BIODEGRADABILITY OF NITROXYLENE ISOMERS

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BIODEGRADABILITY OF NITROXYLENE ISOMERS

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[To Yan Zhang, my dearest mother]

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TABLE OF CONTENTS

9

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

MNX	Mononitroxylene
DNX	Diniotroxylene
MNT	Mononitrotolsuene
DNT	Diniotrotoluene
2,4-DNT	2,4-dinitrotoluene
2,6-DNT	2,6-dinitrotoluene
TNT	2,4,6-trinitrotoluene
BTEX	Benzene, toluene, ethylbenzene and xylene
PTA	Terephthalic acid
2-NT	2-nitrotoluene
4-NT	4-nitrotoluene
3M4NC	3-methyl-4-nitrocatechol
HNOHA	2 -hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid
HPLC	High Performance Liquid Chromatography
GC	Gas Chromatography

SUMMARY

Nitroaromatic compounds are contaminants commonly present in soil and water systems that can have impacts on environment as well as human health. Within the nitroaromatic series of contaminants, the biodegradability of nitroxylene isomers has not been studied. Presently, there is no literature related to the biodegradability of mononitroxylene (MNX) and dinitroxylene (DNX) isomers. Thus, this thesis is primarily focused on the biodegradability and cometabolism of nitro-xylene isomers, in order to provide insights on the potential strategy for bioremediation of DNX.

Microcosm studies were conducted beginning with three xylene isomers: *ortho*-xylene, *meta*-xylene and *para*-xylene; and continued with the four mononitroxylene (MNX) isomers, culminating with testing ten dinitroxylene (DNX) isomers. Soil samples were obtained from a historically contaminated site with high levels of dinitrotoluene (DNT), trinitrotoluene (TNT) and dinitroxylene (DNX) and used as the inoculum for microcosm tests. The microcosm method of different isomers was based on the previous work on biodegradation of nitrotoluene. As it was demonstrated previously that 2,4-DNT degrading bacteria were present at the site, it was hypothesized that these may be capable of transforming or cometabolizing some of DNX isomers. Thus, DNX cometabolism studies were conducted in the presence of 2,4-DNT degrading bacteria. The presence of xylene and 2,4-DNT degraders was confirmed in this thesis. Meanwhile, several MNX and DNX isomers showed degradability in microcosm studies. Cometabolism studies showed that four DNX isomers could be cometabolized by 2,4-DNT enrichment.

CHAPTER 1

INTRODUCTION

Isomers of dinitroxylene (DNX), as by-products in 2,4,6-trinitroluene (TNT) manufacture, can be detected at TNT manufacturing sites. Currently no literature can be found regarding DNX biodegradation. And only a small amount of information exists related to their physical properties. However, as the chemical structure and properties of DNX are similar to those of DNTs, it is reasonable to assume that DNX may represent threat to human health and environment. DNTs are known to be biodegradable and there is interest in biodegradability of DNX isomers to assess the potential for bioremediation at contaminated sites. Considerable efforts of nitrotoluene series biodegradation study, including toluene, mononitrotoluene (MNT) and dinitrotoluene (DNT) isomers, have been performed to illustrate the metabolic pathway of each isomer[26]. Similarly, the purpose of this thesis is to investigate the biodegradability of nitroxylene series, including xylenes, mononitroxylene (MNX) and dinitroxylene (DNX) isomers.

This research is based on the previous bioremediation work at a historically contaminated site in the United States. During the operation of production plants, nitration processes were carried out with aromatic feedstocks other than toluene and nitration products from xylene isomers can be found in a number of locations. Concentrations of 10 dinitroxylene (DNX) isomers have been monitored at one contaminated site in past 4 years. The recent analysis showed that the amount of total DNX was approximately 0.4 g/kg in areas undergoing remediation. Xylene and mononitroxylene (MNX) isomers contamination has not been detected in the soil.

It is hypothesized that intrinsic microorganisms are able to metabolize less-nitrated products such as xylene and MNX under field conditions. If there are such bacteria present in the

site, there may be a potential that these bacteria have evolved to transform DNX isomers. To test the hypothesis, microcosm experiments were set up to confirm the presence of xylene, MNX and DNX degrading or transforming bacteria in the soil. In addition, tests were conducted to evaluate the potential for DNT degraders to metabolize DNX compounds, as soil also contains DNT contaminants.

In this thesis, research focused on nitroxylene degradation is presented with the following objectives.

1) Test the presence of xylene degradation in the soils.
2) Evaluate biodegradation of nitrated xylenes using methods developed for DNT degradation.
3) Establish DNT degrading enrichment from soils and evaluate the potential of DNT degraders to metabolize DNXs.

CHAPTER 2

LITERATURE REVIEW

2.1 Biodegradation Pathway of Xylene Isomers

The series of xylene isomers is a member of the BTEX (benzene, toluene, ethylbenzene and xylenes) class of aromatic compounds, which are commonly distributed in soil and groundwater system as toxic contaminants [1]. Among those, *ortho*-xylene isomers are mainly used in the manufacture of phthalic anhydride, solvent applications and to produce bactericides, soybean herbicides and lubricating oils [2]. One of the main usages of petroleum recovered *para*-xylene is the manufacture of terephthalic acid (PTA), which is used in the production of polyester fiber and p-xylene is used as solvent of herbicides [3]. *meta*-Xylene can be treated as a solvent and an intermediate during synthesis [4]. All three xylene isomers enter the atmosphere easily by volatilization and can be found dissolved in groundwater systems. A number of studies have shown that dissolved xylene isomers can be biodegraded in groundwater[5-11].

Different bacterial strains that can grow on three xylene isomers as sole source of carbon were isolated, identified by 16S rDNA and thoroughly studied concerning their metabolic biodegradation pathways, enzymes and genes. A summary of these strains is presented in **Figure 1**.

Table 1 Identification of Isolated Xylene Degrading Strains

Strain	Identification	Isomers	References
DK17	*Rhodococcus sp.*	o-xylene	5,6
OX1	*Pseudomonas stutzeri*	o-xylene	7,8
B3	Rhodococcus sp.	o-xylene	9
YU6	Rhodococcus sp	o-xylene and p-xylene	10
JMP134	*Ralstonia eutropha*	p-xylene and m-xylene	11

It is shown in **Figure 1** that there are mainly three bacterial classes that can degrade xylenes: those that can degrade both m-xylene and p-xylene, those that can degrade both o-xylene and p-xylene and those that can degrade o-xylene only. The position of methyl group in xylene isomers highly influences the selection of xylene degrading bacteria. In o-xylene and p-xylene degradation pathways, there are two ways of ring cleavage [9, 10, 11]; however, there is only one degradation pathway for m-xylene [11]. On one hand, o-xylene or p-xylene can be degraded through two dioxygenation processes, in which o-xylene and p-xylene transformed to dimehtylcatechol group and the aromatic ring is cleaved by catechol oxidation [10]. On the other hand, all the three xylene isomers can be degraded through the pathway of methylbenzyl alcohol, methylbenzaldehyde, and methylbenzoate, which falls into the toluate degradation by toluate dioxygenase.

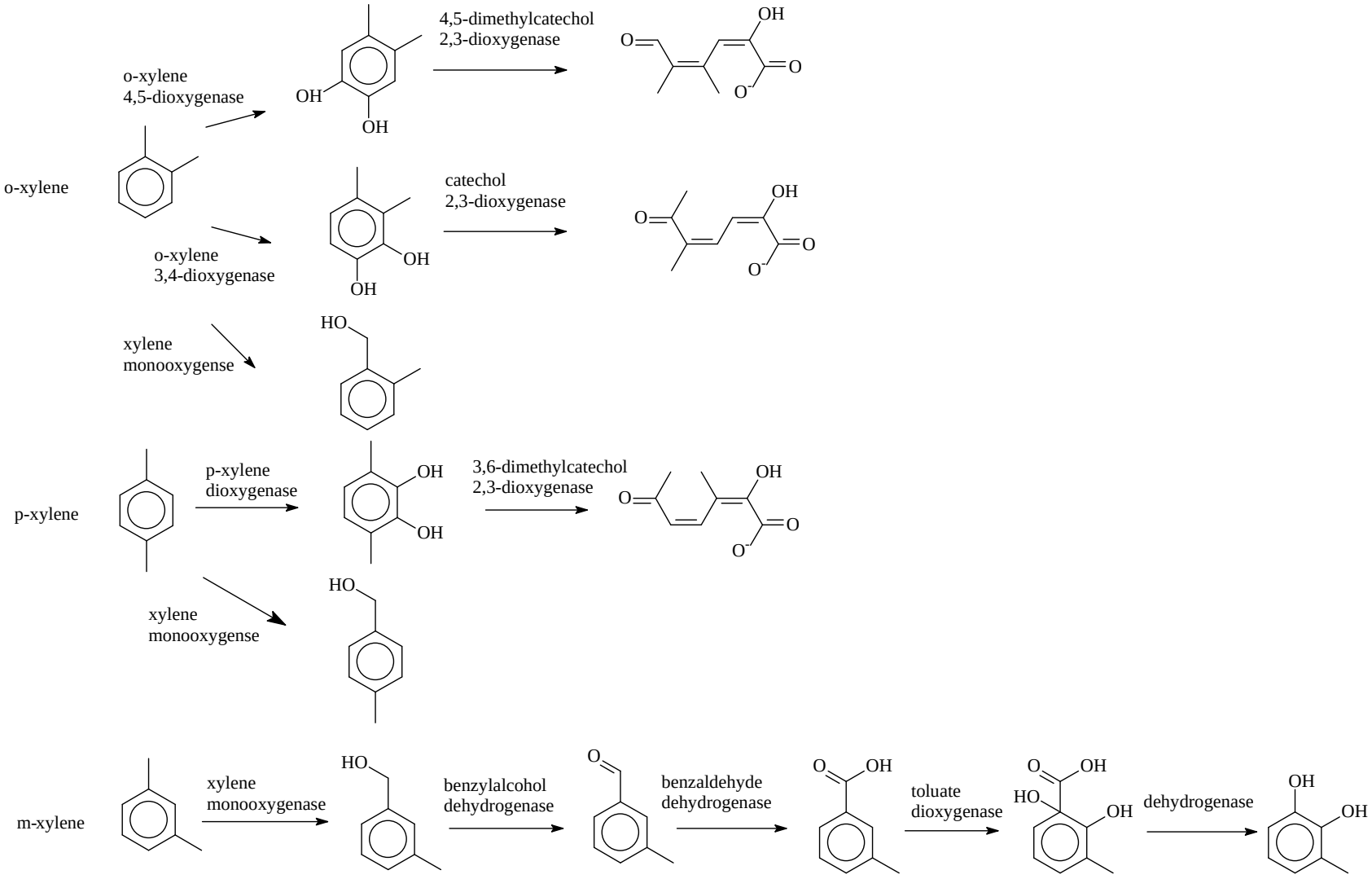
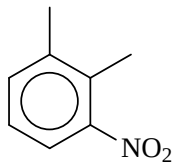


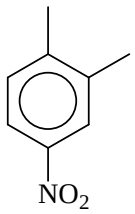
Figure 1 Metabolic Pathway of o-Xylene, p-Xylene and m-Xylene Biodegradation [5-11]

2.2 Physical Properties of Xylene, Mononitroxylene and Dinitroxylene Isomers

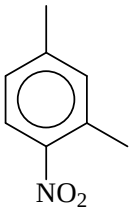
Since untill now no literature can be found related to mononitroxylene and dinitroxylene degradation and toxicity, physical properties of xylene and nitrated xylene are the focus of this section to better support the experiments. Chemical structures of nitrated xylene isomers used in this thesis are shown in **Figure 2**.



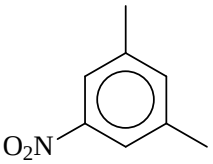
3-nitro-o-xylene



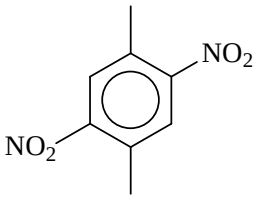
4-nitro-o-xylene



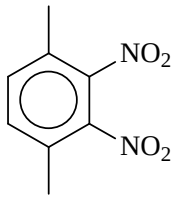
4-nitro-m-xylene



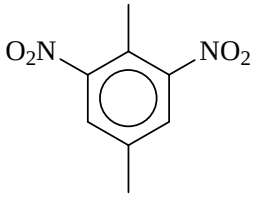
5-nitro-m-xylene



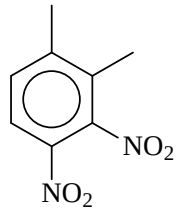
2,5-dinitro-p-xylene



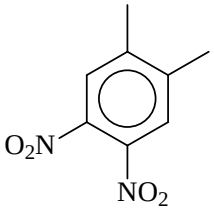
2,3-dinitro-p-xylene



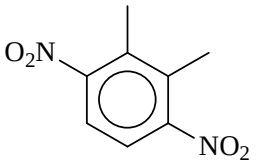
2,6-dinitro-p-xylene



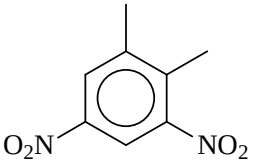
3,4-dinitro-o-xylene



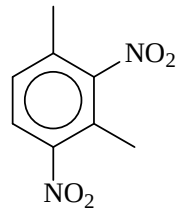
4,5-dinitro-o-xylene



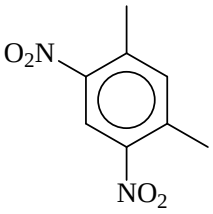
3,6-dinitro-o-xylene



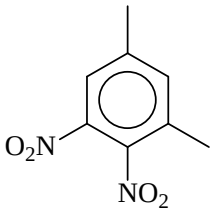
3,5-dinitro-o-xylene



2,4-dinitro-m-xylene



4,6-dinitro-m-xylene



4,5-dinitro-m-xylene

Figure 2 Chemical Structures of Nitrated Xylene Isomers

Table 2 Vapor Pressure of Xylene and Mononitroxylene Isomers Campared to Naphathalene

Name	Vapor Pressure (mmHg)	Temperature (℃)
Naphthalene	0.082	25
p-xylene	10	27
3-nitro-o-xylene	0.0503	25
5-nitro-m-xylene	0.00913	25
4-nitro-o-xylene	0.0163	25
4-nitro-m-xylene	1.000	66
Naphthalene	0.660 (from calculation)	66

From what has been shown in **Table 2**, the vapor pressure of p-xylene as a representative of all three xylene isomers is much higher than that of naphthalene at room temperature, meaning that it is a volatile organic compound as are o-xylene and m-xylene. As a result, xylene degrading systems should be sealed in room temperature, and xylene isomers are better determined by Gas Chromatography (GC) [12]. However, the vapor pressure of four mononitroxylene isomers is lower than that of naphthalene at the same temperature, meaning that they are semi-volatile organic compound. Consequently, mononitroxylene systems should be sealed in room temperature and High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) can both be used to measure their concentration in liquid samples. HPLC was selected in the experiments in order to avoid during liquid-liquid extraction [13]. But the samples should be measured immediately after taking from the systems to avoid volatilization.

For dinitroxylene isomers, neither vapor pressure nor aqueous solubility can be found in the literature. However, it can be predicted from their chemical structure that they are not volatile organic compounds and their solubility should be low (estimated 0.21mg/L) at room temperature [14]. Thus, HPLC rather than GC should be used to analyze their concentration.

2.3 Biodegradation of Related Nitrtoluenes Isomers

Nitrotoluenes constitute an important class of nitroaromatic compounds that is widely used as industrial chemicals for dyes, polymers, pesticides and explosives [15]. The majority of these compounds are introduced by human activities and commonly present in soil and groundwater system. However, certain bacteria that can use these chemicals as carbon and nitrogen source exist under certain circumstances in nature.

2-nitrotoluene (2-NT), 4-nitrotoluene, 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) are by-products in the manufacture of TNT. The process of nitro group removal and aromatic ring cleavage has been studied by others [18, 20, 25, 26]. However, certain compounds with amino group formed during the degradation reaction have higher toxicity, which can be neglected in studies [16, 17].

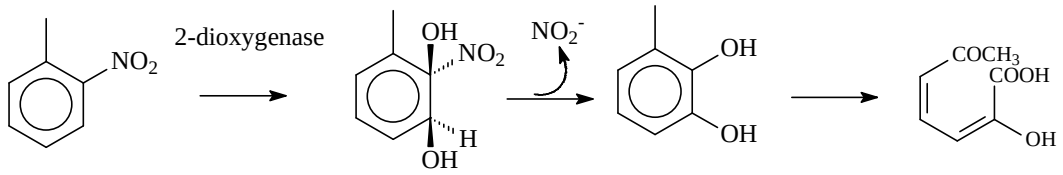
2.3.1 Biodegradation of Nitrotoluenes

Biodegradation pathway of 2-NT by *Pseudomonas* strain JS42 involves dioxygenation via 3-methylcatechol with nitrite release causing ring cleavage[18, 20] (Figure 3). The biodegradation of 2-NT begins with dioxygenation followed by the removal of nitrite, which is similar to the process of 2,4-DNT dioxygenation.

Two different pathways of 4-NT biodegradation exist in different 4-NT degrading strains. 4-NT can be degraded by *Pseudomonas* strains TW3 via oxidation process. 4-NT is first converted to more oxidized aromatic compounds, which can be degraded to protocatechuate with

ammonia removal. Specific genes are responsible for ring cleavage of protocatechuate[19]. Additionally, 4-NT can be degraded through the formation of 4-hydroxylaminotoluene, which is transformed to 6-amino-m-cresol before *meta*-ring cleavage (Figure 3) [20, 21]. It is possible for 4-NT to form 5-methylpicolinic acid, which cannot be fully mineralized by bacteria.

2-Nitrotoluene Degradation Pathway



4-Nitrotoluene Degradation Pathway

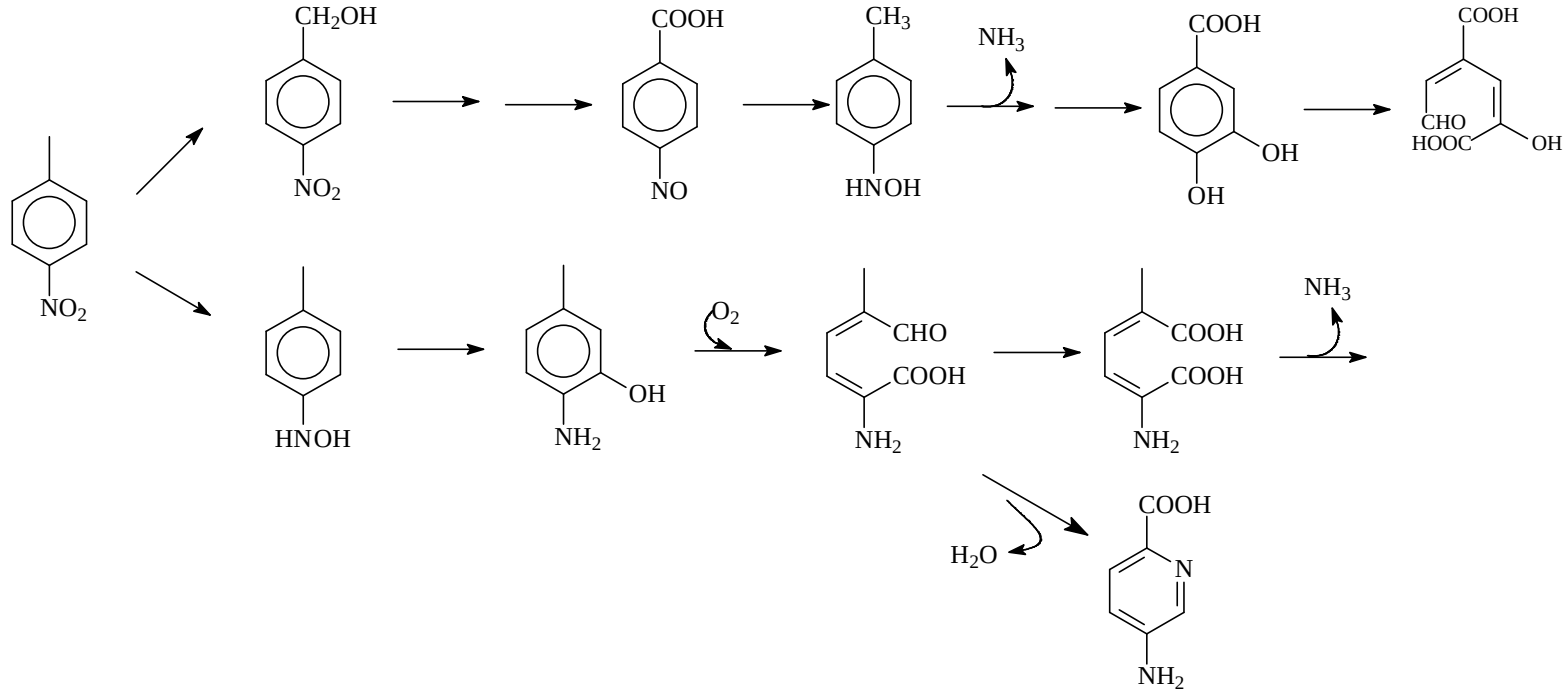


Figure 3 Metabolic Degradation Pathway of NTs [18, 20, 21]

2.3.2 Aerobic Biodegradation of Dinitrotoluenes

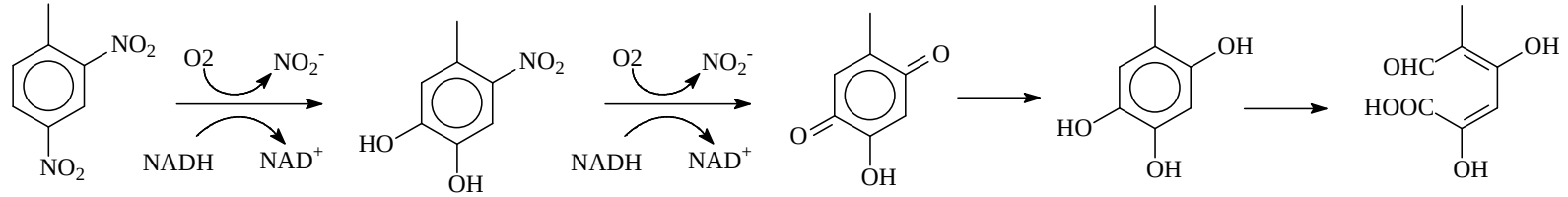
Aerobic biodegradation of 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) has been reported in the literature [22-28], with both serving as sole source of carbon and nitrogen. Figure 4 shows the degradation pathway of 2,4-DNT and 2,6-DNT in those strains which were isolated and identified from soil and water.

One pathway of 2,4-DNT mineralization found in a *Psuedomonas* strain involves dioxygenation and monoxygenation of 2,4-DNT [22, 23], after which 2,4-DNT is transformed to 2,4,5-trihydroxytoluene followed by ring cleavage. In the end, these products can be fully mineralized to carbon dioxide through Krebs cycle [24].

In the degradation of 2,6-DNT, the initial steps of dioxygenation are similar with that of 2,4-DNT degradation, in which 2,6-DNT is transformed to 3-methyl-4-nitrocatechol (3M4NC) with nitrite removal[25, 26]. 3M4NC is then converted to 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid (HNOHA) as ring cleavage proceeds. The reactions that remove the remaining nitro group after the formation of 2-hydroxy-5-nitropenta-2,4-dienoic acid (HNPA) have not been fully demonstrated[27].

In addition, the presence of high concentration of 2,4-DNT or 2,6-DNT does not influence 2,4-DNT degradation[27]. However, the presence of 2,4-DNT can affect the activity of 2,6-DNT degrading bacteria[28]. As a result, even though 2,6-DNT is biodegradable in the environment, it still is difficult to be removed from the site because of 2,4-DNT presence.

2,4-DNT Metabolic Degradation Pathway



2,6-DNT Metoblic Degradation Pathway

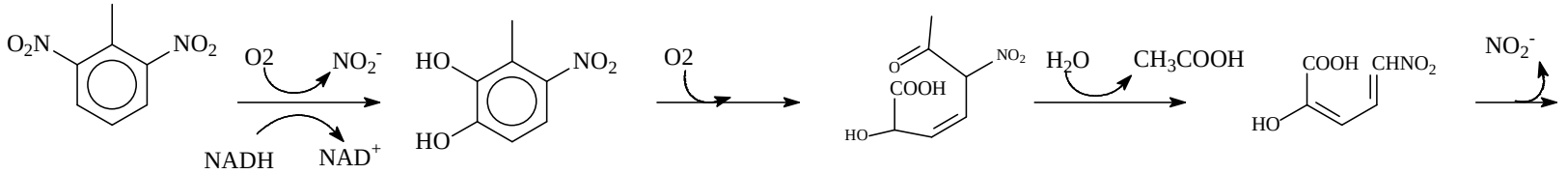


Figure 4 Degradation Pathway of 2,4-DNT and 2,6-DNT[22-28]

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

2,4-DNT (97%) was obtained from Sigma-Aldrich. 10 DNX isomers (Southwest Research Institute), 4 MNX isomers (reagent grade, Fisher Scientific, Acros) and 3 xylene isomers (HPLC grade, 99.9%, Fisher Scientific) were used for the microcosm study. The reagent grade chemicals were used for mineral media constituents as follows: Na_2HPO_4, KH_2PO_4, $CaCL_3 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, H_3BO_3, $CaSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $Na_2MoO_2 \cdot 6H_2O$ (reagent grade, Fisher Scientific, Acros). NaOH (1N) was used to adjust pH (8 ~ 8.5) of the mineral media as required. Acetonitrile (HPLC grade, 99.9%, Fisher Scientific) and reagent grade trifluoroacetic anhydride (Fisher Scientific) were used for HPLC analysis. Methylene Chloride (HPLC grade, 99.9%, Fisher Scientific) was used for GC-MS analysis.

3.2 Analytical Methods

3.2.1 HPLC Analysis

DNX isomers and DNT isomers were analyzed by HPLC (Agilent 1,100 series) equipped with a diode array detector (λ = 213nm 230nm or 246 nm). Hybercarb® porous graphite column (100 × 3 mm, 5 μm, Thermo Hypersil, UK) was used with a mobile phase of 90 % acetonitrile and 10% water with 0.55 mL/L of trifluoroacetic acid at a flow rate of 1 mL/min. Under these operating conditions, 2,4-DNT, MNX isomers, DNX isomers were eluted from 4 ~ 15min.

3.2.2 GC-MS Analysis

For analyzing xylene isomers, gas chromatography analyses were performed with a model 19091J-233 gas chromatograph (Agilent Technologies) with an HP-5 capillary column (30.0m length, 250μm inner diameter, 0.25mm film thickness [J & W Scientific]) coupled to a flame ionization detector. The gas chromatography oven was programmed to increase from 40°C (held for 3 min) to 110°C at 5°C/min, after which 110°C was held for 2 min, and increase from 110°C to 200°C at 15°C/min (held for 1.33min). The gas flow to the detector contained N_2 (30 ml/min), the detector temperature was 300°C, the injection port temperature was 200°C, and 1uL sample was loaded with an auto sampler with a splitless mode. Under this operating conditions, three xylene isomers were eluted from 13 ~15min.

3.3 Soil Experiments

3.3.1 Soil Samples

Composite soil made from C1 ~ C4 cells in the historically contaminated site undergoing remediation was prepared for microcosm study of xylene, mononitroxylene and dinitroxylene isomers. The composite soil containing C1 (4.4g), C2 (4.0g), C3 (4.7g) and C4 (7.4g) mixed with 75ml mineral medium in a flask was put in 30°Cand mixed for 1h at the speed of 180rpm. The same content of composite soil autoclaved for 30 min in 120 °C in two concecutive days was used in the control systems described in the following.

3.3.2 Microcosm Study of Xylene Isomers

Mineral medium (5ml) with addition of 53.8mg/L of NH_4Cl as nitrogen source was added to 50ml serum bottle with 3 drops of the composite soil, in order to observe

the turbidity during microcosm studies. The mineral medium contained the following: Na_2HPO_4 2.66g/L, KH_2PO_4 1g/L, $CaCL_3 \cdot 2H_2O$ 10mg/L, $FeSO_4 \cdot 7H_2O$ 3mg/L, $MgSO_4 \cdot 7H_2O$ 20mg and 1ml trace elements. The trace elements contained the following in mixture: H_3BO_3 100mg/L, $CaSO_4 \cdot 5H_2O$ 50mg/L, $ZnSO_4 \cdot 7H_2O$ 50mg/L and $Na_2MoO_2 \cdot 6H_2O$ 50mg/L. A glass vial (1ml) containing 600uL p-xylene, o-xylene and m-xylene individually was inserted into the serum bottle, which was sealed with Teflon Septa and aluminum crimp top. Each xylene culture has duplicate systems. Soil samples were added to duplicated control systems without any xylene in it. The culture was inoculated at 20°C, incubated in a shake table, and samples were spread on agar plates for enrichment of individual colonies. The agar plates contained the following: mineral medium as described, 53.8mg/L NH_4Cl and 18g/L agarose, which was autoclaved in 115°C for 20min and then was spread in culture inoculation plates to get growth agar. 100uL of a dilution series (1×, 10×, 100×, 1000×) were spread on agar plates, with 1ml insert glass vial containing 400uL each xylene isomers individually placed on the cap of plates sealed with parafilm. The culture on agar plates was incubated at 30°C for 7 days to observe the colonies growth.

To test biodegradability quantitatively, the xylene enrichment in liquid phase and on agar plates were transferred to a Teflon Septa sealed system with 15ml mineral medium with nitrogen source and 50mg/L of each xylene added by micro-syringe inoculating in 30°C, 200rpm. In order to guarantee the oxygen supply during microcosm study, the air/water ratio was set up to 10:1 and air was injected every time when sampling. The negative control systems were designed without any bacterial source. Every time when sampling, 100uL liquid from each system was removed by micro-

syringe and injected to approximately 2ml methylene chloride in GC vials to shake for half an hour before analyzing. The weight of the sampling bottle was measured by scale after every step in order to calculate xylene concentration in the original culture. The ratio between solvent and water and the extraction time was determined according to the literature to obtain approximately over 99% extraction efficiency. The extraction efficiency was measured to be above 99.5% using standard xylene before measuring samples from the systems.

3.3.3 Microcosm Study of Mononitroxylene Isomers

Soil samples (100ml) were added to 150ml serum bottle containing 15ml mineral medium with approximately 200uM 5-nitro-m-xylene, 4-nitro-m-xylene, 4-nitro-o-xylene and 3-nitro-oxylene individually in each of the bottle, sealed with Teflon septa and aluminum crimp cap. The mineral medium was prepared as described above. Each isomer had three systems: one without other carbon sources, one with an additional carbon source and one control system. The composition of the additional carbon sources was prepared by the addition of succinic acid (590 mg/L), glucose (900 mg/L) and glycerol (730 µL/L). The control systems were prepared by adding 100uL autoclaved soil along with approximately 200uM 4 mononitroxylene isomers. The culture was incubated at 30°C with the shaking speed of 230rpm. HPLC samples were collected periodically for mononitroxylene isomers concentration analysis on the same day. The degradation culture was transferred by 10% to fresh mineral medium with a single mononitroxylene isomer 200µM.

3.3.4 Microcosm Study of Dinitroxylene Isomers

Soil samples 100µL were added to 75ml serum bottle containing 12ml mineral medium with approximately 50uM of individual each DNX isomers, sealed with Tyflon

septa and aluminum crimp cap. The mineral medium was prepared as described above. The culture was incubated at 30°C with the shaking speed of 230rpm. HPLC samples were collected periodically for dinitroxylene isomers concentration. The degradation culture was transferred by 10% to fresh mineral medium with a single DNX isomer 50µM.

3.3.5 Cometabolism Study of Dinitroxylene Isomers

2,4-DNT degrading mixed culture was enriched from Barksdale soil to initiate the enrichment. Soil (5g C12) and mineral medium 100ml was added to 250ml Erlenmeyer flask, including 100mg/L 2,4-DNT. The mineral medium was prepared as described previously. The flasks were incubated at 30°C with the shaking speed of 200rpm. HPLC samples were taken every 3 to 5 days after the incubation started. After complete degradation of 2,4-DNT, 10% original culture was transferred to a new 100ml mineral medium with gradually increased concentration of 2,4-DNT. After 6 to 7 transfers of 2,4-DNT degrading enrichment, the culture became soil free and could be used for further experiments. The enrichment culture was kept in an Erlenmeyer flask on a shaker with the speed of 200rpm in 30°C. The 2,4-DNT enrichment was fed with 25mg 2,4-DNT crystals and half volume of the mineral medium was replaced every 4 days.

The 2,4-DNT degrading enrichment from Barksdale soil was used for dinitroxylene (DNX) isomers cometabolism study. Approximately 50µM of individual DNX isomers each dissolved in acetonitrile was added to 50ml serum bottle containing 180mg/L 2,4-DNT in acetonitrile following by complete evaporation of acetonitrile. The mineral medium was prepared according to the previous description. Mineral medium 20ml was added to serum bottle and incubated at 30°C, 200rpm on shaker. The control system was prepared by adding 100uL autoclaved soil sample and approximately 50uM

DNT isomers to the system. Meanwhile, a positive control system with 180mg/L 2,4-DNT and 2,4-DNT degrading bacteria was set up to ensure 2,4-DNT degrading culture activity. HPLC samples were collected periodically for dinitroxylene isomers concentration analysis. After complete degradation of 2,4-DNT, the culture was transferred to a new serum bottle with 180mg/L 2,4-DNT. More HPLC samples were collected to see the concentration change of dinitroxylene isomers.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Biodegradability of Xylene Isomers

The results of microcosm study of xylene isomers are shown in Table 3.

Table 3 Results of Xylene Isomers Microcosm Study

Xylene isomer	Turbidity increase in liquid phase	Colonies growth on agar plates
p-xylene	+	+
m-xylene	+	+
o-xylene	+	+

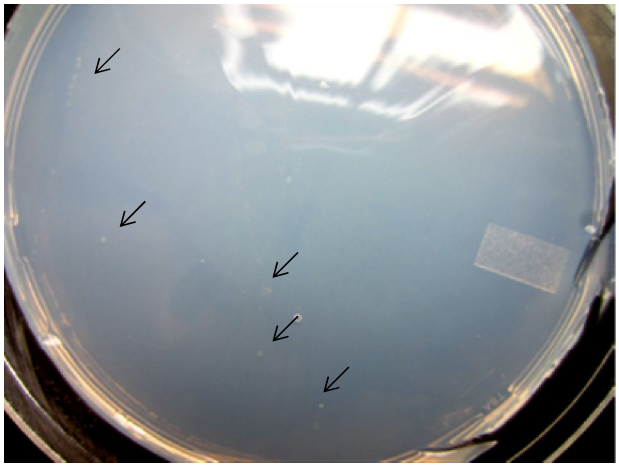


Figure 5 Bacteria colonies growth on the xylene isomer selective agar plates

The liquid microcosm system enriched from soil turned turbid every transfers after 14 days of incubation (**Table 3**). As shown in **Figure 5**, small white colonies were grown on agar plates after about 2 weeks. The turbidity in liquid phase and colonies on plates both indicated the presense of certain bateria that could grow on each xylene

isomer as a sole source of carbon. The observation illustrated that the xylene degrading enrichment can grow on both liquid culture and agar plates. All the colonies from agar plates look similar, indicating that there might exist a novel culture, which can use all three xylenes. However from what has been studied about xylene degradaton [6], there is no pure culture that can degrade all three xylene isomers. As a result, it could be insterested to isolate pure culture from the enrichment to demonstrate its novel biodgradation pathway.

In conclusion, it was confirmed that the enrichment of degradation of each xylene isomer as a sole source of carbon exist and could be selected from soils provided. These results demonstrate that the provided soil contains organisms capable of metabolizing all three isomers of xylene, but what remains unknown is their potential involvement in dinitroxylene, if at all.

4.2 Biodegradability of Mononitroxylene Isomers

The results of MNX isomers microcosm study are shown in **Table 4** below, in which, "+" indicates that this isomer showed degradation while "-" indicates that this isomer did not show any degradation during the microcosm study.

Among 12 microcosm study of MNX isomers, the concentration of 5-NMX and 4-NOX in the system without carbon source dropped to zero in the original culture, indicating that 5-NMX and 4-NOX may have the potential to be biodegraded in the soil. The reason why the control system of 4-NOX was degraded was probably because of the failure of sterilization. However, both the microbial activity in 4-NOX and 5-NMX orginal culture could not be transferred after soil content was largely reduced, indicating that degradation of MNX in these two systems may be due to the following reasons:

1)The additional carbon source or other soil content provided in the orginal culture largely increase the degradation speed of both isomers; 2) The concentration of MNX in the transfer culture exceeds the baterial tolerance of MNX in the original culture.

Table 4 Results of MNX Isomers Microcosm Study

MNX isomer	System without carbon source	System with carbon source	Control System
5-nitro-m-xylene (5-NMX)	+	+	-
4-nitro-m-xylene (4-NMX)	-	-	-
4-nitro-o-xylene (4-NOX)	+	+	+
3-nitro-o-xylene (3-NOX)	-	-	-

The other two MNX isomers, 4-NMX and 3-NOX, did not show any biodegradability during incubation because MNX concentration did not change from the beginning to the end of the microcosm study in all the three systems. The reason why they cannot be biodegraded can be summarized into: 1) There's no such bacteria that can degrade these two isomers in the provided soils; 2) The concentration of two MNX is high enough to cause toxicity to degrading culture; 3) The degradation products might be toxic to MNX degrading bacteria.

In short, certain bacteria that can use 5-NMX and 4-NOX as a sole source of carbon and nitrogen source may exist in the provided soil. In order to create a new remediation strategy for MNX, more efforts should be conducted to isolate certain strains and illustrate the biodegradation pathway.

4.3 Biodegradability of Dinitroxylene Isomers

The results of DNX isomers microcosm study are shown in **Table 5** below, in which, "+" indicates that this isomer showed degradation in microcosm system and the concentration remained stable in control system, while "-" indicates that this isomer did not show any degradation during the microcosm study.

Table 5 Results of DNX Isomers Microcosm Study

DNX isomer	Biotic Transformation
2,5-dinitro-p-xylene (1,4-dimethyl-2,5-dinitrobenzene)	+
2,3-dinitro-p-xylene (1,4-dimethyl-2,3-dinitrobenzene)	-
2,6-dinitro-p-xylene (1,4-dimethyl-2,6-dinitrobenzene)	-
4,5-dinitro-o-xylene (1,2-dimethyl-4,5-dinitrobenzene)	+
3,6-dinitro-o-xylene (1,2-dimethyl-3,6-dinitrobenzene)	-
3,4-dinitro-o-xylene (1,2-dimethyl-3,4-dinitrobenzene)	+
3,5-dinitro-o-xylene (1,2-dimethyl-3,5-dinitrobenzene)	-
4,6-dinitro-m-xylene (1,3-dimethyl-4,6-dinitrobenzene)	-
4,5-dinitro-m-xylene (1,3-dimethyl-4,5-dinitrobenzene)	-
2,4-dinitro-m-xylene (1,3-dimethyl-2,4-dinitrobenzene)	-

The situation of DNX biodegradability test was similar with that of MNX biodegradability test. Among 10 different DNX isomers, three out of ten isomers (2,5-dinitro-p-xylene, 4,5-dinitro-o-xylene, 3,4-dinitro-o-xylene) showed promising degradation in the original culture in the first 30 days, however, slowed down in the next 50 days. And the first transfer culture did not work after 30 days inoculation. It might

because that the bacteria in the original culture are still in lag phase. It is also likely that the degradation in the original culture depends on the soil content. When soil content is largely reduced during the first transfer, the degradation stopped simultaneously.

In short, DNX degraders may exist in the provided soil but are difficult to be selected or isolated. And three DNX isomers (2,5-dinitro-p-xylene, 4,5-dinitro-o-xylene, 3,4-dinitro-o-xylene) appeared to have higher potential to be biodegraded than the other seven isomers.

4.4 DNX Isomers Cometabolism Study

The table below (**Table 6**) summaries the results of cometabolism study of DNX isomers with 2,4-DNT degrading enrichment from the site in Wisconsin, in which, "+" indicates that this isomer showed degradation in microcosm system and 2,4-DNT in positive control was able to be degraded simultaneously, while "-" indicates that this isomer did not show any degradation during the microcosm study.

Table 6 DNX Cometabolism Study with 2,4-DNT Degrading Enrichment

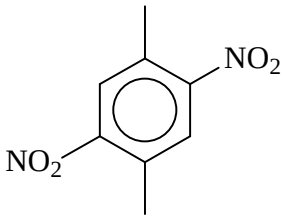
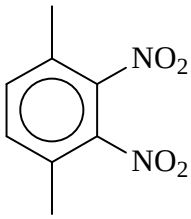
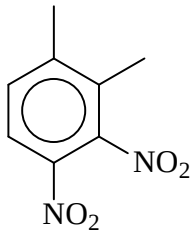
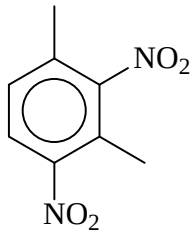
DNX isomer	Biotic transformation
2,5-dinitro-p-xylene	+
2,3-dinitro-p-xylene	+
2,6-dinitro-p-xylene	-
4,5-dinitro-o-xylene	-
3,6-dinitro-o-xylene	-

3,4-dinitro-o-xylene	+
3,5-dinitro-o-xylene	-
4,6-dinitro-m-xylene	-
4,5-dinitro-m-xylene	-
2,4-dinitro-m-xylene	+

Cometabolism study of DNX isomers with 2,4-DNT degrading enrichment showed that four out of ten DNX isomers were capable of being cometabolized by 2,4-DNT degrading culture enriched from the soil provided. These DNX isomers including 2,5-dinitro-p-xylene, 2,3-dinitro-p-xylene, 3,4-dinitro-o-xylene and 2,4-dinitro-m-xylene, were degraded at an initial containing 50μM by 2,4-DNT degrading enrichment in 16~30 days while 2,4-DNT was degraded from 180mg/L to zero in the first 7 days. Some of them (2,5-dinitro-p-xylene, 2,3-dinitro-p-xylene, 3,4-dinitro-o-xylene) started to be degraded after 2,4-DNT was consumed and some of them (2,4-dinitro-m-xylene) was degraded when 2,4-DNT was being degraded. It might because that certain enzyme or intermediates generated from 2,4-DNT degradation activated the cometabolic process of DNX isomers. From their structures shown in

Table 7, it is likely that the similar dinitro- and dimethyl- structure in the three isomers (2,3-dinitro-p-xylene, 3,4-dinitro-o-xylene and 2,4-dinitro-m-xylene) helps to improve the cometabolism of 2,4-DNT degrading enrichment.

Table 7 Chemical Structures of DNX Cometabolizable Isomers by 2,4-DNT Enrichment

2,5-dinitro-p-xylene	2,3-dinitro-p-xylene	3,4-dinitro-o-xylene	2,4-dinitro-m-xylene
			

CHAPTER 5

CONCLUSIONS

This research is the first attempt of biodegradability and cometabolism study of nitroxylene series isomers under aerobic condition. Specific conclusions are as follows.

- The microcosm study of 2,4-DNT showed that after 5 to 6 transfers of 2,4-DNT degrading culture, saturated concentration of 2,4-DNT (200mg/L) dissolved from 2,4-DNT crystals can be fully biodegraded within 4 days, indicating the presence of 2,4-DNT degrading bacteria which are capable to use 2,4-DNT as a sole source of carbon and nitrogen existed in the soil provided.
- The microcosm study of three xylene isomers showed that the xylene degrading system turned turbid in 2 weeks after 5 to 6 transfer of xylene degrading culture to a new clear mineral medium with nitrogen source, demonstrating the presence of p-xylene, m-xylene and o-xylene degrading bacteria which are able to use each xylene isomer as a sole source of carbon existed in the soil provided.
- The microcosm study of 4 mononitroxylene (MNX) isomers showed that two out of four MNX isomers, 5-nitro-m-xylene and 4-nitro-o-xylene, were able to be fully degraded in 40 ~ 60 days in the original culture however this activity could not be transferred after soil content was largely reduced, which illustrated that biodegradability of MNX might depend on the presence of soil content.

- The microcosm study of 10 dinitroxylene (DNX) isomers showed that three out of ten DNX isomers, 2,5-dinitro-p-xylene, 3,4-dinitro-o-xylene, 4,5-dinitro-o-xylene, exhibited biodegradation activity in 80 days in the original culture, but lost activity in the next transfer where soil content was largely reduced as similar to the MNX degrading culture in the next transfer, indicating that soil content may play an important role in the biodegradation process of DNX isomers.
- The cometabolism study of 10 dinitroxylene (DNX) isomers with 2,4-DNT degrading bacteria enriched from the historically contaminated site showed that four out of ten DNX isomers, 2,5-dinitro-p-xylene, 2,3-dinitro-p-xylene, 3,4-dinitro-o-xylene and 2,4-dinitro-m-xylene, were capable to be cometabolized in 16 ~ 30 days by 2,4-DNT degrading enrichment. Additionally, further experiments suggested that this activity could be transferred, potentially creating a strategy for bioremediation of DNX isomers in contaminated sites.

In summary, the first attempt of nitroxylene series biodegradability and cometabolism study showed the presence of 2,4-DNT and xylene isomers degrading bacteria at the site. Evidence of MNX and DNX biodegradation was observed for several isomers, but not all. Four DNX isomers could be cometabolized by 2,4-DNT degrading enrichment.

CHAPTER 6

FUTURE WORK

This research is the first attempt to demonstrate the biodegradability of nitroxylene isomers, therefore, more detailed research need to be done in the future. Concerning degradation of xylene isomers, in order to better evaluate biodegradation rate of xylene, quantatitive experiments can be conducted using GC-MS. Both the liquid culture and colonies on plates can be transferred to new sealed system to measure xylene concentration change. However, it should be concerned that sampling using regular needles will affect the sealed system. As a result, using sacrificed systems might be one of the solutions to get valid results.

To further the understanding of the biodegradability of MNX and DNX, it is recommended to start with those that can be degraded in the original culture. In order to get stable enrichment of these isomers, different conditions can be controlled during the transfer experiments. For example, variable initial concentration of MNX and DNX can be used for the transfer system, in case toxicity by MNX or DNX inhibits the bacterial activity.

Further research can be designed to demonstrate the cometabolism of DNX with 2,4-DNT experiments. In order to select 2,4-DNT enrichment with better performance, every transfer can contain less 2,4-DNT and more DNX. But it is a concern that DNX solubility is much less than 2,4-DNT, also it is unknown that if certain amount of DNX exhibits toxicity to 2,4-DNT degrading bacteria. As a result, solubility tests and toxicity tests of DNX are recommended. In addition, cometabolism of DNX can be attempted with pure 2,4-DNT culture, which can be isolated from the current enrichment. If the

process of cometabolism is due to the function of pure culture, it can be clearly illustrated in such experiments.

APPENDIX A

The information hereby in Appendix A includes the concentration change of the two biodegradable MNX isomers (5-NMX, 4-NOX) mentioned in Table 4 in Chapter 4.2.

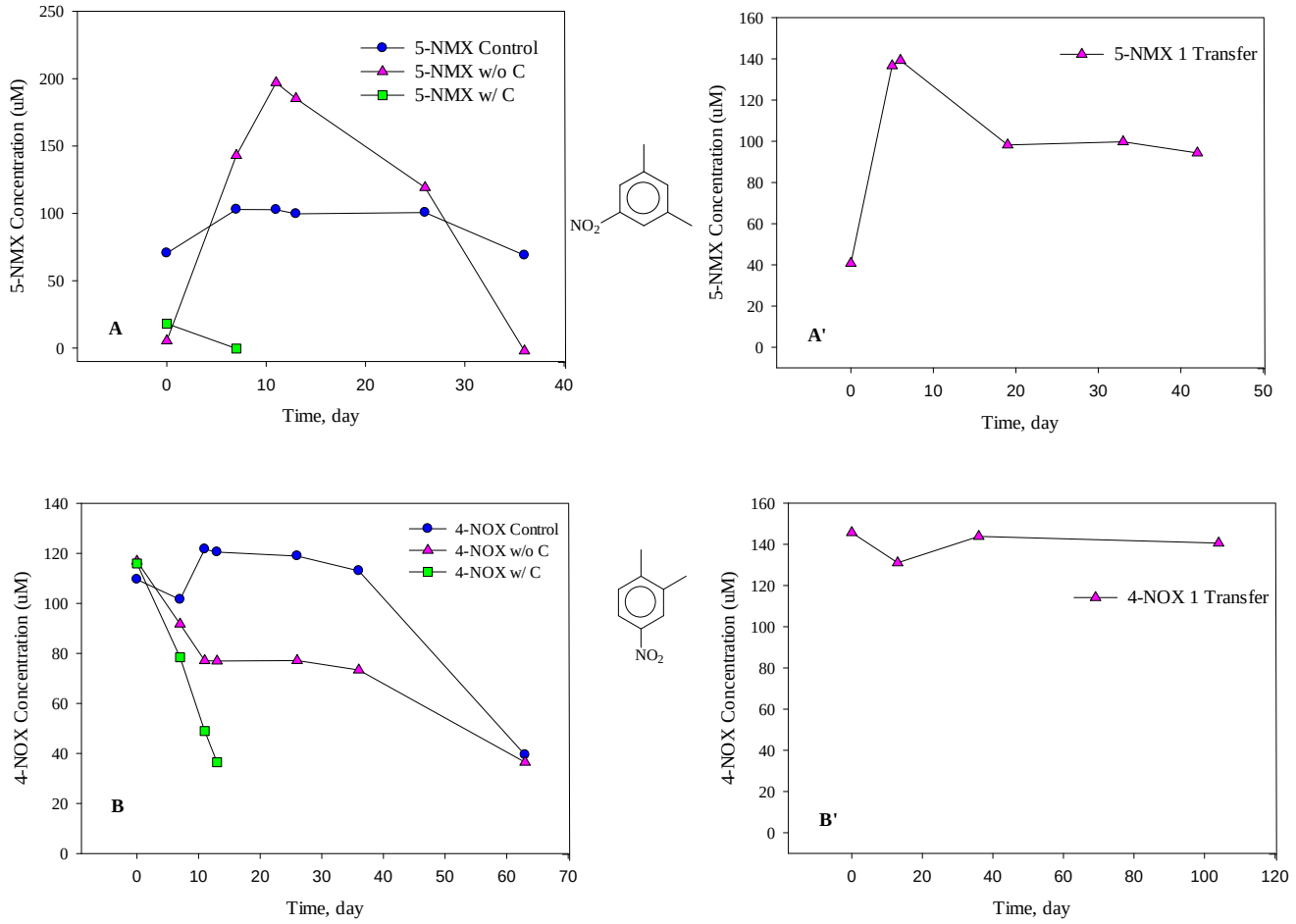
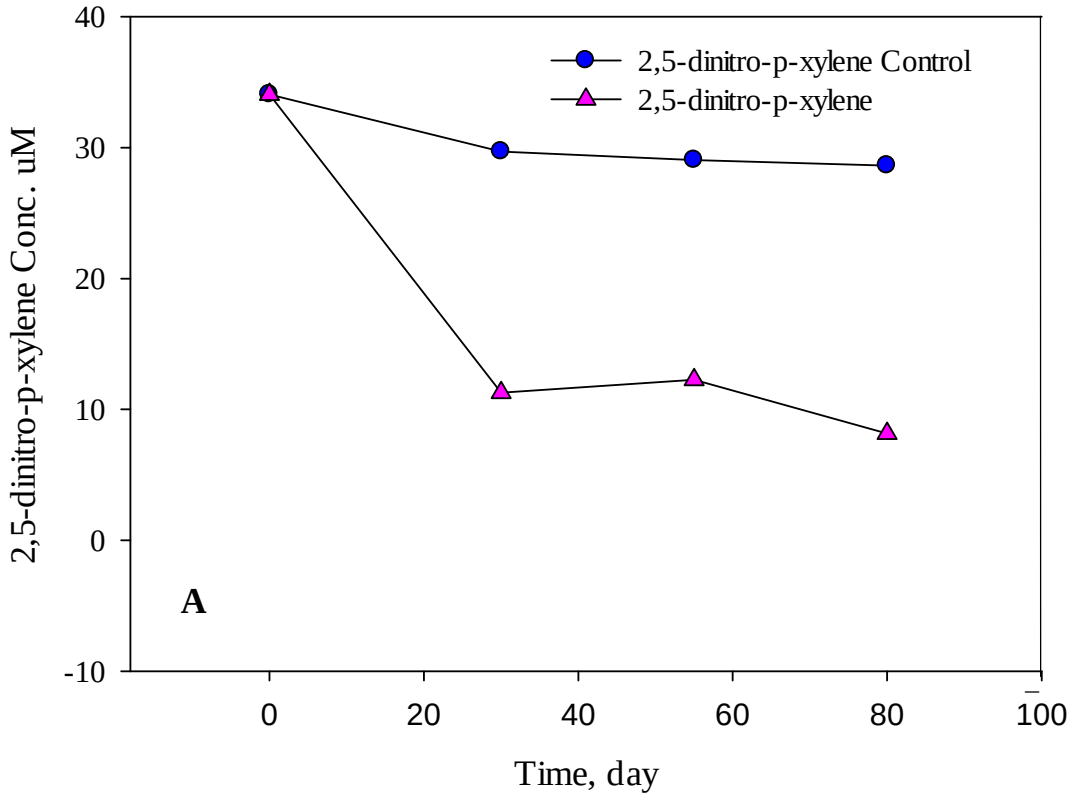
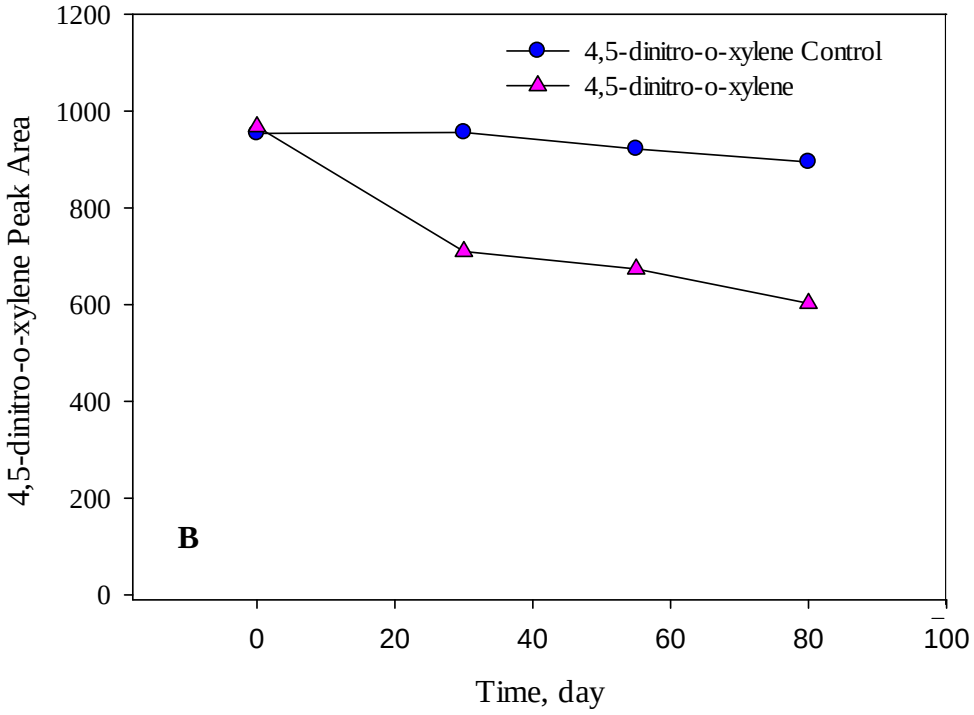


Figure A Chemical structures and Concentration Changes of 2 MNX Isomers in Microcosm Studies: 5NMX Original Culture (A), 5NMX 1 Transfer Culture (A'), 4NOX Original Culture (B), 4NOX 1 Transfer Culture (B')

APPENDIX B

Appendix B provides the supplementary information for Chapter 4.3, showing the concentration change of 3 degrading DNXs listed in Table 5.





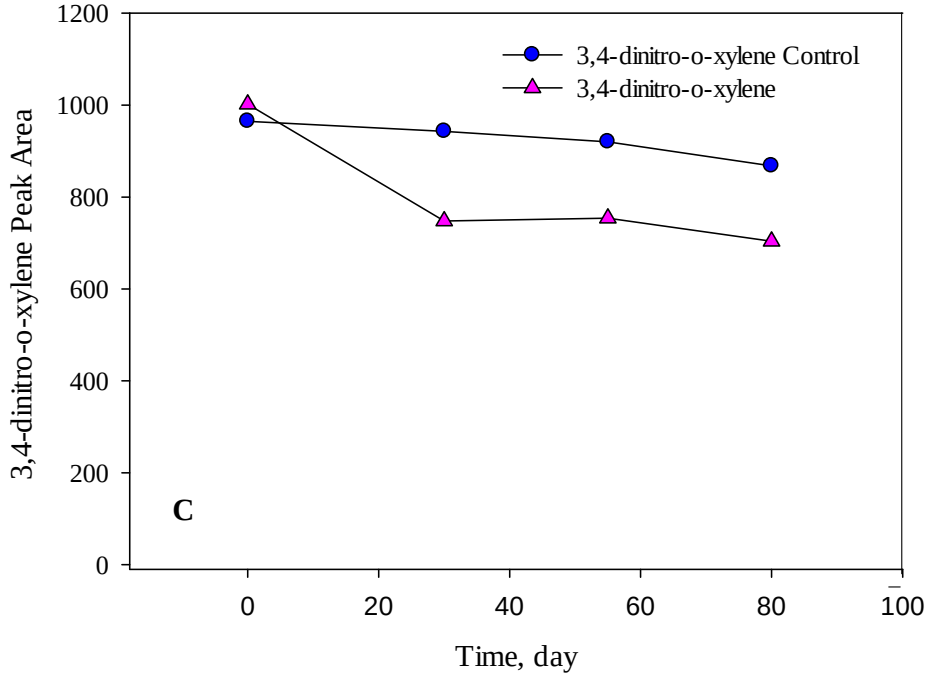
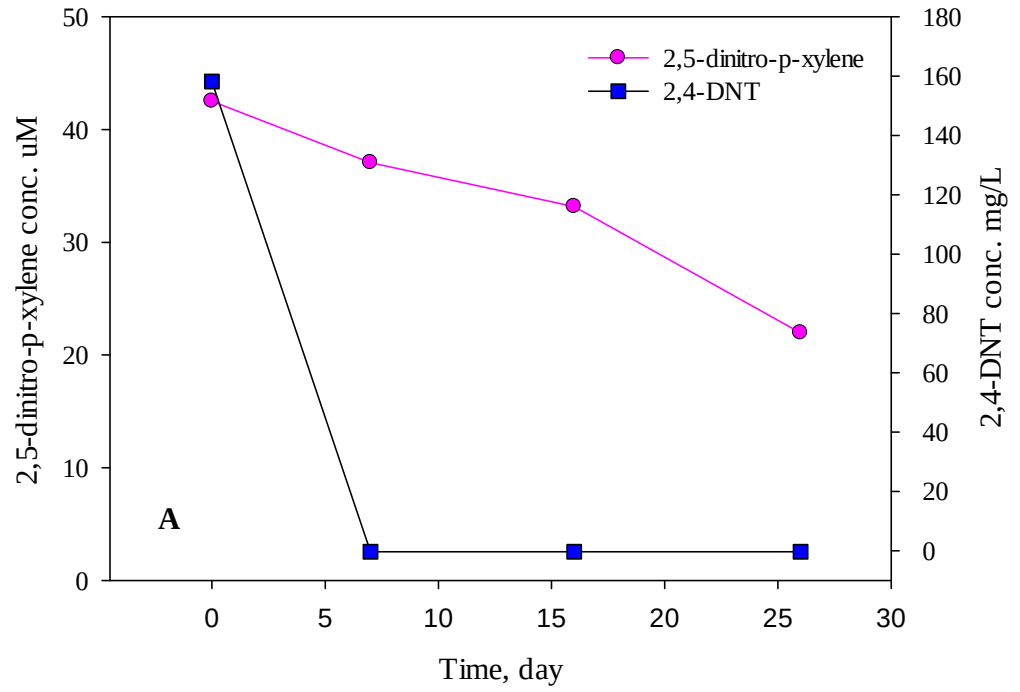
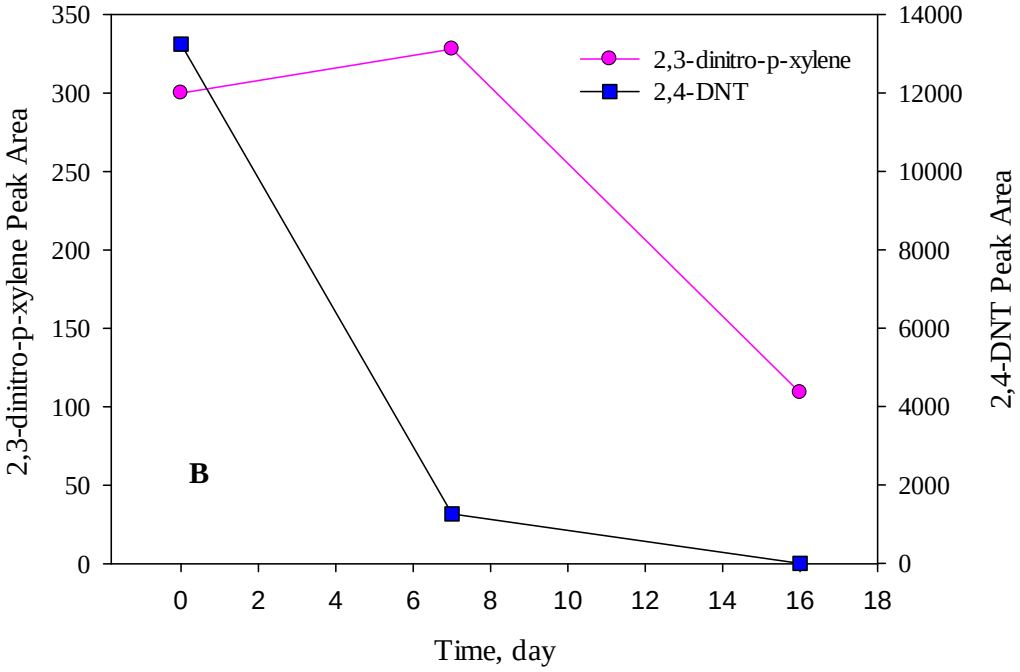


Figure 6 DNX Concentration Change in 2,5-dinitro-p-xylene (A), 4,5-dinitro-o-xylene (B) and 3,4-dinitro-o-xylene (C)

APPENDIX C

The figure C shown in Appendix C provides the concentration change of 4 working DNX isomers mentioned in Table 6 in Chapter 4.4.


2,5-dinitro-p-xylene
2,4-DNT
2,5-dinitro-p-xylene conc. uM
2,4-DNT conc. mg/L
A
Time, day


2,3-dinitro-p-xylene
2,4-DNT
2,3-dinitro-p-xylene Peak Area
2,4-DNT Peak Area
B
Time, day

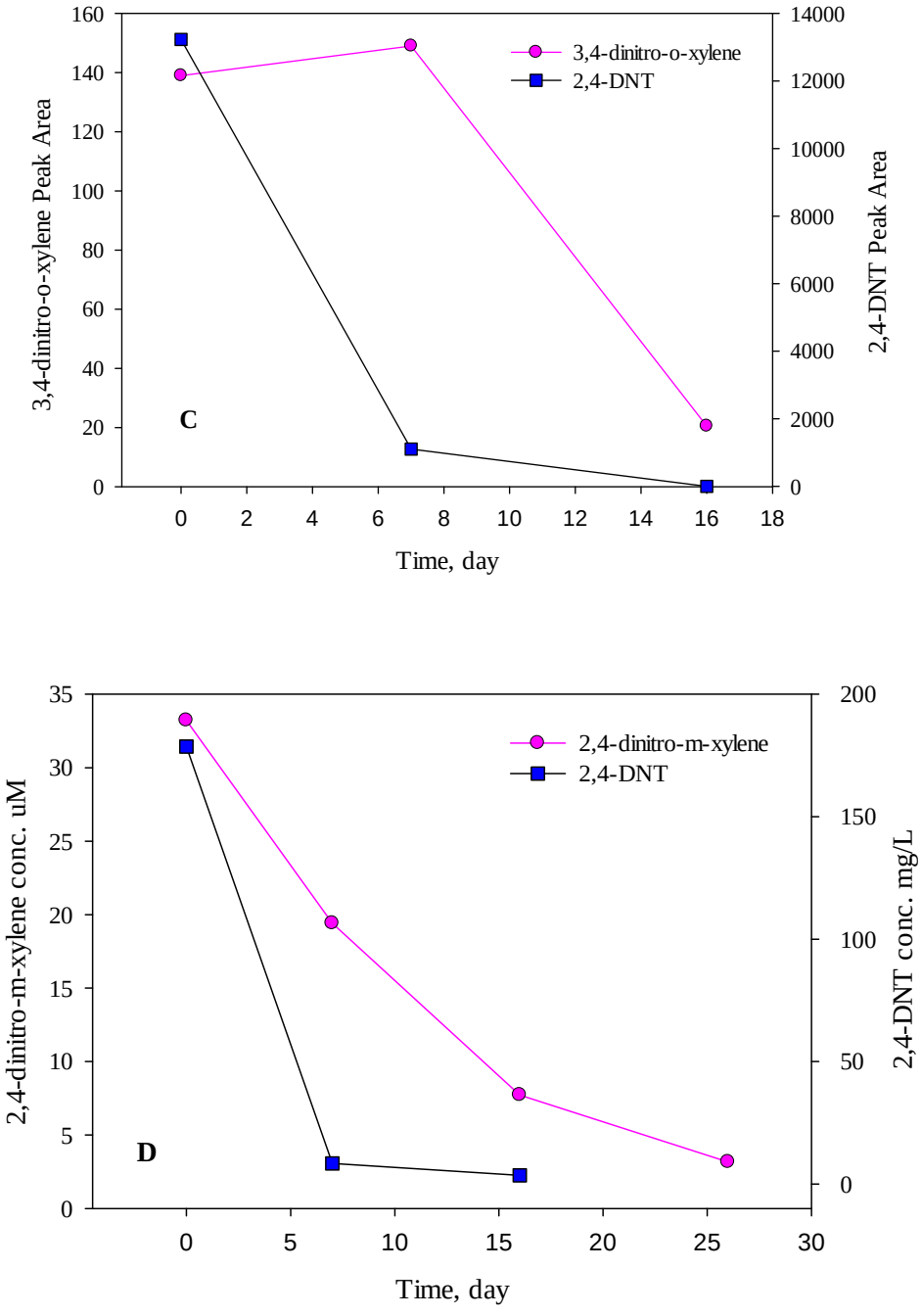


Figure C DNX and 2,4-DNT Concentration Change in DNX Cometabolism Systems: 2,5-dinitro-p-xylene (A), 2,3-dinitro-p-xylene (B) and 3,4-dinitro-o-xylene (C) and 2,4-dinitro-m-xylene (D)

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