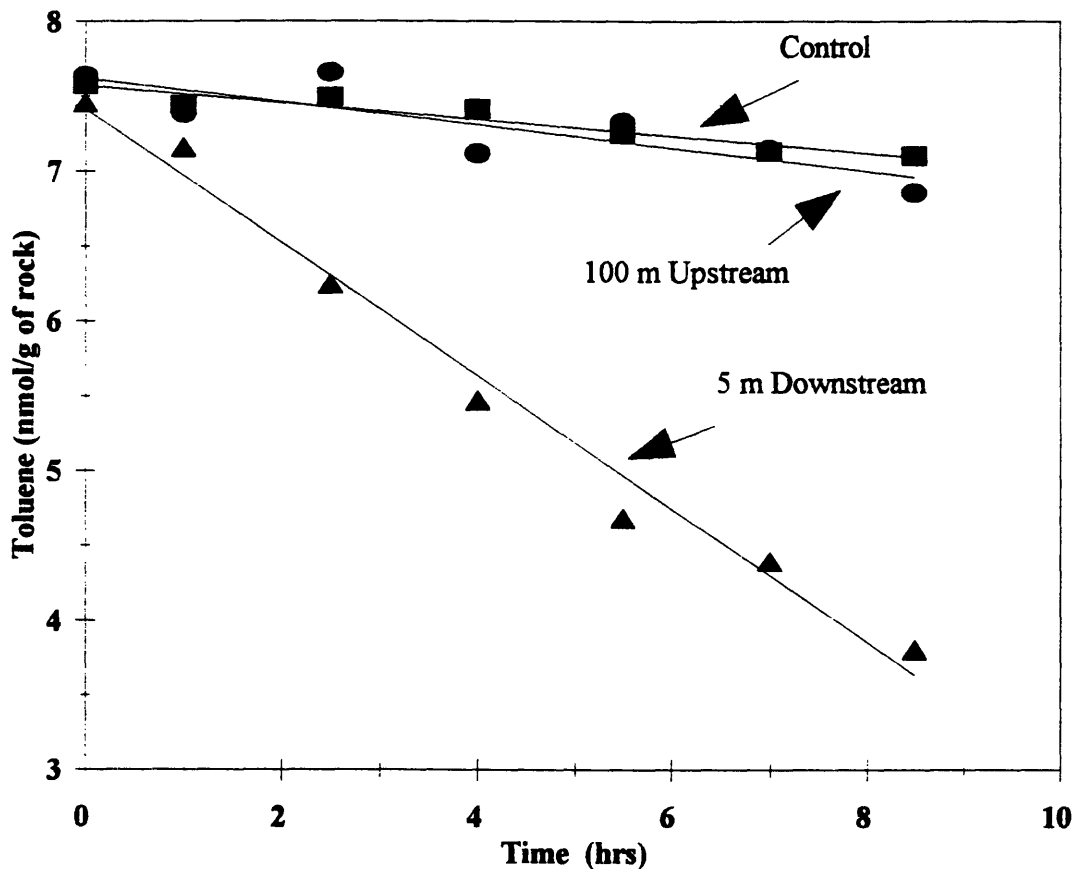


Figure 2.6.- Toluene loss vs. time plot for the determination of biodegradation rates. Data from samples collected on September 19, 1993. For the control the best fit line equation is $Y = -0.060 X + 7.5$, $r^2 = 0.92$. For samples collected 100 m upstream from the culvert's entrance, the equation is $Y = -0.078 X + 7.5$, $r^2 = 0.70$. For samples collected 5 m downstream from the culvert's end, the equation is $Y = -0.445 X + 7.4$, $r^2 = 0.98$.

date are plotted in figures 2.4 to 2.6. In these plots, each point in the time series for a given sampling site represents the average toluene concentration for the duplicate bottles at a given moment. The standard deviation between the two measurements that conform each point was never larger than 5% of the corresponding average.

These results indicate that for those samples collected from 100 m upstream from the toluene source, where no toluene was found in the water, average degradation rates are not significantly different from the killed controls (t-test, 90% confidence level), suggesting that toluene losses on those reactors were mainly due to abiotic mechanisms in the eight and a half hours incubation period. Those samples collected 50 m upstream from the culvert, where toluene concentration range from 0 to 6 ppb, the average degradation rate is ca. three times that of the previous site, but is still almost one order of magnitude lower than the average rates for points downstream from the source. Both of the sampling points located downstream from the culvert, 5 and 50 m downstream, had similar average degradation rates. It can also be observed that degradation rates do not seem to change as the toluene concentration in the bottles decreases, indicating rates that are zero order with respect to toluene concentration.

2.3.3.- ENUMERATION OF EPILITHIC MICROORGANISMS. TOTAL DIRECT COUNTS

Rock samples for the determination of total bacteria numbers were collected on September 7, 1993, and September 19, 1993. Figure 2.7 compares the average results and relates them with average toluene concentrations in the water of the East Drainage Ditch for each sampling site (see also Appendix A, Table A.3). These results suggest that

total bacterial numbers, per unit weight of biomass, have not been significantly influenced by the presence of toluene in the water, since results are comparable for all four sites.

2.3.4.- TOTAL HETEROTROPHIC BACTERIAL COUNTS

From the samples collected on September 7 and September 19, numbers of cultivable heterotrophic bacteria were also obtained. The results for each sampling date and the corresponding average are shown in Table A.4, Appendix A. The averages are also graphed in Figure 2.7. As for the total bacterial numbers, the level of toluene contamination in the water does not seem to have a significant influence in the numbers of heterotrophic bacteria per gram of biomass in the epilithic communities.

2.3.5.- PLATE COUNTS OF TOLUENE DEGRADING BACTERIA

Minimal salts plates were inoculated for determination of the number of cultivatable toluene degraders only once, on September 7, 1993. In this experiment we could not detect a toluene degrading population 100 m upstream from the toluene source (See Table A.5, Appendix A). The numbers of toluene degrading bacteria as obtained with this method follow the trend of toluene levels in the EDD water (Figure 2.7). No colonies were isolated from the site with 0 toluene concentration, and the number of colonies increases as the concentration increases.

2.3.6.- MOST PROBABLE NUMBER OF TOLUENE DEGRADING BACTERIA

Also on September 7, the vials for the determination of the most probable number (MPN) of toluene degraders were inoculated. Six dilutions were used (ie. 10^{-2} to 10^{-7}). According with the MPN results (See Appendix A, Table A.5) the toluene degraders make up 0.12 % of the heterotrophic population 100 m upstream from the source and

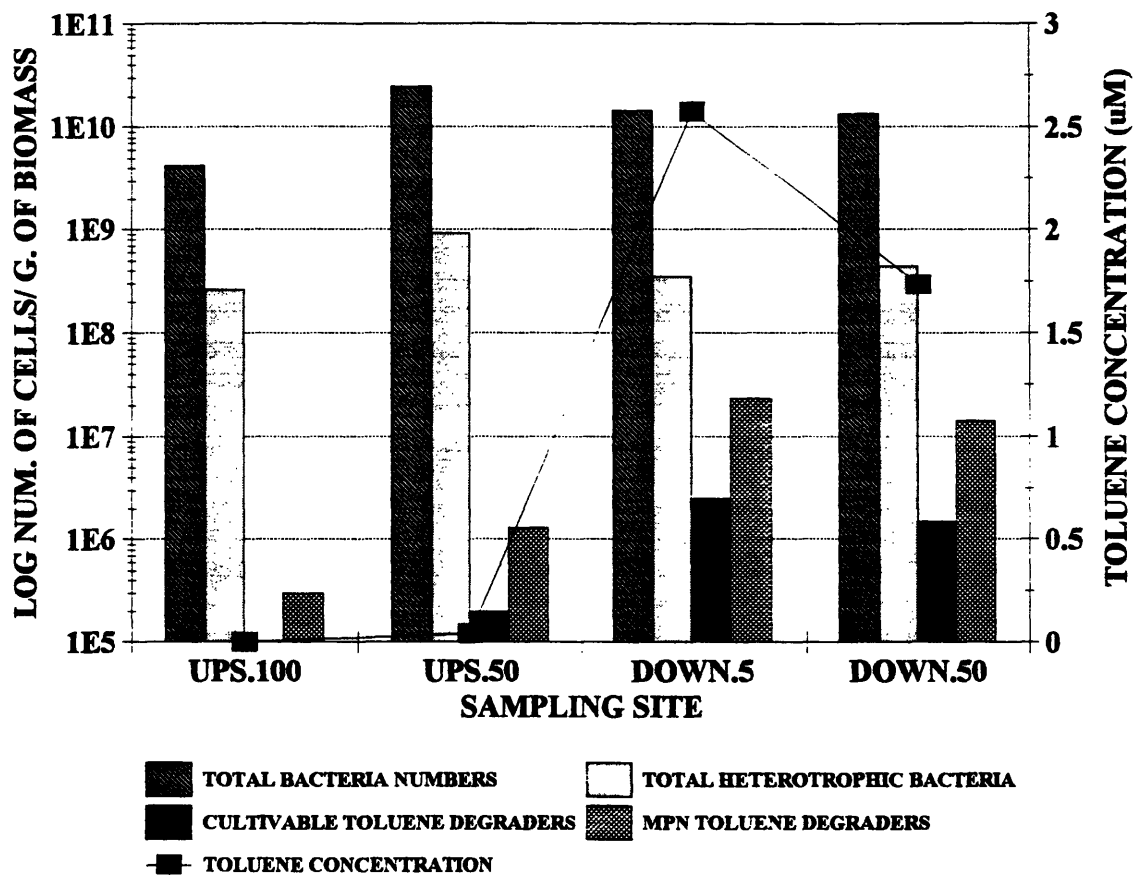


Figure 2.7.- Graphic comparison of average bacterial numbers for each site and the relationship between toluene degrading bacteria numbers and average toluene concentrations at each site.

0.14 % of that 50 m upstream from the source. However, the same experiment shows that toluene degraders are approximately a 7.0 % of the heterotrophic population 5 m downstream from the source, and form a 3.4 % of the heterotrophic population 50 m downstream from the source. This indicates a strong influence of the toluene aqueous concentration on the numbers of toluene degrading bacteria in relation with total heterotrophic bacteria within the communities (Figure 2.7). The same kind of trend is seen in relation to total bacterial numbers.

2.4.- DISCUSSION

Using the information collected through the preceding experiments to assess the overall impact of toluene contamination on the epilithic bacterial community of the East Drainage Ditch, one major conclusion can be drawn: higher toluene concentrations seem to correlate with enhanced ability of the community to degrade the contaminant and with a larger toluene degrading population. On the other hand, toluene levels do not seem to have a significant effect on total bacteria numbers or on the total heterotrophic bacterial numbers as a function of biomass weight. (Figure 2.7)

How the presence of a contaminant affects the total numbers of bacteria and the total heterotrophic population strongly depends on the specific characteristics of the environment in which the communities exist, and on the characteristics of the communities themselves (Pfaender and Buckley, 1984). Studies by Spain et al. (1984), and by Pignatello et al. (1986) have reported viable heterotrophic counts unaffected by the presence of a xenobiotic contaminant (*p*-nitrophenol and pentachlorophenol, respectively). Pignatello et al. (1986) interpret this result as an indication that the contaminant is not toxic to most of the members of the bacterial community at the

concentrations present at their study site. In a related paper no significant increases in total bacterial numbers were observed due to the adaptation of the community to pentachlorophenol (Pignatello et al., 1983).

Increased biodegradation rates where the bacterial communities have had the opportunity to adapt to the presence of a contaminant have been reported in many different studies (Atlas, 1984; Bossert and Bartha, 1984; Leahy and Colwell, 1990; Pignatello et al., 1983; Spain et al., 1980, 1984). In the case of those samples from the contaminated site in the East Drainage Ditch the epilithic communities have probably been exposed to toluene for many years, thus they are likely to be well adapted to the contaminant (Kim et al., 1994). Our results indicate that samples from the contaminated site are capable of degrading toluene approximately ten times faster than samples from the pristine site. In field studies, Spain et al. (1984) reported an increase of two orders of magnitude in the biodegradation rate of *p*-nitrophenol by adapted communities in comparison to previously unexposed ones. Similar results were obtained in laboratory studies of *p*-cresol biodegradation (Van Veld and Spain, 1983); also, a ten fold increase in biodegradation rates was observed for *p*-nitrophenol and for methyl parathion.

Our biodegradation rate results indicate that the reaction is zero order with respect to concentration (the toluene loss vs. time plot is a straight line with a constant slope). This indicates that the process is not limited by the amount of substrate present in the flask. Cohen et al. (1994) reported biodegradation rates for samples from the EDD that were independent of the concentration (only enzyme or biomass dependent) for concentrations above 250 µg/L. In biological processes, reactions only appear to be first order with respect to substrate concentration when the number and activity of the

microorganisms responsible for the degradation remain constant (Van Veld and Spain, 1983). Thus, what we are observing may be the result of growth occurring in the reaction vessels.

Elevated numbers of toluene degrading bacteria in samples from the contaminated sites as compared with those from the uncontaminated sites also points towards the existence of a well adapted community (Pignatello et al., 1983, 1985, 1986; Spain et al., 1980, 1983, 1984). Spain et al.(1980, 1983, 1984) have reported that , on average, the number of organisms capable of degrading the contaminant of interest increases by three orders of magnitude after adaptation. Pignatello et al. (1986) reported that the pentachlorophenol degrading population increased by two orders of magnitude after adaptation with respect to a background population found prior to the addition of the contaminant. The latter studies with *p*-nitrophenol and pentachlorophenol (Spain et al., 1980, 1983, 1984;and Pignatello et al, 1986) were obtained using the MPN determination as was used for this study. Our MPN results indicate a two orders of magnitude increase in the toluene degrading populations from the pristine to the contaminated site. The larger population of toluene degrading bacteria at the site 50 m upstream from the culvert with respect to the pristine site (100 m upstream) may be due to the sporadic presence of small quantities of toluene at this site.

The fact that toluene degrading bacteria can be enumerated from the site 100m upstream from the culvert (the pristine site) suggests that the large numbers of toluene degrading bacteria now present in the contaminated sites grew out of initially small populations of organisms with the innate ability to degrade toluene or similar hydrocarbons, ie. a selective enrichment process took place (Spain et al., 1983). But our

experiments can not be used to categorically describe which was the adaptation mechanism for this communities.

In summary, our results suggest that the epilithic bacterial communities from the toluene contaminated part of the East Drainage Ditch have adapted to its presence by developing a large population of toluene degrading bacteria. This toluene degrading segment of the community forms a considerably larger proportion of the heterotrophic population of the contaminated sites than the same segment does of the corresponding heterotrophic populations in the uncontaminated site. Exposure to toluene has not, however, affected the total bacterial numbers and the heterotrophic bacterial numbers with respect to biomass weight, since all four site gave similar results.

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APPENDIX A: SUMMARY OF EXPERIMENTAL DATA

Table A.1: Aqueous toluene concentrations in the East Drainage Ditch as measured using headspace gas chromatography.

SAMPLING DATES	June 25	September 19
SITE	Concentration (uM)	Concentration (uM)
100 m Upstream	0.00	0.00
50 m Upstream	0.00	0.04(0.022)
25 m Upstream	0.07(0.003)	-
1 m Upstream	1.39(0.041)	2.91(0.022)
5 m Downstream	1.46(0.018)	2.58(0.016)
50 m Downstream	1.19(0.007)	1.74(0.026)
400 m Downstream	0.05(0.003)	-

note: values in parentheses are standard deviations

Table A.2 : Biodegradation of toluene by rocks in the East Drainage Ditch. Average degradation rates.

SITE	RATE (nmol/hr/g of rock)
Control	0.078 (0.025)
100 m Upstream	0.061 (0.023)
50 m Upstream	0.18 (0.06)
5 m Downstream	0.68 (0.37)
50 m Downstream	0.58 (0.20)

note: numbers in parentheses are standard deviations

Table A.3: Enumeration of total epilithic bacteria obtained by the DAPI staining technique

SAMPLING DATE	September 7	September 19	Average
SITE	(cells/g of biomass)	(cells/g of biomass)	(cells/g of biomass)
100 m Upstream	1.68(0.12)e09	6.75(0.19)e09	4.22(3.40)e09
50 m Upstream	2.81(0.06)e10	2.15(0.15)e10	2.48(0.47)e10
5 m Downstream	1.47(0.11)e10	1.40(0.17)e10	1.44(0.14)e10
50 m Downstream	1.64(0.14)e10	1.04(0.10)e10	1.34(0.42)e10

note: numbers in parentheses are standard deviations

Table A.4: Enumeration of Heterotrophic Bacteria from epilithic communities as determined by 1% PTYG plate counts

SAMPLING DATE	September 7	September 19	Average
SITE	(cells/g of biomass)	(cells/g of biomass)	(cells/g of biomass)
100 m Upstream	1.95(0.49)e08	3.25(1.48)e08	2.60(0.92)e08
50 m Upstream	7.65(0.67)e08	1.07(0.07)e09	9.20(2.2)e08
5 m Downstream	2.30(0.28)e08	4.65(0.35)e08	3.48(1.7)e08
50 m Downstream	4.25(1.35)e08	4.50(0.71)e08	4.38(0.18)e08

note: numbers in parentheses are standards deviations

Table A.5: Most Probable Number and MS plate counts of epilithic toluene degrading bacteria in the EDD.

SITE	PLATE COUNTS	MPN	95% Confidence limits (lower;upper)
	(cells/g of biomass)	(cells/g of biomass)	(cells/g of biomass)
100 m Upstream	0.00	3.0 e05	1.0 e05; 1.2 e06
50 m Upstream	2.0(1.41)e05	1.3 e06	0.5 e06; 3.9 e06
5 m Downstream	2.5(0.71)e06	2.3 e07	0.9 e07; 8.6 e07
50 m Downstream	1.5(0.71)e06	1.4 e07	0.6 e07; 3.6 e07

note: numbers in parentheses are standard deviations