AN ABSTRACT OF THE DISSERTATION OF

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Cometabolic biodegradation processes are potentially useful for the bioremediation of hazardous waste sites. In this study the potential application of phenol-oxidizing and nitrifying bacteria as "priming biocatalysts" was examined in the degradation of polycyclic aromatic hydrocarbons (PAHs), aryl ethers, and aromatic ethers. We observed that a phenol-oxidizing *Pseudomonas* strain cometabolically degrades a range of 2- and 3-ringed PAHs. A sequencing batch reactor (SBR) was used to overcome the competitive effects between two substrates and the SBR was evaluated as a alternative technology to treat mixed contaminants including phenol and PAHs. We also have demonstrated that the nitrifying bacterium *Nitrosomonas europaea* can cometabolically degrade a wide range polycyclic aromatic hydrocarbons (PAHs), aryl ethers and aromatic ethers including naphthalene, acenaphthene, diphenyl ether, dibenzofuran, dibenzo-*p*-dioxin, and anisole. Our results indicated that all the compounds are transformed by *N. europaea* and that several unusual reactions are involved in these reactions. In the case of naphthalene oxidation, *N. europaea* generated predominantly 2-naphthol whereas other monooxygenases generate 1-naphthol as the major product. In the case of dibenzofuran oxidation, 3-hydroxydibenzofuran initially accumulated in the reaction medium and was then further transformed to 3-hydroxy nitrodibenzofuran in a

pH- and nitrite-dependent abiotic reaction. A similar abiotic transformation reaction also was observed with other hydroxylated aryl ethers and PAHs. We also characterized the role of AMO in the degradation of aromatic ethers. Our results indicated that aromatic ethers including anisole were transformed by both O-dealkylation or hydroxylation reactions. This research has led to the development of a rapid colorimetric assay to detect AMO activity.

Cometabolic Degradation of Polycyclic Aromatic Hydrocarbons (PAHs) and Aromatic Ethers by Phenol- and Ammonia-Oxidizing Bacteria

by

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Chair of Dept. of Civil, Construction, and Environmental Engineering

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Chapter 1

Introduction

This dissertation contains four manuscripts dealing with the cometabolic degradation of aromatic and polycyclic aromatic hydrocarbons (PAHs) by phenol utilizing bacteria *Pseudomonas* strain and nitrifying bacterium *Nitrosomonas europaea.* Initially, We were concerned with developing treatment technology to reduce the concentration of PAHs found in the waste water from a coking plant operated by Pohang Iron and Steel Company (POSCO) in South Korea. In general PAHs are relatively stable and recalcitrant in soils and groundwater and less degradable than many other organic compounds in contaminant environments. The characterization of using phenol-oxidizing bacteria to degrade PAHs was conducted and potential application of Sequencing Batch Reactor (SBR) as an alternative technology was evaluated. However, we also noticed the presence of NH_3 in the POSCO wastewater steam. As a next step, the nitrifying bacteria, *Nitrosomonas europaea* which is a widely distributed in soil, water, and sewage, are examined for the potential application as "priming catalysts" in the bioremediation of chemical pollution. Nitrifying bacterium can cometabolize a large number of non-growth supporting substrates using the catalytic activity of their respective monooxygenases. If these organisms could be used in bioremediation schemes, it is important to understand the contamination ranges a meanable to biodegradation and the limitation of the

technology. The things must be considered that aryl ethers including dibenzofuran (DBF), dibenzodioxin (DD), and other lignin compounds are often present in complex mixtures with PAHs. The problems of the PAHs and other related compounds are expanded by the wide spread use and accidental release of coal and petrochemicals for industrial processes. The second manuscript examines the PAHs degradation by nitrifying bacterium *N. europaea.* The kinetics of cometabolic degradation, substrate ranges of *N. europaea*, and potential toxic effects of cosubstrate are determined. As described in previously, the results of second manuscripts addressed the examination of related compounds with PAHs. Many of polyaromatic ether-bonded compounds are toxic and known to resist to biological degradation. The third manuscript examine the degradation of aryl ethers including dibenzofuran (DBF) and dibenzo-*p*-dioxin (DD) by *N. europaea.* In this study, biological transformations of aryl ethers are characterized and also abiotic reaction following the biological transformation is discussed. The fourth manuscript describes the transformation of aromatic ethers including anisole by *N. europaea*, presenting AMO activity for substrates through O-dealkylation and hydroxylation reactions. Colorimetric assay to detect ammonia monooxygenase activity (AMO) was examined for the potential applications.

Chapter 2

Co-metabolic Degradation of Polycyclic Aromatic Hydrocarbons by a Phenol-Oxidizing *Pseudomonas* Strain in Batch, Continuously Stirred Tank- and Sequencing Batch Reactors.

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ABSTRACT

A phenol-oxidizing *Pseudomonas* strain was isolated from an activated-sludge-treated coking-plant wastewater. Batch experiments demonstrated that the phenol-oxidizing organism was capable of cometabolically degrading 2- and 3-ringed polycyclic aromatic hydrocarbons including naphthalene, 2-methyl naphthalene and anthracene similar to other fungal and bacterial systems. The *Pseudomonas* sp. generated 1-naphthol as the major product of naphthalene oxidation (> 90 % conversion). In batch cultures the removal efficiencies of naphthalene were over 90 % and 69.7 % with and without acetate additions, respectively. Based on batch experiments, a sequencing batch reactor (SBR) was proposed as a alternative system to minimize competitive inhibition between phenol and PAHs. A SBR achieved a removal efficiency of 98 % and a transformation yield of 0. 056 mg naphthalene transformed/mg phenol utilized, as compared to 57 % and 0.022 mg naphthalene/mg phenol utilized, respectively, for a continuously stirred tank reactor (CSTR). Naphthalene, 2-methyl naphthalene and anthracene are degraded simultaneously, although anthracene slightly inhibits degradation of naphthalene and 2-methyl naphthalene in the SBR.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants. The main anthropogenic sources of PAHs are processes such as petroleum processing and coal coking which involve the pyrolysis of organic compounds (37). Remediation of previous PAH contamination and prevention of further releases of PAHs into the environment is important because of their varied toxic, carcinogenic, and mutagenic properties (14). Currently 16 PAHs are listed as Priority Pollutants by the United States Environmental Protection Agency (27). Recent studies have shown that microbiological degradation of PAHs is the major removal process from contaminated environments, although other mechanisms such as volatilization, leaching, and photodegradation may be important (6).

Studies of the microbial degradation of PAHs have demonstrated that many species of bacteria (7, 34, 38, 39, 41) and fungi (15) are capable of oxidizing PAHs. In some instances PAHs are fully metabolized and used as carbon and energy sources for growth (5, 34). In other instances PAHs are transformed by microorganisms growing on other carbon and energy sources and in these cases the products of the PAH oxidation reactions are not further assimilated by the microorganism. This latter process is most commonly known as co-metabolism. The initial reactions in both the aerobic metabolism and co-metabolism of PAHs are catalyzed by a variety of oxygenase-type enzymes which generate mono- and di-hydroxylated products, respectively (8, 39). The microbial detoxification of PAHs through mineralization is often limited by the low solubility and bioavailability of these compounds. To overcome this limitation it has been suggested that the partial degradation of PAHs through simple oxygenase-catalyzed hydroxylation

reactions could increase the availability of PAHs which would then be expected to lead to substantially increased rates of PAH mineralization (7, 38, 39).

In the present study we have been concerned with developing methods which allow for the reduction in concentrations of PAHs found in the waste water stream from a coking plant operated by Pohang Iron and Steel Company (Pohang, South Korea) (POSCO). This coking plant is used to generate coke for steel manufacturing and the waste stream from the coking process contains high concentrations of phenol (400 mg/L), ammonia (25 mg/L), and a variety of PAHs. This study describes the PAH co-metabolizing activity of a phenol-oxidizing pseudomonad isolated from activated sludge at the coking plant wastewater treatment facility. Our results demonstrate that this organism is capable of rapidly co-metabolizing a variety of PAHs and this process has been characterized in a variety of reactor configurations. Our results suggest that the activities of this phenol-oxidizing bacterium could be utilized to decrease the current levels of PAHs found in the waste water from the coking process.

MATERIALS AND METHODS

Materials

PAHs were purchased from Aldrich Chemical Co. (Milwaukee, WI). Solvents and other reagents were commercial products of the highest purity available. Stock solution of naphthalene, 2-methyl naphthalene, acenaphthalene, and fluorene were prepared at 10 mg/ml in methanol; anthracene, phenanthrene, fluoranthene, and pyrene were prepared at 1 mg/ml in methanol.

Organisms and Growth Conditions

The phenol-utilizing bacterium utilized in this study, *Pseudomonas* TJ-1, was isolated from an aerobic enrichment culture established using activated sludge from Pohang Iron and Steel Co. (POSCO). The initial enrichment was conducted in a chemostat (2 L) fed continuously (2 L/d) with a Basal Salts Medium (BSM) containing phenol (200 mg/l) as the sole source of carbon and energy. The BSM contained (per liter) 1.1 mg $FeSO_4 \cdot 7H_2O$, 2.02 mg $ZnSO_4 \cdot 7H_2O$, 4.5 mg $CaCl_2 \cdot H_2O$, 7.18 mg NH_4Cl, 32.4 mg $MgCl_2 \cdot 6H_2O$, 23.4 mg KCl, 0.36 mg $MnCl_2 \cdot 4H_2O$, 0.1 mg $CoCl_2 \cdot 6H_2O$, 0.1 mg H_3BO_4, 0.05 mg $Na_2MoO_4 \cdot 2H_2O$, and 37.8 mg $ZnCl_2$. The pH of the medium was adjusted to 7.0 by the addition of phosphates (2.4 g Na_2HPO_4 and 2.2 g KH_2PO_4) added from sterile aqueous solution. After 14 days an aliquot (1 ml) of the effluent was used to inoculate mineral malts medium (100 ml) in a serum bottle (125 ml) sealed with a butyl rubber stopper. Further additions of either O_2 or phenol (0.21 mmole) were added to the incubations as needed. After growth for 14 days an aliquot (1 ml) of this enriched culture was transferred to agar plates (mineral salts medium, phenol (100 mg/L) and agar (1.5%).

Individual colonies detected after 7 days growth were subsequently picked and transferred to fresh plates. The purity of organisms isolated by this procedure was determined by microscopic analysis after growth in phenol-containing liquid media. After isolation, the bacterium was maintained by growth at 30 °C in continuous culture in a fermenter (1 L) fed with BSM containing phenol (200 mg/l).

Batch Culture Experiments

The potential for co-metabolic PAH degradation by phenol-grown *Pseudomonas* TJ-1 was initially determined in batch culture incubations. All reactions were conducted in glass serum bottles (160 ml). The PAHs were added to the serum bottles from stock solutions in methanol. The methanol then was evaporated under a stream of N_2 gas. Growth medium (BSM with phenol and phosphates; 100 ml) then was added and the remaining gas phase was briefly flushed (20 s) with O_2 gas. The flushed bottles then were sealed with Teflon-lined butyl rubber stoppers and aluminum crimp seals and the sealed bottles were then autoclaved. After cooling the sterilized media was inoculated with phenol-grown cells (1 ml) obtained from the continuous culture described above. The inoculated serum bottles were incubated on a rotary shaker (150 rpm) in a constant temperature room (30 °C).

Sequencing Batch Reactor Experiments

Based on the results of batch culture experiments, a Sequencing Batch Reactor (SBR) system was tested to study the removal of PAHs without potential competitive effects arising from the presence of the growth substrate, phenol. An SBR system is a

periodically operated, fill-and-draw reactor.

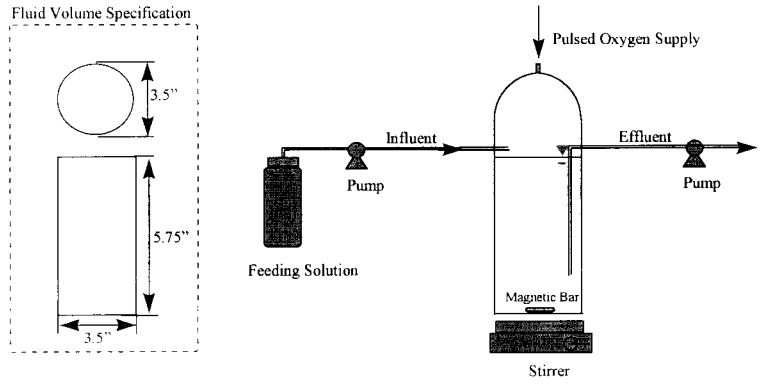

Figure 2-1. Schematics of Sequencing Batch Reactor (SBR)

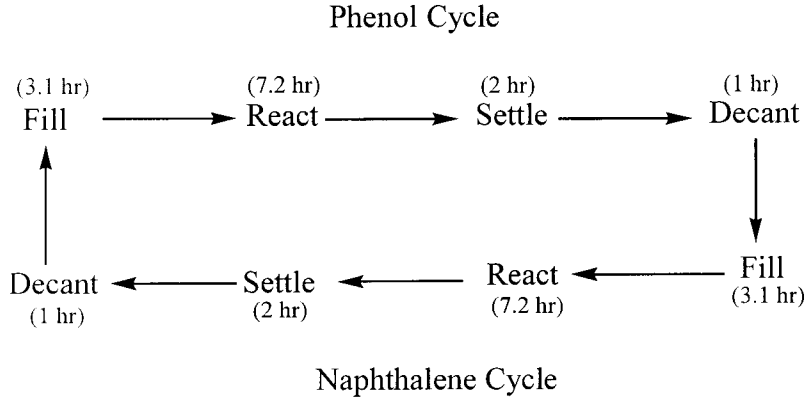

Figure 2-2. Schematics of reaction cycle of Sequencing Batch Reactor

The modified system used in this study had a reaction volume of 1 liter and was operated with four discrete periods in each cycle: fill, react, settle, and draw. The schematic description and reaction cycle of SBR was illustrated in Fig. 2-1 and Fig. 2-2. The SBR was operated for 14 days until steady-state conditions were reached. Subsequently the reactor was operated continuously with a 13.3 h reaction cycle. After reaching steady-state conditions of cell density, the reactor contents were settled for 2 hours and 70 % of the reaction medium (700 ml) was decanted within 1 h. The SBR then was refilled to a final volume of 1 L with sterile medium containing phenol (200 mg/l) added at a flow rate of 1.62 ml/min for 3.1 hours. After filling, individual PAHs were added to the reactor and treatment occurred for the next 7.2 hours. Oxygen was supplied to the headspace with pulsed injection for 2 min for every 2 hr period during the filling and reaction periods. A Continuous Stirred-Tank Reactor (CSTR) was also operated as a control to compare with the results of SBR. The CSTR was operated with same retention time as the SBR (13.3 hr). The CSTR was operated in a 1 L glass reactor that was baffled, stirred, and continuously fed with sterile medium containing phenol. Oxygen was supplied by adding air every 2 hr interval for 1 min.

Analytical Methods

High-performance liquid chromatographic (HPLC) analysis of phenol and individual PAH was performed by using the Altex Model 110A pump fitted with a reversed-phase Ultremex C_{18} column (150 mm x 4.60 mm; Phenomenex) and a UV detector. The solvent system consisted of methanol/water (70%:30% v/v) for the analysis of phenol and acetonitrile/water (50%:50% v/v) for all PAHs. The flow rate was 1.5

ml/min. The elution of phenol and PAHs were monitored at 274 and 254 nm respectively. Cell density was determined spectrophotometrically by measuring absorbance at 600 nm.

The co-metabolic oxidation products of naphthalene were identified by GC-MS. Samples (10 ml) of the reaction medium were concentrated 10-50-fold by evaporation under vacuum. These samples were then evaporated to dryness under a gentle stream of N_2 and the residue was finally redissolved in hexane (200 µl). A 1 µl sample of this material was analyzed. The GC-MS analysis was performed by using a Hewlett Packard 5988 mass spectrometer connected to a 5890 gas chromatograph (GC) fitted with an XTI-5 fused silica capillary column. The GC was operated with injection temperature of 250° C. The column temperature was set at 50° C for 1 min and then increased linearly at 10° C/min until a final temperature of 300° C was reached. Injector and detector temperature were set at 290° C and 315° C, respectively. Helium was used as the carrier gas at flow rate of 20 ml/min. Chemical structures of metabolites were suggested on the basis of their mass spectra, an instrument library search, and literature data. Where authentic samples were available, metabolites were identified by comparing mass spectra and GC retention time (R_t) with those observed for authentic compounds.

RESULTS

Co-metabolism in the biological transformation of a compound which cannot be utilized as a source of energy or carbon by an organism grown on another substrate. Our hypothesis in this study was that the structural similarities between phenol and PAHs might allow phenol-oxidizing organisms to co-metabolically degrade PAHs. To test this hypothesis we attempted to isolate phenol-oxidizing organisms from activated sludge and then examine these organisms for their ability to degrade PAHs after growth on phenol. Using the approaches described in the Methods section we isolated several bacterial strains capable of growth on phenol as a sole source of carbon and energy. The studies reported here were conducted with a single isolate designated *Pseudomonas* TJ-1. This isolate was a gram-negative rod which was identified by the Biolog procedure as a *Pseudomonas cepacia.*

Batch Experiments

The PAH-degrading activity of *Pseudmonas* TJ-1 was examined for a range of PAHs under standard conditions (Table 2-1). For each PAH four incubations were conducted: Incubation #1 served to follow growth of *Pseudomonas* TJ-1 on phenol. Incubation #2 served to follow the extent of PAH consumption when each PAH was provided as a sole source of carbon and energy for *Pseudomonas* TJ-1. Incubation #3 served to follow PAH consumption by *Pseudomonas* TJ-1 grown the presence of phenol. Finally, incubation #4 served to follow the abiotic depletion of both phenol and the tested PAH over the time course of the experiment. Of the 10 PAH compounds tested by this approach, only three compounds (naphthalene, 2-methyl naphthalene and anthracene) were found to be degraded in the presence of phenol (Fig. 2-3).

The results obtained for naphthalene are typical of the results obtained for all degradable PAHs. For example, in the absence of naphthalene (Fig. 2-3A) the phenol growth substrate was completely consumed within 4 days and this was associated with prolific microbial growth. When phenol and naphthalene were added simultaneously, the time course of phenol consumption was delayed and complete consumption was only observed after 7 days (Fig. 2-3A). Naphthalene degradation was only observed once the concentration of phenol had been substantially reduced and complete consumption of the added naphthalene occurred only after 9 days (Fig. 2-3B). In contrast, low rates of naphthalene degradation were observed in inoculated incubations containing naphthalene alone and these rates were equivalent to the rate of loss observed in the uninoculated, abiotic controls (Fig. 2-3B). Further studies of the effects of naphthalene and phenol were also conducted to investigate the causes of the lag phases in phenol consumption and the unusual kinetics of naphthalene degradation observed in the experiment described in Fig. 2-3. We observed that progressive increases in the initial naphthalene concentration present in the phenol-containing growth medium led to commensurate increases in the lag phase of phenol consumption, as compared to cultures grown on phenol in the absence of naphthalene (Fig. 2-7A). As in our previous experiment (Fig. 2-3), we consistently observed that naphthalene consumption only occurred once the concentration of phenol had been substantially reduced in each incubation (Fig. 2-7B). Finally, we also observed that naphthalene-dependent lag phases in phenol consumption were associated with very similar lag phases in the development of microbial biomass (Fig. 2-7C) although in all incubations the final level of biomass production was very similar.

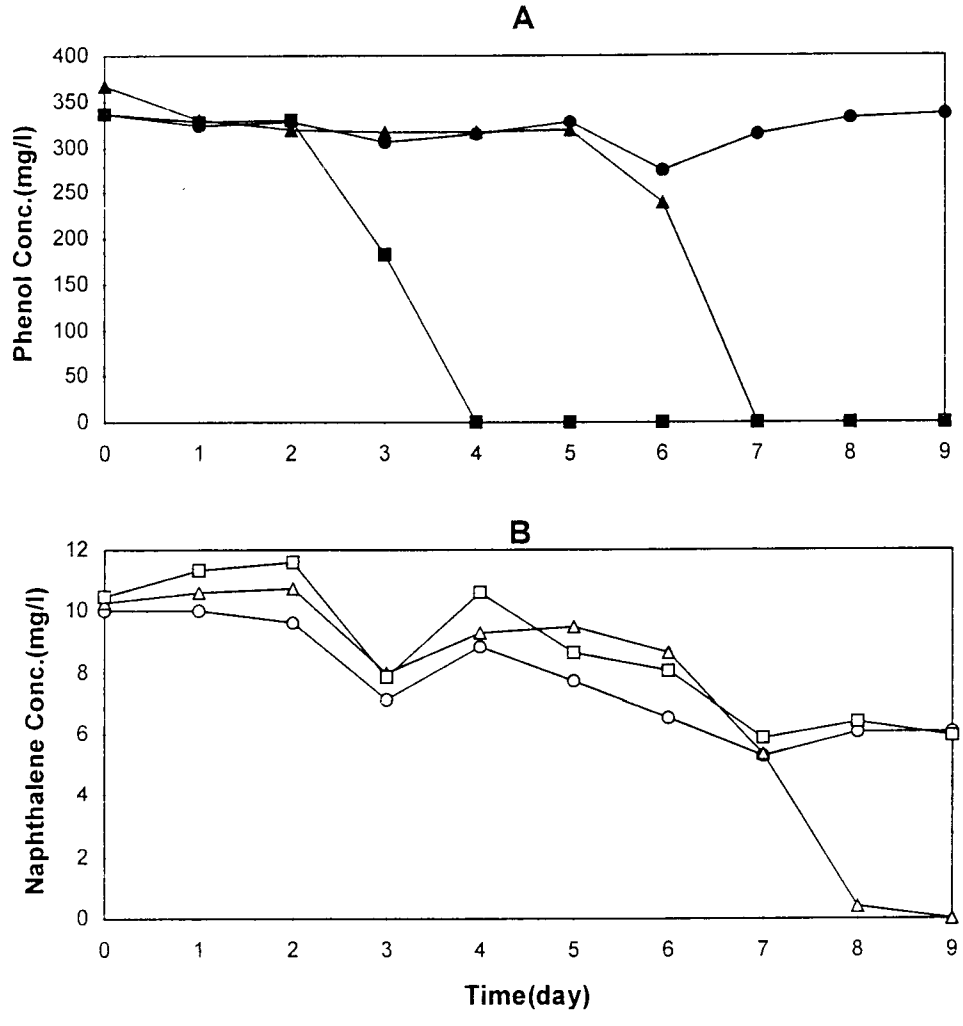

Figure 2-3. Time course for naphthalene degradation in phenol-grown batch culture. Initially naphthalene and phenol were fed simultaneously, and detail descriptions for preparation are described in Materials and Methods. (A) Time course of phenol consumption incubated with cells and (■) phenol alone, (▲) phenol and naphthalene, and (●) phenol control without cells. (B) Time course of naphthalene degradation in the medium incubated with (□) naphthalene alone, (△) naphthalene and phenol, and (○) naphthalene control without cells.

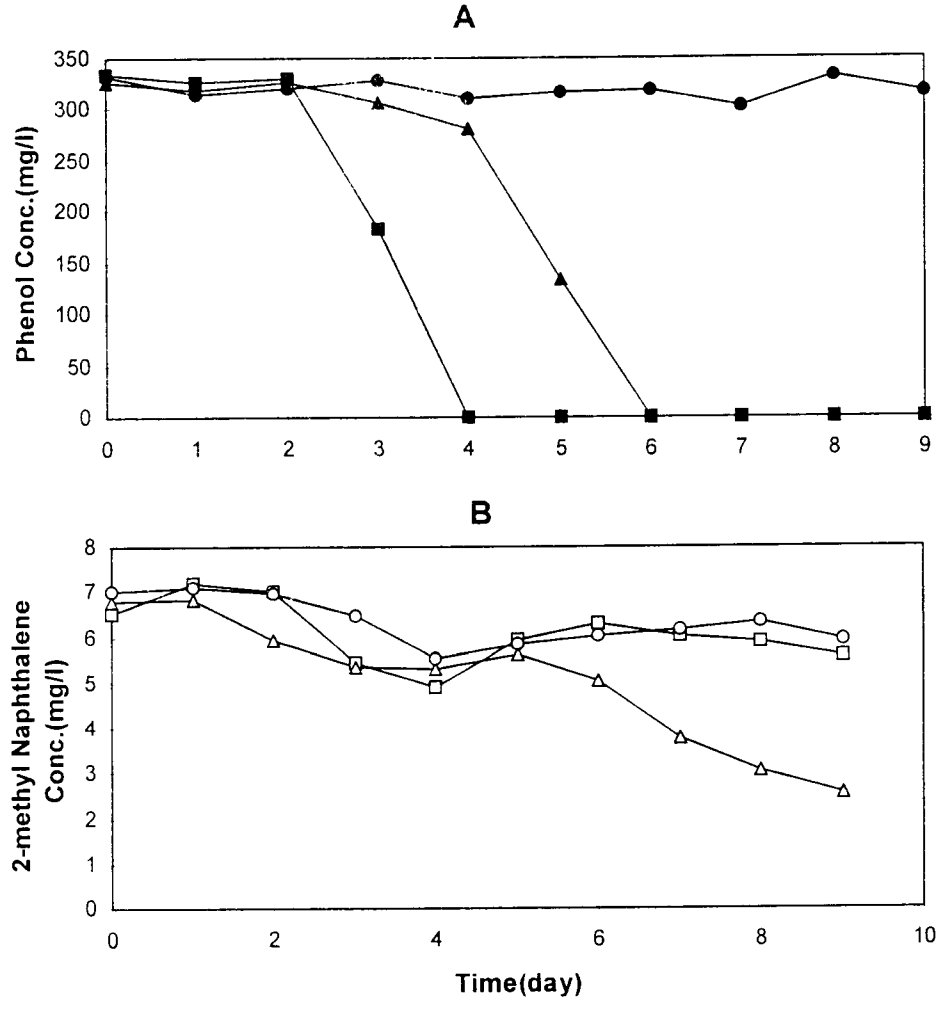

Figure 2-4.Time course for 2-methyl naphthalene degradation in phenol-grown batch culture. Initially 2-methyl naphthalene and phenol were fed simultaneously, and detail descriptions for preparation are described in Materials and Methods. (A) Time course of phenol consumption incubated with (■) phenol alone, (▲) phenol and 2-methyl naphthalene, and (●) phenol control. (B) Time course of 2-methyl naphthalene degradation incubated with cells and (□) 2-methyl naphthalene alone, (△) 2-methyl naphthalene and phenol, and (○) 2-methyl naphthalene control.

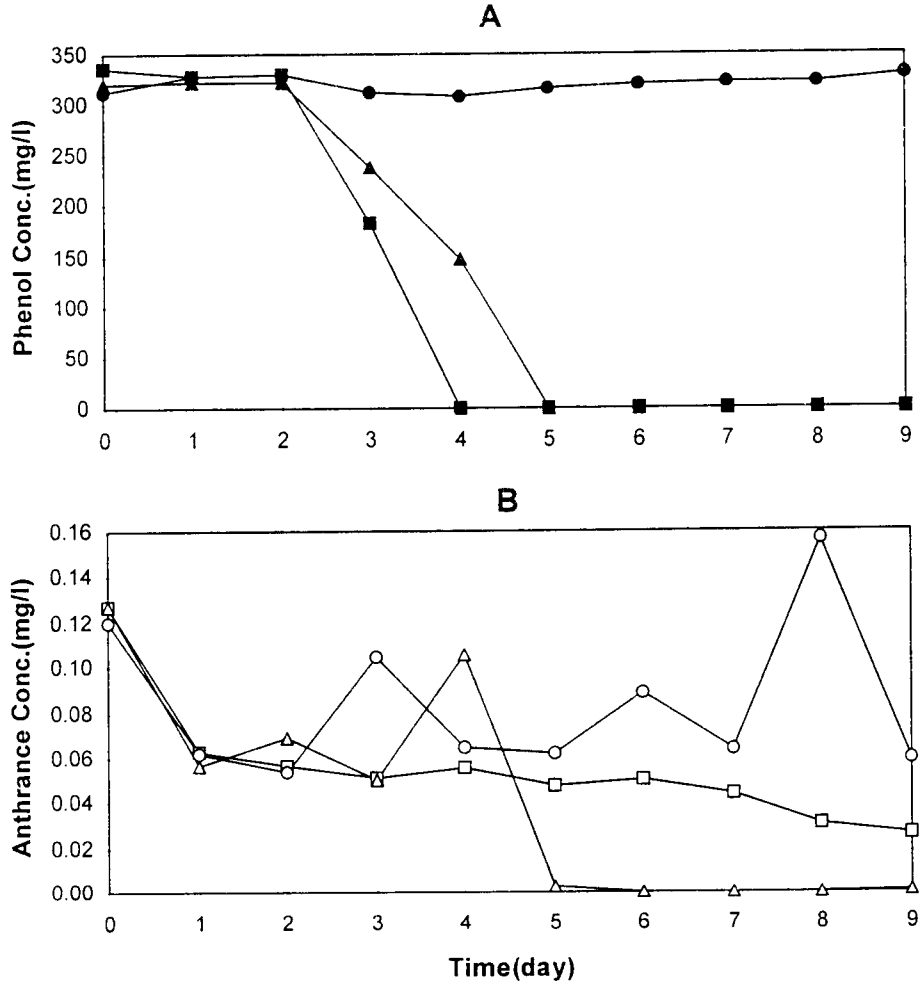

Figure 2-5.Time course for anthracene degradation in phenol-grown batch culture. Initially anthracene and phenol were fed simultaneously, and detail description for preparation are described in Materials and Methods. (A) Time course of phenol consumption incubated with cells and (■) phenol alone, (▲) phenol and anthracene, and (●) phenol control. (B) Time course of anthracene degradation incubated with cells and (□) anthracene alone, (Δ) anthracene and phenol, and (○) anthracene control.

Our results to this point suggest that naphthalene was co-metabolically oxidized by phenol-grown *Pseudmonas* TJ-1 and that this activity involved the same enzyme required for initiating phenol oxidation. If correct, this conclusion would imply that the products of naphthalene co-metabolism should be compatible with a reaction catalyzed by a phenol-oxidizing monooxygenase, assuming no further transformation of these co-metabolites occurs. Our results (Fig. 2-6) confirm this hypothesis and demonstrate that naphthalene oxidation by phenol-grown *Pseudomonas* TJ-1 resulted in the extracellular accumulation of a mixture of mono-hydroxylated products, of which 1- naphthol was the predominant product (Fig.2-6). Further studies demonstrated that total naphthol production accounted for > 90% of total naphthalene consumption in batch experiments (data not shown). In addition, we also examined the effect of exogenous reductant sources on naphthalene co-metabolism by phenol-grown *Pseudomonas* TJ-1. The quantity of naphthalene which was degraded by phenol grown cells was significantly increased by the addition of acetate as a potential electron donor. For example, greater than 90% of the total added naphthalene (13 mg/l) was degraded when acetate (3.2 mM) was added with naphthalene (Fig. 2-8). In contrast, only 60% of the added naphthalene was consumed when phenol-grown cells were incubated with naphthalene in the absence of acetate (Fig. 2-8) whereas no naphthalene degradation was observed for cells grown on acetate alone. The removal efficiencies and transformation yields for the experiment described in Fig. 2-8 are summarized in Table 2-2. The stimulating effect of acetate suggests that naphthalene degradation is limited by the availability of reductant. This conclusion is compatible with the apparent inability of *Pseudomonas* TJ-1 to obtain reductant either by utilizing naphthalene as a sole carbon and energy source or by further oxidizing the products of naphthalene oxidation (*e.g.* 1-naphthol). These results also demonstrate that

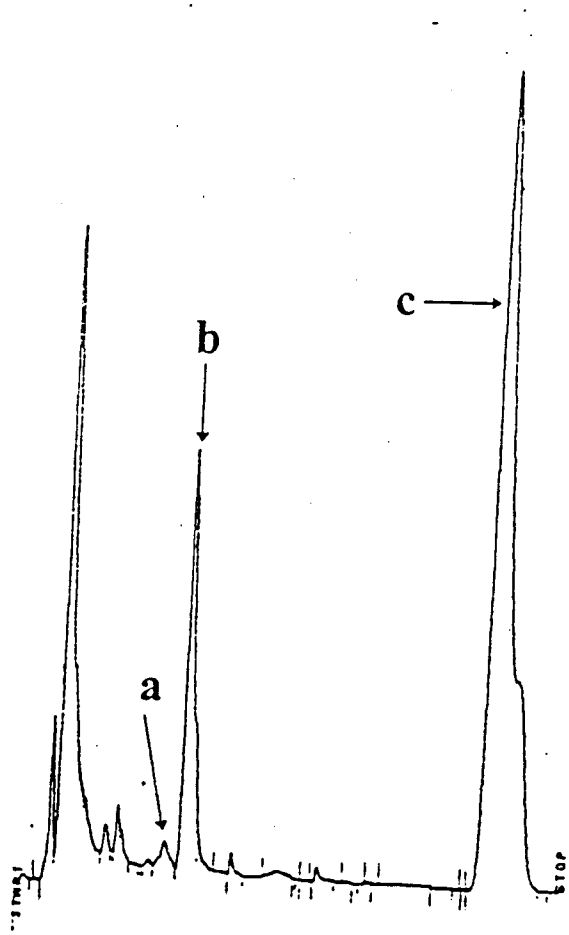

Figure 2-6. HPLC Chromatogram showing the oxidation of naphthalene to 1- and 2-naphthol by phenol-utilizing bacteria. Sample was taken after 1 hr incubation as described in Materials and Methods. Peak (a) 2-naphthol, (b) 1-naphthol and (c) naphthalene.

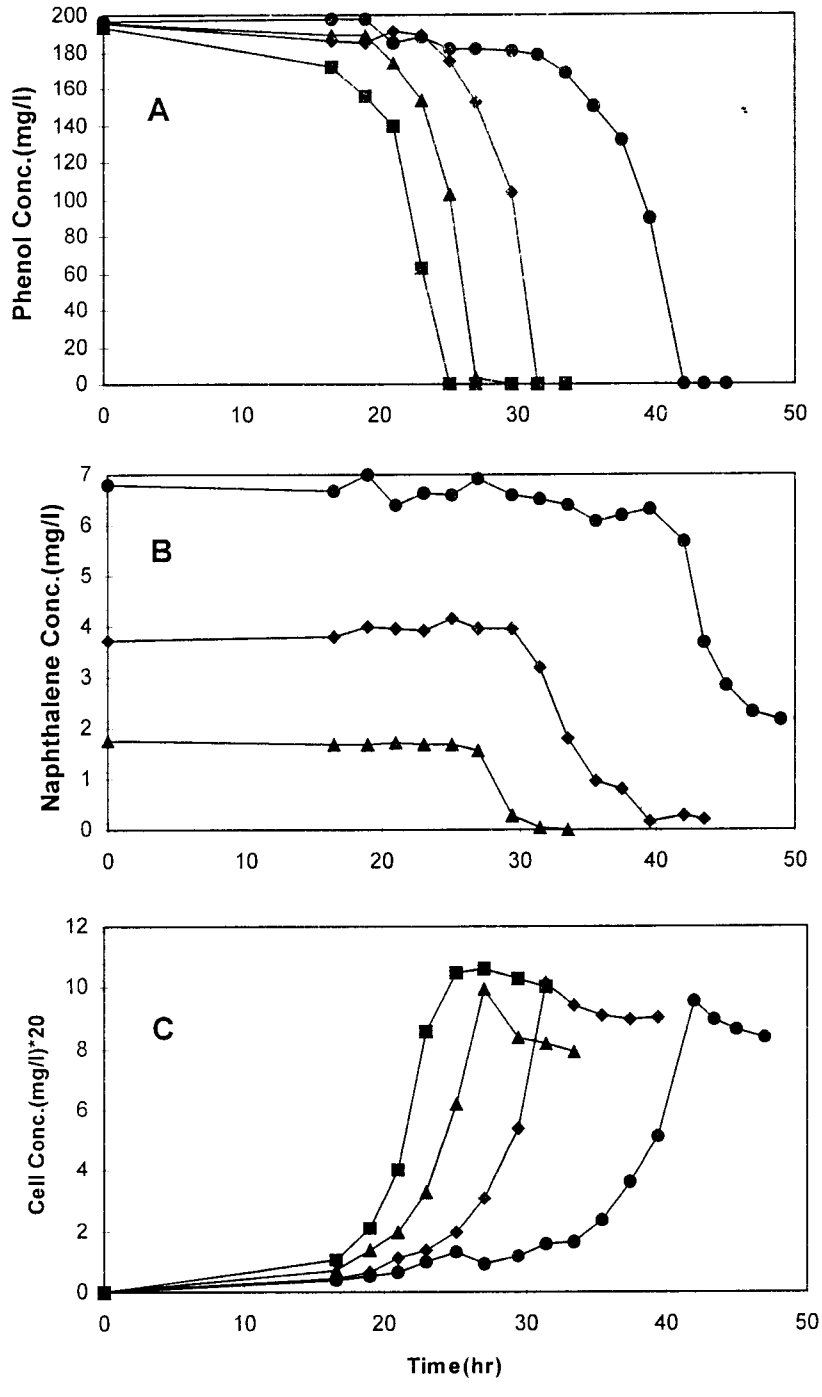

Figure 2-7. Time course for lag period due to naphthalene concentration. Figure (a), (b), and (c) represent phenol, naphthalene degradation and cell growth with 0 (■), 1.7(▲), 3.7(◆), and 6.7(●) mg/l naphthalene.

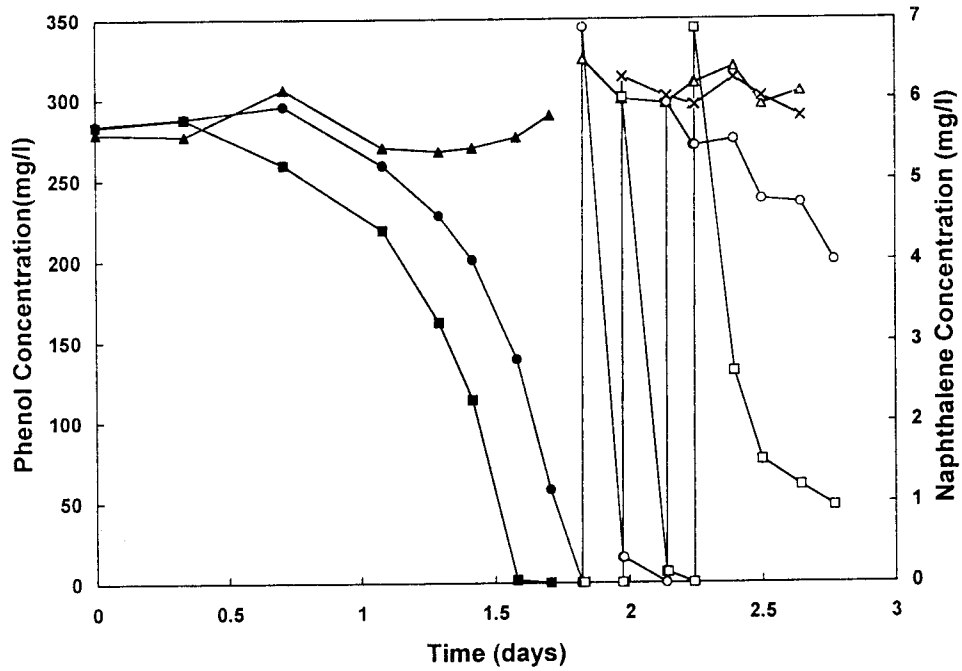

Figure 2-8. Time course for naphthalene degradation with/without pulsed acetate addition in the medium for phenol grown cells. Phenol was fed to bottle #1 (•) and bottle #2 (▪). After phenol was completely consumed, only naphthalene(○) was added twice to bottle #1 and naphthalene & acetate was added to bottle #2 (□). Phenol and naphthalene controls without cells at bottle #3 (▲), bottle #4 (×) also were conducted.

acetate-grown cells do not express the enzymes required for naphthalene degradation and this observation again supports our hypothesis that the co-metabolism of naphthalene and other PAHs requires the enzymes normally expressed for phenol oxidation.

Table 2-1. Removal efficiencies and transformation yields of naphthalene with/without acetate in batch culture experiment

Phenol added (mg/l)	Naphth added (mg/l)	Final naphth (mg/L)	Acetate added (mg/l)	Removal efficiency (%)	Trans. Yields mg naphth / mg phenol
281	13.2	0.9	180	93.2	0.044
284	13.1	4.1	0	68.7	0.031

Reactor Experiments

Our results with batch cultures presented above (Figs 2-3 ~ 2-6) suggest that the simultaneous presence of both phenol and PAHs results in an inhibition of phenol oxidation and a delay in the onset of PAH degradation. We considered it likely that this effect could be attributed largely to a competitive interaction between phenol and PAHs for oxidation by the same monooxygenase type enzyme. To overcome this potential competitive effect a Sequencing Batch Reactor (SBR) system was developed and operated as described in the Methods section. Our results demonstrate that a very reproducible rate of phenol consumption could be demonstrated in the reactor once steady state conditions had been established (Fig. 2-9). After 5 days of operation the reactor contents were partially drained (700 ml removed) and an equivalent volume of a solution of naphthalene (4.5 mg/l influent concentration) was added to give a theoretical initial diluted concentration of 3.2 mg/l. However, during this "fill" phase some 73 % (2.2 mg/l)

of the naphthalene was rapidly oxidized by the remaining phenol-utilizing cells in the reactor. During the subsequent "reaction" phase the remainder of the naphthalene was consumed although the rate of naphthalene degradation had declined, as compared to the rate observed during the earlier "fill" phase. At Day 7 the reactor was recycled with a further addition of phenol and the reactor was subsequently challenged with a further exposure to naphthalene with added acetate as a source of reductant. No substantial differences in the rates of naphthalene degradation were observed between the unamended and acetate-containing reactor cycles. Similar experiments were also conducted with the SBR using concentrations of naphthalene as high as 7.5 mg/l (Data not shown).

As a control for the SBR, simultaneous experiments were also conducted with a CSTR (Continuous Stirred-Tank Reactor) which was operated as described in the Methods section (Fig. 2-10). In the CSTR the influent phenol concentration was maintained constant at approximately 180 mg/l. At Day 15 naphthalene was added at a constant concentration of 3.7 mg/l. A constant rate of naphthalene degradation was established in which 56.8 % of the influent naphthalene was removed with a transformation yield of 0.011 mg naphthalene/ mg phenol. At Day 17 the influent concentration of naphthalene was increased to 7.2 mg/l. After this the removal efficiency was decreased to 50 % of the influent naphthalene with a transformation yield of 0.022 mg naphthalene /mg phenol. The summarized results of removal efficiencies and transformation yields for both the SBR and CSTR are shown in Table 2-2. These results demonstrate that much higher removal efficiencies and transformation yields were achieved in the SBR as compared to the CSTR. Having established the SBR as the more

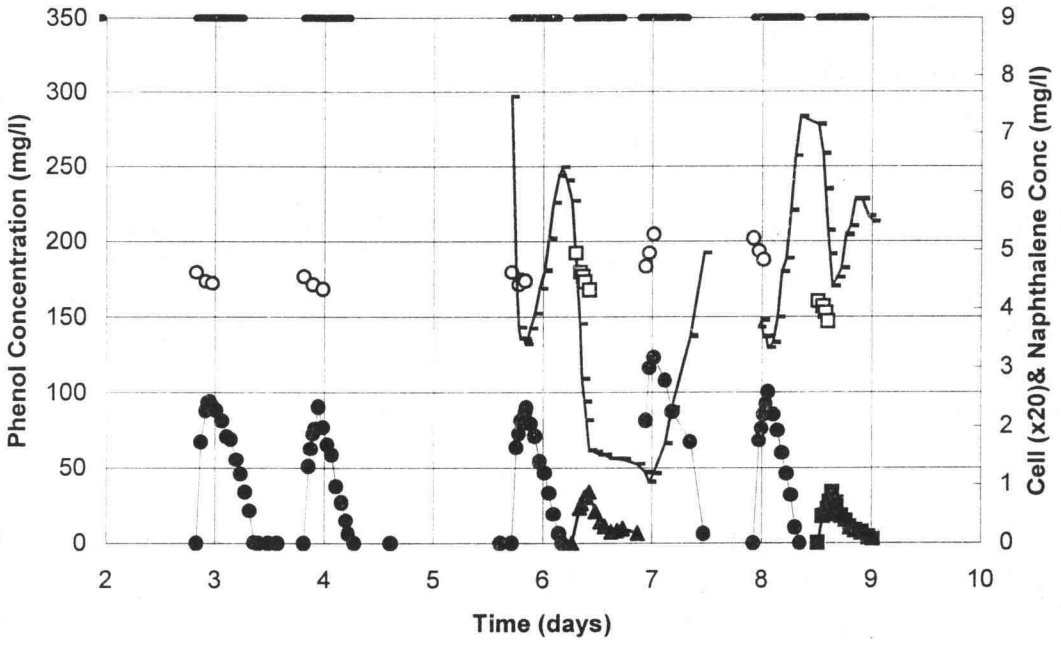

Figure 2-9. Time course of sequencing batch reactor (SBR) experiment. Experimental conditions are described in Materials and Methods. The (-) line at the top of figure represent the retention time of CSTR as control of SBR. Symbol : Influent phenol(○), effluent phenol(●), effluent naphthalene(▲), influent naphthalene(□), and effluent naphthalene with acetate addition(■).

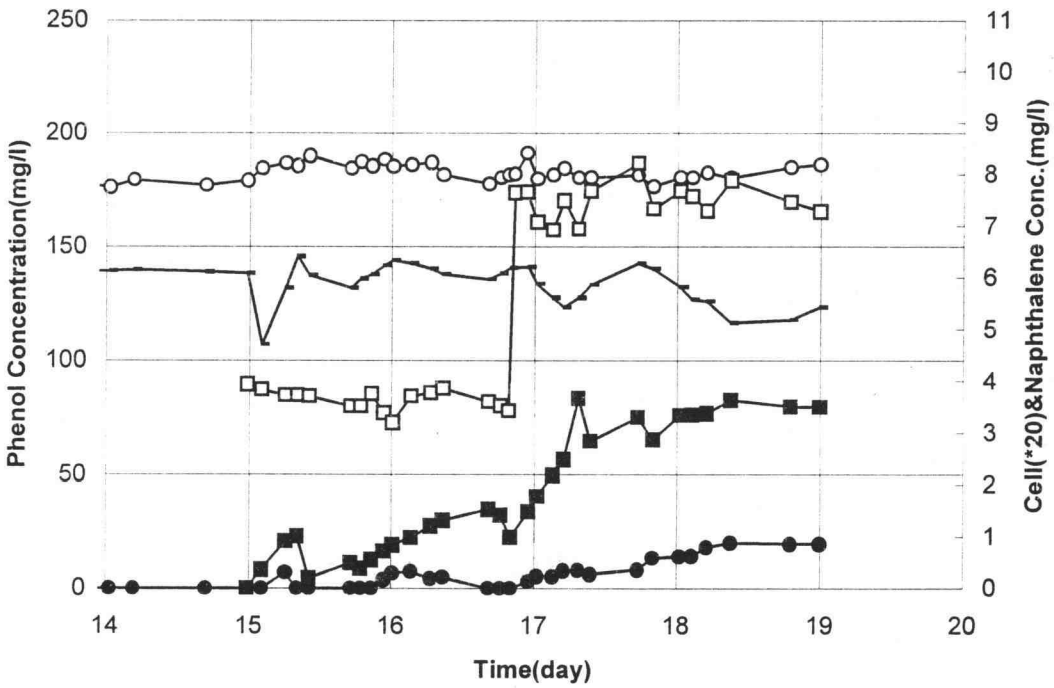

Figure 2-10. Time course of CSTR experiment to compare with SBR. Symbol : Influent phenol (○), effluent phenol(●), influent naphthalene(□), and effluent naphthalene(■).

efficient reactor configuration for PAH degradation, we were also interested to determine whether the SBR could be used effectively for the concurrent removal of mixtures of PAHs. The simultaneous degradation of naphthalene, 2-methyl naphthalene, and anthracene by phenol-grown cells was achieved using the SBR system (Fig. 2-11).

Table 2-2. Removal efficiencies and transformation yields of Sequencing Batch Reactor (SBR) and Continuous Stirred-Tank Reactor (CSTR)

	Influent Phenol (mg/l)	Effluent Phenol (mg/L)	Influent Naphthalene (mg/l)	Effluent Naphthalene (mg/L)	Removal efficiency (%)	Trans. Yields $\frac{\text{mg naphth}}{\text{mg phenol}}$
SBR	122	0	3.2	0.17	94.6	0.025
	136	0	2.8*	0.06	97.8	0.02
	127	0	6.2	0.52	91.6	0.045
	126	0	7.5*	0.43	94.3	0.056
CSTR	182	0	3.7	1.6	56.8	0.011
	181	19	7.2	3.6	50.0	0.022

* with 78 mg/l acetate added.

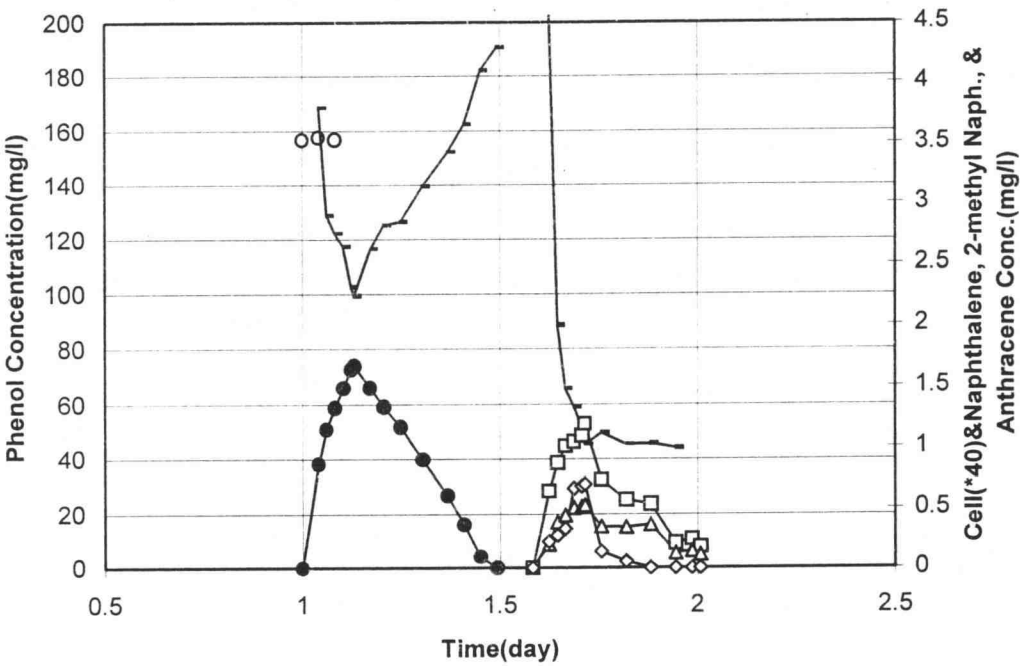

Figure 2-11. Time course of sequencing batch reactor (SBR) experiment with simultaneous PAHs addition. Symbol: Influent phenol(○), effluent phenol(●), effluent naphthalene (□), effluent 2-methyl naphthalene (Δ), effluent anthracene (✧). Anthracene concentration represented was 100 times higher than actual concentration.

DISCUSSION

The results indicated that microorganisms grown on phenol can degrade PAHs cometabolically. The ability of microorganisms to effectively co-metabolize compounds under aerobic conditions is dependent on many factors. One important physiological and regulatory factor is which growth substrates allow for the expression of the enzyme systems responsible for the cometabolic activity. Another important kinetic factor, especially for monooxygenase-catalyzed co-metabolic reactions, is how the growth substrate and the co-substrate utilization patterns influence the supply of reductant to the non-specific monooxygenase responsible for substrate oxidation reactions.

Our results (Figs 2-3 ~ 2-5) demonstrated that PAHs are only degraded by *Pseudomonas* TJ-1 when cells are grown in the presence of phenol. These results strongly support the hypothesis that naphthalene degradation requires catabolic enzymes associated with phenol utilization. Moreover, our results indicated that the presence of naphthalene initially inhibits phenol consumption although naphthalene consumption is only initiated when the ambient phenol concentration has been significantly depleted. In addition, naphthalene was a stronger inhibitor than 2-methyl naphthalene and anthracene.

These results illustrate that the higher inhibitory effect observed for naphthalene than for other compounds is probably due to a higher affinity of naphthalene for phenol monooxygenase. This result is similar to the results of other researches who have shown phenol utilizing organisms degrade TCE effectively (25), but with significant competitive inhibition. Inhibition of cell growth and phenol degradation due to variations in the concentration of naphthalene added is clearly shown in Figure 2-6. The other compounds, 2-methyl naphthalene and anthracene, possibly cause less degrees of inhibition of phenol

oxidation than naphthalene because of their lower affinity for the phenol-oxidizing enzymes as shown in Figure 2-8.

Our results demonstrated that the addition of acetate supported the phenol and naphthalene-oxidizing activities in batch systems although similar results were not observed in the SBR studies. The effect of acetate in batch experiments probably reflects the used for reductant to maintain enzyme activity and to improve removal rate effectively. Formate was used very effectively as a reductant to support the methane monooxygenase (MMO) activity of methanotrophs (30).

Phenol-utilizing bacteria generally do not have broad nongrowth substrate ranges like methane- and ammonia-utilizing bacteria, but, they can catalyze the oxidation of phenolic compounds (31, 42) and chlorinated solvents including TCE and DCE (25). The results of this work indicated that phenol monooxygenase can also catalyze the oxidation of 2- and 3-ring PAHs to hydroxylated PAHs. Menke *et al.* (31) observed the degradation of mixtures of monochlorophenols by phenol-utilizing bacteria cometabolically, with a different degree of transformations. A variety of di- and tri-chlorophenols were degraded in the presence of phenol (42) which suggested that a different degradation rate might be due to both the number of chlorines and their position on the phenolic ring. Phenol-oxidizing bacteria also cometabolically degrade several chlorinated solvents including c-DCE, TCE and t-DCE (25). Brusseau *et al.* (1990) demonstrated naphthalene oxidation by *Methylosinus trichosporium* OB3b which oxidized naphthalene to 1- and 2-naphthol.

Cometabolic degradation of PAHs using lower molecular weight PAH as a carbon and energy source has been observed by several researchers (7, 34, 38, 39). A *Rhodococcus* species has been shown to use pyrene as a sole carbon and energy source and pyrene grown cells cometabolically degraded naphthalene, dibenzofuran, fluorene

and dibenzothiophene (38). Similarly, Weissenfels *et al.* (39) observed that *Alcaligenes denitrificans* WW1 utilized naphthalene, 1-and 2-methyl naphthalene, phenanthrene and anthracene as sole carbon sources and cometabolized fluorene, and pyrene. Bouchez *et al.*(7) also showed that *Pseudomonas* strains were capable of degrading PAHs cometabolically.

The products of naphthalene oxidation by phenol-utilizing bacterium were determined by HPLC (Fig. 2-7) and GC-MS. We observed both 1- and 2-naphthol production from naphthalene oxidation, although 1-naphthol accounted more than 90 % of the total products. A similar predominant production of 1-naphthol occur with other monooxygenases including fungal cytochrome P-450 enzymes (12, 13, 21) and methane monooxygenases (8, 18). Substrate interactions during the biodegradation are important for the understanding of the environmental behavior of PAHs. In our study (Fig. 2-11), we observed that anthracene inhibited naphthalene and 2-methyl naphthalene degradation. Further degradation of naphthalene and 2-methyl naphthalene were accelerated after anthracene was completely transformed. Similar inhibitory effects on acclimation of benzene were observed when both toluene and *p*-xylene were present (1).

The effects of competitive interaction between phenol and PAHs in our batch experiments led us to examine the sequencing batch reactor (SBR) as an alternative system to minimize inhibition effects between the substrates. Previously, a number of reactor studies were investigated to evaluate the possibility of PAH degradation in contaminated soil or industrial waste water. A slurry-phase bioreactor was operated successfully to remove a creosote-contaminated soil containing semivolatile compounds including PAHs and volatile compounds including toluene, xylene, and benzene (28). A bench scale rotating-drum bioreactor demonstrated PAH degradation using mixed

cultures (3). Cardinal *et al.* (10) showed enhanced removals of naphthalene and phenanthrene under aerobic condition. Our results demonstrated that significantly higher removal efficiencies and transformation capacities were observed in the SBR operation compared to in CSTR. These results have implications for the future treatment of hazardous waste at POSCO and other plants treating complex PAHs mixtures.

Cometabolism is an attractive procedure for bioremediation when toxic compounds are present at low concentrations. In our study, the transformation of PAHs by phenol-utilizing bacteria generated hydroxylated products which might be more soluble than parent compounds. This increased solubility will result in increased bioavailability for other microorganisms in the waste stream.

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Chapter 3

Cometabolic Degradation of Naphthalene and Other Polycyclic Aromatic Hydrocarbons (PAHs) by *Nitrosomonas europaea*

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ABSTRACT

The soil nitrifying bacterium *Nitrosomonas europaea* has shown the ability to cometabolically transform a range of 2- and 3- ringed polycyclic aromatic hydrocarbons (PAHs). A strong inhibitory effect of naphthalene on ammonia oxidation by *N. europaea* was observed. Naphthalene was readily oxidized by *N. europaea* and 2-naphthol was detected as the major oxidation product (> 85 %) of naphthalene oxidation. The rates of naphthol and nitrite production were dependent on the both concentrations of ammonia and naphthalene. The maximal rate of naphthol production was 1.65 nmole/mg protein-min in the presence of 240 μM naphthalene and 10 mM NH_4^+. With fixed NH_4^+ and varied naphthalene concentrations, naphthalene was oxidized greater than 2200-folder slower than ammonia at lowest concentration of naphthalene tested (15 μM) whereas at the highest concentration (240μM) naphthalene was only oxidized 100-fold slower than ammonia. NH_4^+ - and N_2H_2 -dependent O_2 uptake measurement demonstrate irreversible inhibitory effects of the naphthalene and subsequent oxidation products on AMO and HAO activity.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are ubIquitous compounds that originate from natural and anthropogenic pyrolysis of organic matter such as forest fires, automobile exhaust, coal-refining process, and the oil industry (3). Since many PAHs and substituted PAHs have been implicated as probable human carcinogens (19), there is considerable interest in processes which can either remove existing PAHs from contaminated environments or minimize further environmental contamination by these compounds.

Biological processes have considerable potential for the degradation of PAHs. Under aerobic conditions a wide variety of organisms can utilize PAHs as carbon and energy sources for growth (28). In contrast to the complete metabolism of PAHs, PAHs are known to be cometabolically transformed by microorganisms growing on other carbon and energy sources, such as phenol (6), methane (4), and even PAHs (3, 32, 33). In the present study, we have examined the PAH-oxdizing activity of the soil nitrifying bacterium *Nitrosomonas europaea* as an example of a cometabolically active organism grown on a substrate structurally unrelated to PAHs. As an obligate lithoautotrophic soil nitrifying bacterium *N. europaea* obtains all of its energy for growth from the oxidation of ammonia to nitrite. Ammonia is initially oxidized to hydroxylmine by the enzyme ammonia monooxygenase (AMO) (34) as follows: $NH_3 + O_2 + 2[H] \rightarrow NH_2OH + H_2O$. The further four-electron oxidation of hydroxylamine to nitrite is catalyzed by the hydroxylamine oxidoreductase (HAO) as follows: $NH_2OH + O_2 \rightarrow NO_2^- + H_2O + H^+$. This second reaction is the only source of the two electrons required to maintain steady-state AMO activity. The remaining two electrons are utilized for ATP synthesis through a

conventional electron transport chain (34). AMO transforms many chemicals that are known to contaminate wastewater, soil and groundwater. Previous studies have shown that whole cells of *N.europaea* can oxidize a wide variety of alternative hydrocarbon substrates, such as alkanes, alkenes, and aromatic compounds, through the action of AMO (12, 14, 15, 17, 21). More recently , it was shown that *N. euopaea* can also oxidize a wide variety of chlorinated aliphatic compounds, including TCE (25, 26, 27), alkyl ethers (18) and thioethers (20). In this study, we have examined the PAH oxidizing activity of *N. euroapea*, and we have studied in detail the interactions between ammonia and naphthalene as cosubstrates.

MATERIALS AND METHODS

Materials

Naphthalene, 1-naphthol, 2-naphthol, 2-methyl naphthalene, 2-methyl naphthalenol, 2-methyl naphthaledehyde, acenaphthalene, acenaphthene, fluorene, anthracene, phenanthrene and acenaphthenone were obtained from Aldrich (Milwaukee, WI). Allythiourea was obtained from Eastman Kodak Co. (Rochester, N.Y.). All other chemicals were of reagent grade.

Growth of *Nitrosomonas europaea*

Cells of *N.europaea* (ATCC 19178) were grown in batch cultures (1.5 liters) and harvested by centrifugation and finally resuspended in phosphate buffer (50 mM sodium

buffer[pH 7.8], 2 mM $MgSO_4$), as described previously (12). In all cases, cell suspensions (0.2 g[wet weight] per ml) were stored on ice and used within 24 h of harvesting.

PAH degradation experiments

Experiments examining the degradation of PAHs by *N. europaea* were conducted in serum vials (37 ml) sealed with Teflon-lined silicone septa (Sun Brokers™, Wilmington, NC). The incubation medium (10 ml) consisted of phosphate buffer (50 mM potassium phosphate, pH 7.8, 2 mM $MgSO_4$) and 5 mM $(NH_4)_2SO_4$. In experiments with naphthalene required concentrations of naphthalene were added from stock solution (0.164 M) prepared in dimethyl sulfoxide (DMSO). The vials were placed in a sonicator for 10 min to allow complete dissolution of naphthalene. With all other PAHs examined saturated concentrations of each compounds were added. In all cases the reaction was initiated by addition of cells (100 µl; approximately 1 mg of protein). The vials was then returned to the shaker. To determine the consumption of PAHs and accumulation of oxidation products, 0.5 ml sample in liquid phase was removed and filtered with a syringe filter to remove cells before the analysis by high performance liquid chromatography (HPLC). To establish the role of AMO in PAH transformations control incubations were conducted as above contained cells suspensions treated with acetylene (1 %), a specific and irreversible inactivator of AMO (16).

O_2 uptake measurement

The effects of PAHs and their oxidation products on the activities of the two enzymes involved in ammonia oxidizing activity were determined by measuring rates of

NH_4^+ - and N_2H_4 - dependent O_2 uptake, as described previously (13). O_2 uptake measurements were made using a Clark style O_2 electrode (Yellow Springs, Ohio) mounted in a glass water-jacked reaction vessel (1.8 ml). Cells exposed to PAHs, or their oxidation products, were sedimented by centrifugation in a microfuge (10,000 rpm for 1 min) and resuspended in fresh buffer (1.5 ml). Samples of these washed cells (50 µl) were added to buffer in the O_2 electrode reaction chamber and the rate of O_2 uptake activity was measured after the addition of 5 mM $(NH_4)_2SO_4$. Once a steady rate of ammonia-dependent O_2 uptake had been established, allythiourea (100 µM) was then added inhibit further NH_4^+-dependent O_2 uptake. Hydrazine was then added to a final concentration of 600 µM and the steady state rate of the residual hydrazine-dependent O_2 uptake was used as an estimate of residual HAO activity. All substrates and inhibitors were added from aqueous stock solutions by means of gas-tight microsyringes. The solubility of O_2 in air-saturated buffer was taken 230 µM (30).

Analytical Methods

The consumption of PAHs and the accumulation of PAH oxidation products was determined using high performance liquid chromatography (HPLC). The HPLC apparatus consisted of Altex Model 110A pump, a UV detector, reversed-phase ultramex C_{18} column (150 mm * 4.60 mm; Phenomex). Samples (200 µl) of the reaction mixtures were transferred into 1.5 ml Eppendorf tubes, and cells were removed by centrifugation. Samples (100 µl) of supernant were then injected into the HPLC. The PAH substrates and their oxidation products were eluted under isocratic conditions using acetonitrile:water mobile phase (50:50) at a flow rate of 1.5 ml/min. The eluted compounds were detected

by UV absorption at 254 nm and were identified by coelution with authentic compounds and by GC-MS analysis of eluted compounds.

GC-MS analysis was conducted using the fractions (20 ml) that were eluted from the HPLC column. The samples were evaporated to drynesss under a gentle stream of N_2, and the residue subsequently redissolved in hexane (5 ml). The hexane mixture was dried over anhydrous Na_2SO_4, and then evaporated to a final volume of 100 µl under a stream of N_2. Samples (2 µl) of the hexane solution were then injected into a Hewlett Packard model 5988 connected to a 5890 gas chromatograph (GC). The GC column was an XTI-5 fused silica capillary column. Helium, at flow rate of 20 ml/min, was used as the carrier gas. The GC was operated with an initial column temperature of 50 °C for 1 min and then increased linearly at 10 °C/min to a final temperature of 300 °C. The injector and detector temperatures were set at 290 and 315° C, respectively. The oxidation products were identified by comparison mass spectra and GC retention time with those observed for authentic compounds.

Nitrite concentrations were determined colorimetrically as described previously (10). Protein concentrations were determined by the biuret assay (9) after solubilization of cell protein in aqueous 3N NaOH for 30 min at 60° C, and sedimentation of insoluble material by centrifugation (14,000 × g, 5 min).

RESULTS

Inhibitory effects on nitrite production

Several previous studies with *N. euroapea* have indicated that alternative substrates for AMO can be detected through their inhibitory effects on ammonia oxidation. Theses inhibitory effects occur because of competitive interactions between ammonia and alternative substrate for simultaneous oxidation by AMO. To examine the effects of PAHs on ammonia oxidation we compared the effects of different concentrations of naphthalene on nitrite production during 2 hr incubations (Fig. 1). In the absence of naphthalene, cells consumed all of the added ammonia and produced nearly 10 mM nitrite within 1 hr. However, in the presence of 80 μM naphthalene, only 4.2 mM total nitrite accumulated after 2 hr. Increasing the naphthalene concentration to 240 μM resulted in less than 2 mM nitrite being generated over the same time period. No nitrite production was observed for the same incubation conditions with acetylene-treated cells which have no residual AMO activity (16).

Transformation of naphthalene

A subsequent HPLC analysis of the samples generated during the experiment described in Fig. 3-1 confirmed that naphthalene was oxidized by *N. europaea* and that the inhibitory effects on nitrite production were inversely related to the quantity of naphthalene oxidized (Fig. 3-2.). For example, for cells incubated with 80 μM naphthalene (Fig. 3-2B). we observed both 1- and 2-naphthol as products of naphthalene oxidation; 2-naphthol accounted for more than 85 % of the total products. In contrast, no

oxidation products were detected in the incubation with cells which were pretreated with acetylene. This result indicates that AMO is required for naphthalene oxidation.

The rate of naphthol productions

We were interested to determine the conditions that would support the maximal rate of naphthalene oxidation. To establish the actual rates of naphthalene degradation it

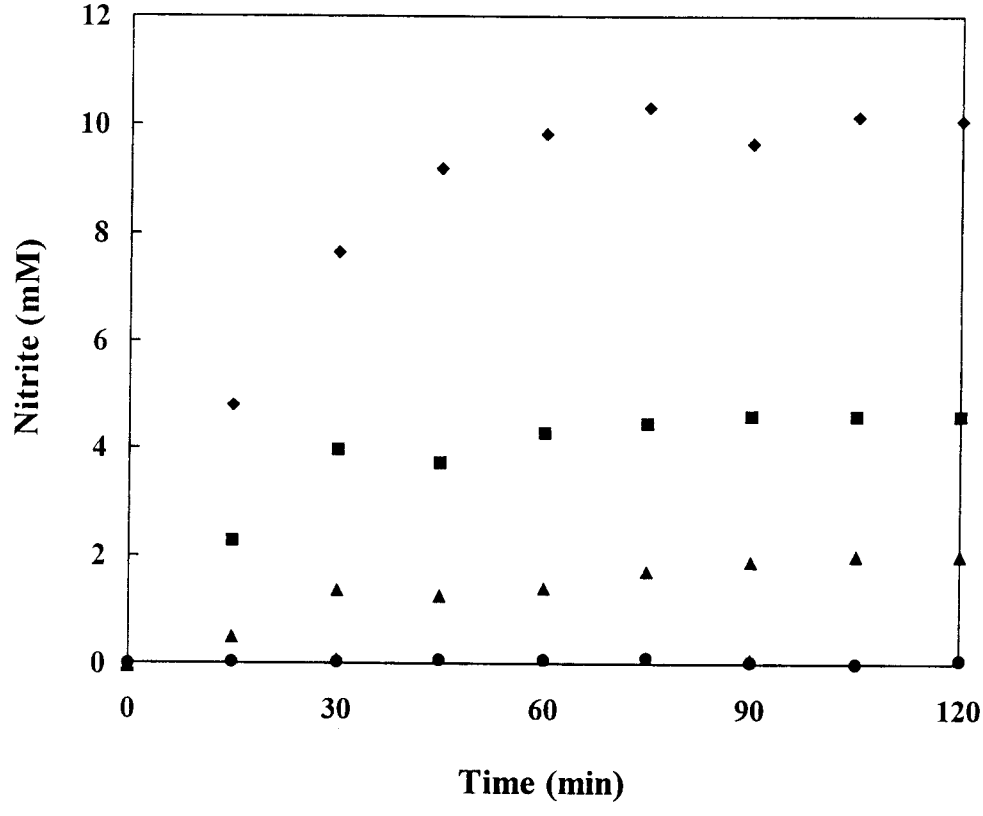

Fig. 3-1. Time course of nitrite production by *N. europaea* in the presence of varying naphthalene concentrations. Cells were incubated in serum bottles with 10 mM NH_4^+ and a range of naphthalene concentrations of 0 μM (◆), 80 (■), 240 (▲), and 240 (●). In the case of symbol • acetylene (1 %[vol/vol]) was also added. Aliquots (0.3 ml) of the reaction bottles were removed at the indicated time points, and the reaction was terminated with ATU (up to 100 μM) as described in Materials and Methods.

was necessary to determine the period of time over which the rate of naphthol production was constant. A time course experiment of naphthol production was initially conducted in the presence of 10 mM NH_4^+ and a range of naphthalene concentrations up to 240 μM, the limit of aqueous solubility for the compound. Our results (Fig. 3-3) demonstrate that the rate of naphthol production increased with increasing initial naphthalene concentration over the range of naphthalene concentrations examined. However, naphthol production rate was only constant during the initial portion (0 - 10 min) of the reaction and tended to decrease as the reaction progressed.

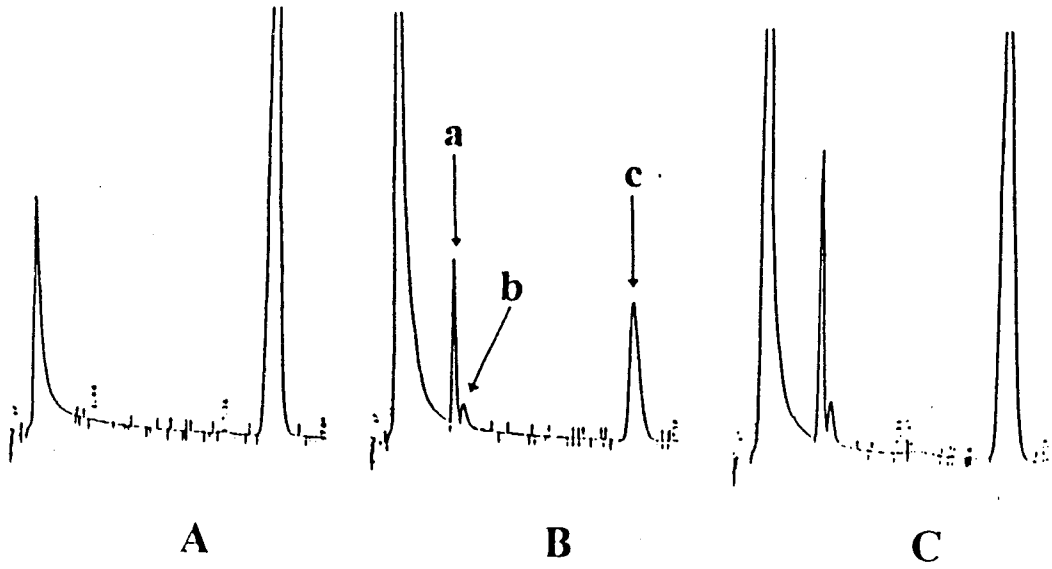

Fig 3-2. HPLC chromatograms showing oxidation of naphthalene by *N. europaea*. The samples were analyzed after 2 hr incubation from previous experiment as described in Materials and Methods. The each chromatograms were represented the samples incubated in the presence of cells with (A) 240 μM naphthalene and C_2H_2 (1 % v/v) (B) 80 μM naphthalene (C) 240 μM naphthalene. (a) 2-naphthol, (b) 1-naphhtol, (c) naphthalene

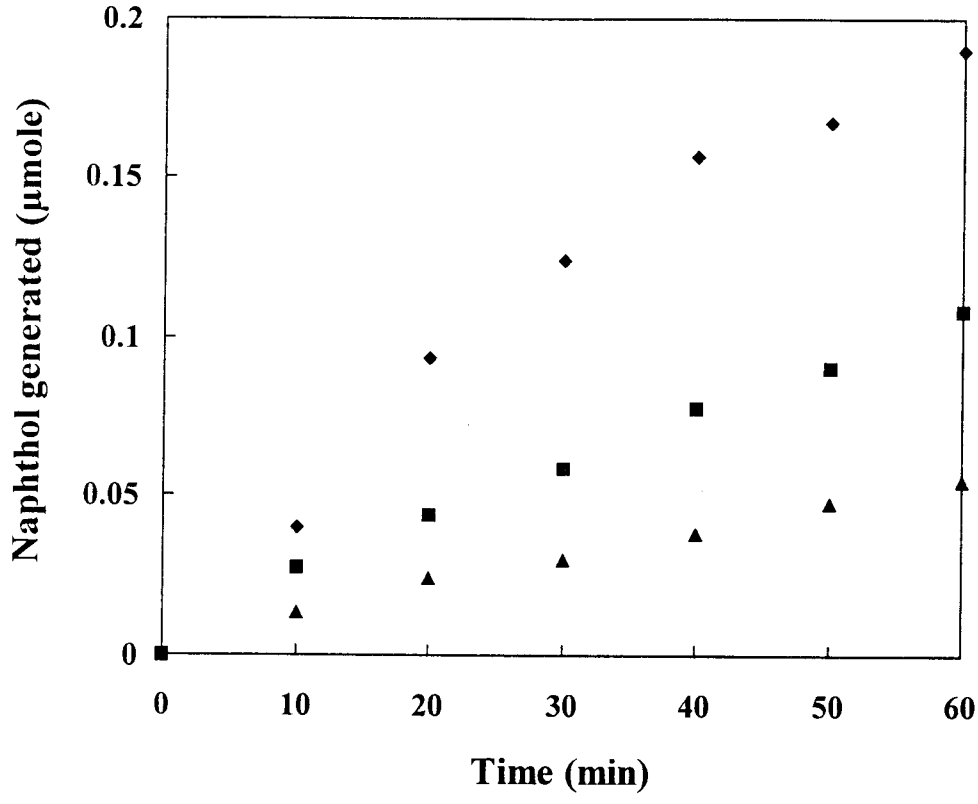

Fig. 3-3. Time course of naphthalene oxidation to naphthols by *N. europaea*. Assays were conducted as described in Materials and Methods in the presence of 80(▲), 120(■), and 240(◆) µM naphthalene with 10 mM NH_4^+ and cells.

Effects of naphthalene concentrations of naphthol and nitrite productions

Based on the previous experiment (Fig. 3-3), we examined the effect of naphthalene concentrations on both the rate of nitrite and naphthol production using the initial rates of naphthol production over the initial stages (0 - 10 min) of incubations. The experiment was conducted using varied naphthalene concentrations from 0 to 240 μM and a fixed concentration of NH_4^+ (10 mM). In the absence of naphthalene, the maximal rate of nitrite production (38 nmole/mg protein-min) was observed. Increasing concentrations of naphthalene led to a concomitant decrease in the amount of nitrite produced from ammonia oxidation and an increase in the rate of naphthol production from naphthalene oxidation. A plot of the relative rates of ammonia and naphthalene oxidation (Fig. 3-4B) demonstrate that the naphthalene was oxidized 2200-fold slower than ammonia at the lowest concentration of naphthalene examined (15 μM) whereas naphthalene was oxidized only 100-fold slower than ammonia oxidation at the highest concentration of naphthalene tested (240 μM).

Effects of NH_4^+ concentrations on naphthol productions

We also determined the effect of ammonia concentration on the rate of naphthol production. To prevent the complete oxidation of low concentrations of ammonia we examined naphthol production using a fixed concentration of napthalene (150 μM) in 10 min incubations using ammonia concentrations between 0 to 50 mM. This result (Fig. 3-5) demonstrates that the rate of naphthalene oxidation progressively increased with increases in ammonia concentration up to 10 mM NH_4^+ and then the rate of naphthol

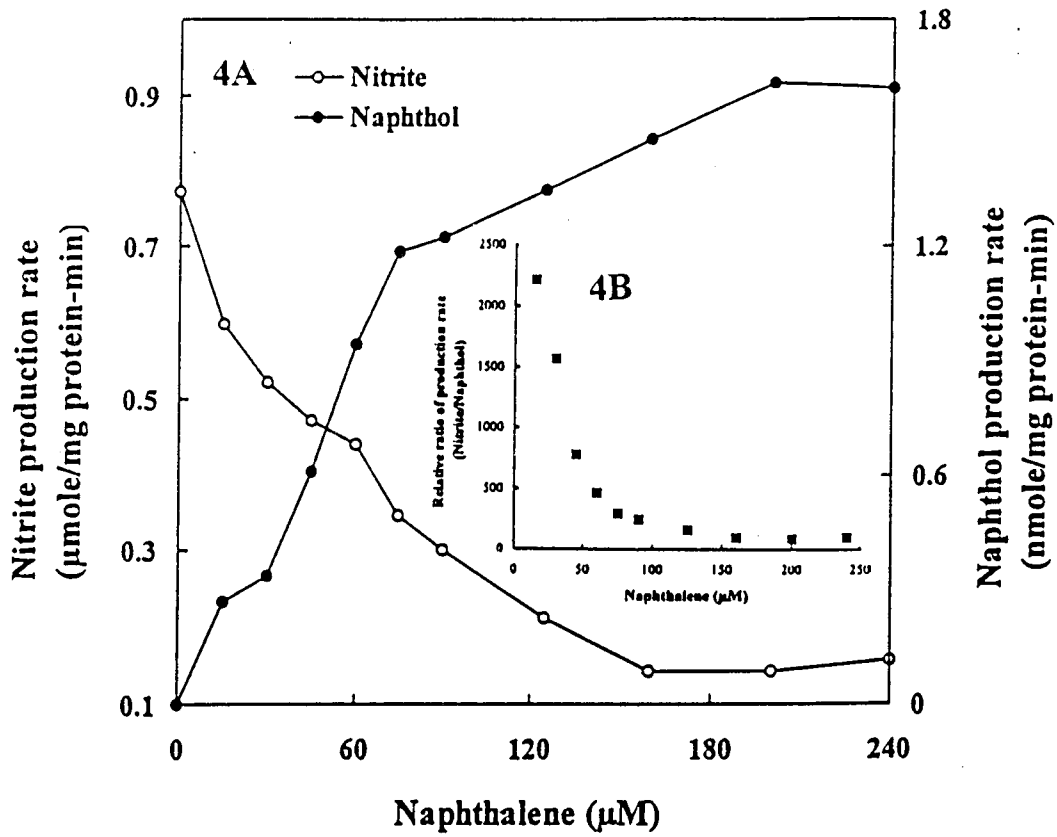

Fig. 3-4. Effects of naphthalene concentrations on the rate of naphthol and nitrite productions. Cells were exposed to a range of naphthalene concentrations from 0 to 240 μM and 10 mM NH_4^+. Naphthol(▪) and nitrite(▲) productions generated in the same incubations were measured as described in Materials and Methods.

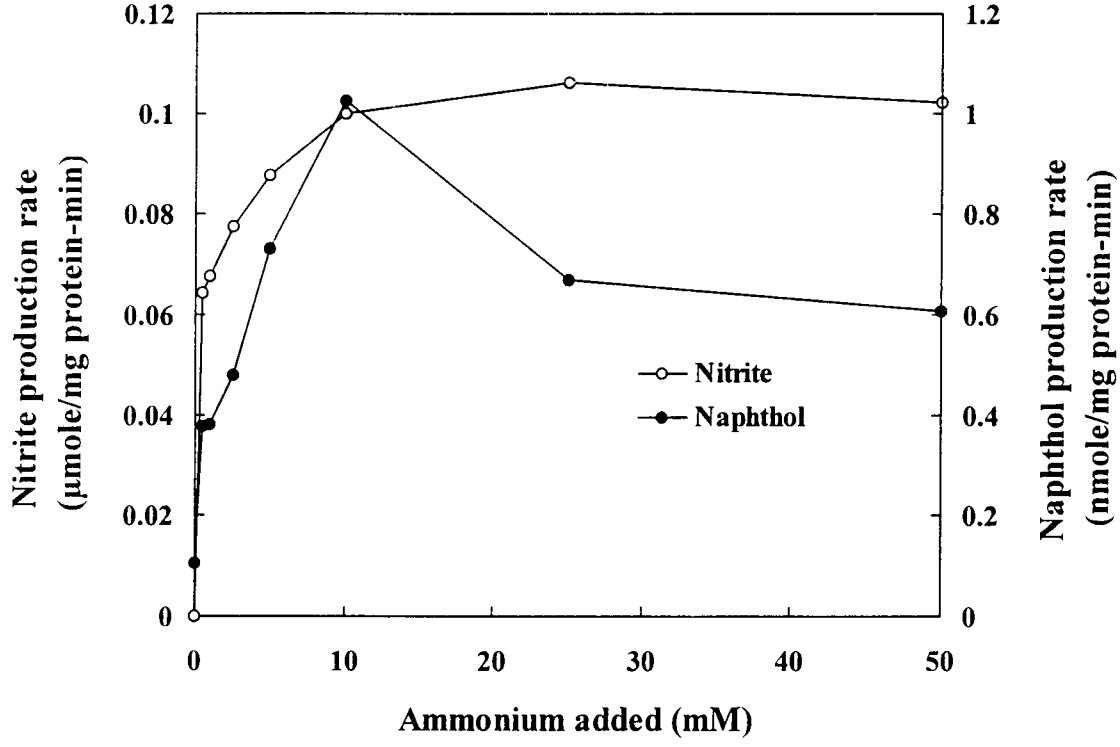

Fig. 3-5. Effects of NH_4^+ concentrations on the rate of naphthol production. Cells were exposed to fixed naphthalene concentration (150 μM) and a range of NH_4^+ concentrations from 0 to 50 mM. Naphthol(▪) and nitrite(▲) generated in the same incubations were measured, as described in Materials and Methods.

production to a lower but almost constant rate as the ammonia concentration was increased up to 50 mM NH_4^+.

Toxic effects of naphthalene and oxidation products

Potential toxic effects associated with naphthalene oxidation were examined by incubating cells with a range of concentrations of naphthalene and an equivalent range of concentrations of the two naphthalene oxidation products, 1-and 2-naphthol. Toxic effects were investigated by monitoring AMO and HAO-dependent O_2 uptake activites after preexposure of cells to these compounds. O_2 consumption in the presence of ammonium reflects AMO activity in addition to terminal oxidase activity. Both of these O_2-consuming reactions utilize electrons derived from the oxidation of hydroxylamine, which itself is generated from ammonia oxidation. When ammonia oxidation is inhibited by the addition of the AMO-specific inhibitor allythiourea, the capacity of hydroxylamine oxidation can be monitored separately by adding hydrazine, a competitive, alternative substrate for hydroxylamine oxidoreductase (11). Our results (Fig. 3-6) demonstrate that naphthalene and both naphthols produced an irreversible inhibitory effect on both AMO and HAO activities (Fig.3-6). The maximal inhibitory effects were observed in the incubations conducted with 200 μM naphthalene or naphthols. Both naphthalene and the naphthols cause larger effect on HAO activity than on AMO activity (Fig. 3-6B).

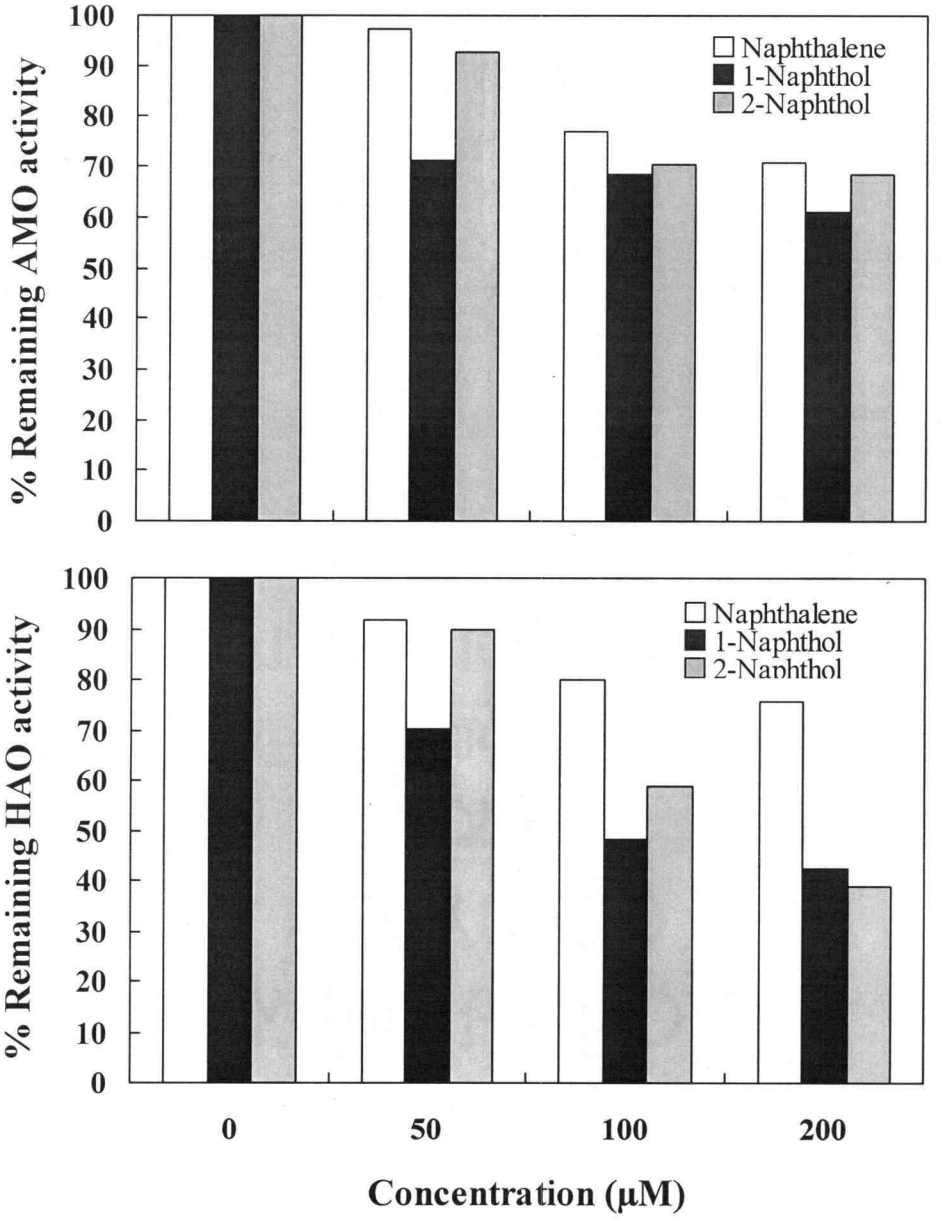

Fig. 3-6. Inhibition of NH_4^+ - and N_2H_2 -dependent O_2 uptake in whole cells of *N. europaea*. To determine the effects of naphthalene and its oxidation products, 1-and 2-naphthols, on the activities of two enzymes involved in ammonia oxidizing activity, cells were exposed to varying concentrations of naphthalene and 1-and 2-naphthols for 2 hr. After incubation, cells was washed and resuspended in buffered solution (50 mM sodium buffer[pH 7.8], 2 mM $MgSO_4$). Samples of the washed cells (50 μl) were examined to analyze NH_4^+ - and N_2H_2 - dependent O_2 uptake, as described in Materials and Methods.

Oxidation products of PAHs by *N. europaea*

Having examined the oxidation of naphthalene, we were interested to further characterize the PAH substrate range by *N. europaea*. Our investigation was conducted by incubating individual PAHs with *N. europaea* for 24 hr. The incubation of cells with 2-methyl naphthalene yielded 2 metabolites. One of them (35 % of the total ion chromatogram) possessed an R_t (19.5 min), mass spectrum (M^+ at m/z 158), and fragmentation ions (130 [M^+ -CO]) identical to those observed for authentic 2-methyl naphthalenol. The other metabolite detected was identified as 2 methyl naphthaldehyde on the basis of molecular weight (M^+ at m/z 156), mass spectrum (128 [M^+ - CO], 101 [M^+ - COOH]) and R_t (17.4 min). Incubations with both acenaphthalene and acenaphthene yielded one major metabolite in netural extraction. The metabolite possessed an R_t (19.9 min), mass spectrum (M^+ at m/z 168), and fragmentation ions (at m/z 140 [M^+ - CO], 113, and 98) identical to those observed for authentic acenaphthenone. None of the oxidation products described here were observed in the presence of acetylene (Data not shown). We obtained no evidence to suggest fluorene, anthracene and phenanthrene were oxidized by AMO.

Table 3-1. Oxidation products of other PAHs by *N. europaea*.

Substrate	Amount (μM) added	Product detected	Transf-ormation	% of O_2 uptake activity remaining after incubation with individual PAH and ammonia for 2 hr	
				NH_3- dependent	N_2H_4-dependent
Naphthalene	27.3	1-Naphthol (14 %) 2-Naphthol (86 %)	Yes	81.1	88.9
2-Methyl naphthalene	24.3	2-Methyl naphthalenol (84 %) 2-Methyl aphthaldehyde (16 %)	Yes	85.8	79.0
Acenaphthalene	23*	Acenaphthenone	Yes	82.8	79.3
Acenaphthene	23*	Acenaphthenone	Yes	73.6	74.7
Fluorene	9	ND	No	95.0	87.0
Anthracene	0.45*	ND	No	86.3	86.9
Phenanthrene	8.4*	ND	No	89.4	98.6

*Saturated amount were added
ND: Not detected

DISCUSSION

The ability of whole cells of *N. europaea* to oxidize polycyclic aromatic hydrocarbons (PAHs) was investigated. The results of this study extended the known substrate range of AMO to PAHs including naphthalene and other PAHs.

Inhibitory effects of PAHs on nitrite production

Our results demonstrated that naphthalene exerts a strong inhibitory effect on ammonia oxidation by *N. europaea*. These effects are similar to the inhibitory effects that have been shown to occur with other classes of alternative AMO substrates. Although there are several mechanisms to account for the inhibitory effects of alternative substrates on ammonia oxidation these effects usually reflect the combined effects of the mutually

exclusive binding of ammonia and alternative substrates and the effects of this interaction on the supply of reductant required to maintain AMO activity. Our results (Fig. 3-4) demonstrate naphthol production increased with increases in naphthalene concentration. In contrast, the effects of NH_4^+ concentration on the naphthalene oxidation involved an initial increase in the rate followed by a decline (Fig. 3-5). The initial increase in the rate of naphthalene oxidation is likely due to the effect of increased reductant supply from ammonia oxidation. In the higher NH_4^+ concentration (25 - 50 mM), small decline of oxidation rate of naphthalene observed compared to previous study (Fig. 3-4) suggests that the binding of NH_3 and naphthalene are not be entirely mutually exclusive.

Transformations of PAHs by *N. europaea*

Previous studies with *N. europaea* have demonstrated some unusual reactions catalyzed by AMO. For example, the relative proportions of 1- and 2-ols generated by *N. europaea* during the oxidation of n-alkanes is the opposite to that encountered with sMMO-catalyzed reactions (17). We also observed an unusual reaction in the case of naphthalene oxidation where 2-naphthol was the major hydroxylated product. This result contrasts with methanotrophic- (4), phenol-oxidizing bacteria (6) and many fungi containing cytochrome P-450 enzymes that generate 1-naphthol as the dominant product of naphthalene oxidation. In the case of methanotrophs it is also important to note that naphthalene-oxidizing activity is restricted to organisms expressing the soluble methane monooxygenase system, not the particulate form of this enzyme which is structurally similar to AMO in *N. europaea* (2).

Potential applications

It is important to address the potential application of organisms for the remediation of PAHs. In general it was known that unsubstituted PAH compounds are largely resistant to biodegradation under anaerobic conditions despite the fact that anaerobic processes could be advantageous for economic reasons. This resistance to degradation is thought to reflect the stability conferred to the unsubstituted rings by the symmetrical electron distributions. A potentially effective route to promote anaerobic PAH degradation would be to biologically introduce ring substituents such as hydroxyl groups into PAHs. In this study we have demonstrated ammonia-oxidizing bacteria are incapable of mineralizing PAHs under aerobic conditions but are readily capable of transforming certain PAHs to hydroxylated products. Such oxygen incorporation into hydrophobic compounds could increase partitioning of contaminants from soil surfaces into the aqueous phase, making them more available to other microorganisms (21). If their reactions were to be catalyzed by ammonia-oxidizing bacteria it us is conceivable that the oxidized nitrogen species generated by this process could be subsequently used as electron acceptors for denitrifying organisms capable of utilizing hydroxylated PAHs as electron donors. In combination, these aerobic and anaerobic processes could fully mineralize NH_4^+ and PAHs to N_2 and CO_2.

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Chapter 4

Cometabolic Degradation of Dibenzofuran and Other Aryl Ethers by the Soil Nitrifying Bacterium *Nitrosomonas europaea*

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ABSTRACT

In this study evidence for the transformation of six aryl ethers by the soil nitrifying bacterium *N. europaea* is presented and the subsequent abiotic transformations suggest these transformations involved two separate reactions. First, our results indicate that *N. europaea* has the ability to cometabolically oxidize dibenzofuran (DBF), dibenzo-*p*-dioxin (DD), and other aryl ethers. Control experiments using acetylene indicated that AMO is the enzyme involved in oxidation of these compounds although none of these substances show an inhibitory effect on ammonia oxidation. DBF oxidation by AMO yielded 3-hydroxydibenzofuran (3-OH DBF) as an initial oxidation product. This transformation product was verified using co-elution with authentic 3-OH DBF on HPLC and GC/MS analysis. The time course of DBF degradation using different ammonia concentrations indicated competitive interactions between ammonia and DBF. The production of 3-OH DBF was almost doubled with increase in ammonia concentrations from 10 to 20 mM. Second, our results also indicated that hydroxylated products of all the aryl ethers by AMO action are further transformed abiotically, generating nitro-hydroxy compounds. Experiments using 3-OH DBF indicate that the nitration reactions are strongly affected by pH with smaller effects due to nitrite concentration.

INTRODUCTION

Because of the extreme toxicity of halogenated dibenzo-*p*-dioxins (DD) and dibenzofuran (DBF), areas contaminated with these compounds are potentially hazardous and require remediation. These halogenated heterocycles are formed during the manufacture of pesticides, bleaching of pulp paper (22, 24, 3), incineration of halogen-containing chemicals and industrial and domestic waste. Biological processes have considerable potential for the degradation of polyaromatic hydrocarbons. However, compounds such as DD and DBF are generally resistant to microbial attack because of the stable central oxygen link between the two aromatic nuclei (25). A number of microorganisms have been isolated which can oxidize DBF (25, 26, 28, 29), DD (7, 8, 25, 26, 30, 31), diphenyl ether (DPE) (32), and other aryl ether compounds (10) using dioxygenase enzymes. However, very little is known about the microbial degradation of aryl ethers by monooxygenase enzymes. In this study we have examined the oxidation of DBF and other aryl ether compounds by the nonspecific monooxygenase AMO, found in the soil nitrifying bacterium *N. europaea*. *N. europaea* is a lithoautotroph which obtains all of its energy for growth from the oxidation of ammonia to nitrite, *via* hydroxylamine as an intermediate. The energy derived from ammonia oxidation is used to support both ATP generation and CO_2-fixation (33). The initial oxidation of ammonia is catalyzed by a membrane-bound enzyme known as ammonia monooxygenase (AMO). The reductant required to support AMO activity is provided through the oxidation of the product of ammonia oxidation (hydroxylamine) using the enzyme hydroxylamine oxidoreductase (HAO) (21). AMO in *N. europaea* is capable of oxidizing a range of hydrocarbons (11, 17), halogenated hydrocarbons (18), aromatics (14), PAHs (15) and alkyl ethers (13).

AMO catalyzed the substrates by several mechanisms such as hydroxylation (11), epoxidation (11) and O-dealkylation (13). In this present study we have examined the oxidation of a range of aryl ether compounds and have demonstrated those compounds undergo both aerobic biotic and abiotic transformation reactions ultimately leading to the formation of nitro hydroxy derivatives.

MATERIALS AND METHODS

Materials

Diphenyl ether, 4-chloro- and 4-bromo diphenyl ether, dibenzofuran, 2-hydroxy dibenzofuran, and xanthene were purchased from Aldrich Chemical C., Inc., Milwaukee. Dibenzo-*p*-dioxin was purchased from EST Inc. (Florida). All chemicals were more than 97.5 % pure by manufactures analysis. All other chemicals were of reagent grade. The 3- and 4-hydroxy dibenzofurans were the gift from Dr. Franke at Hamburg University in Germany.

Growth and preparation of the cells

N.europaea (ATCC 19178) was cultured as described previously (12). Cell suspensions were harvested by centrifugation (10,000 × g, 10 min) after 3 days of growth. Cells were washed and resuspended in 1.5 ml of the assay buffer (50 mM NaH_2PO_4, 2 mM $MgCl_2$ [pH 7.8]). Cell suspensions were stored on ice and used within 24 hr of harvesting.

Assays for inhibition of NH_3 oxidation to NO_2^-

All stock solutions of the aryl ether compounds were prepared in dimethyl sulfoxide (DMSO). The incubations were conducted in 37 ml glass serum vials containing 10 ml assay buffer and ammonium sulfate (5 mM). Aryl ether compounds were then added to the assay vials. The vials were then sealed with Teflon-lined silicone septa (Sun Brooks™, Wilmington, NC). The aryl ether was allowed to dissolve by placing the sealed vials in a sonic water bath for 10 min. The reactions were initiated by adding 100 µl of the cell suspension (*ca.* 1 mg of protein) to the assay vial. The reactions were conducted at 30 °C in a shaking water bath (300 rpm). To compare the inhibitory effects on nitrite production by several aryl ethers, equal amounts of four aryl ether compounds, which corresponded to 5 mg/l, were added although they have different solubility. Liquid samples (5 µl) of the reaction mixtures were removed to determine the nitrite content using the colorimetric analysis as described previously (20).

Transformations of aryl ethers

To examine the transformations of the aryl ether compounds, the reaction mixtures were prepared as described above, and the reaction mixtures were incubated for 24 hr. To follow the time course of changes in substrate and oxidation product concentrations samples (300 µl) were removed from the incubation vials at various time. The samples were transferred to 1.5-ml Eppendorf tubes, and cells were removed by microcentrifugation. The supernant (100 µl) was then injected into the HPLC.

Abiotic transformation of hydroxylated aryl ethers

Two experiments were conducted to establish the abiotic transformation of hydroxy DBF. First, To compare the abiotic disappearance rate of 3-OH DBF that was accumulated following biological transformation of DBF by *N. europaea*, DBF was incubated in the presence of cells for 5 hr with DBF to examine 3-OH DBF production. Five mg/l DBF was initially incubated in the presence of 10 and 20 mM NH_4^+ for 5 hr, the time after which maximum 3-OH DBF production was observed. The liquid medium was subdivided into 3 separate incubation vials. In one incubation the reaction mixture was left unchanged, to another incubation C_2H_2 (1 % v/v) was added. In the third incubation the cells were removed by centrifugation. In all cases the concentration of residual 3-OH DBF was monitored by HPLC. In the second experiment we examined whether the same products were generated in the presence and absence of cells. In these experiments 2- and 3-OH DBF (5 mg/l) were incubated in phosphate buffer (50 mM sodium buffer, 2 mM $MgSO_4$) over a range of pH values from 4.9 to 7.8 and two different NO_2^- concentrations for 12 hr.

Analytical procedures

The oxidation of aryl ethers and the accumulation of oxidation products were determined using high-performance liquid chromatograph (HPLC). The HPLC system equipped with a reversed-phased ultramex C18 column (150 mm × 4.60 mm; phenomenex).The eluent was composed of acetonitrile (HPLC grade; Mallinckrodt) (varied as necessary from 40 to 60% by volume) and deionized water, and the flowrate was set at 1 ml/min. Eluted compounds were detected by UV detector at 254 nm using a

Hewlett Packard integrator. Further characterization of the oxidation products of the aryl ether compounds was conducted using a Finnigan 4023 GC/MS with a Varian 3400 GLC, coupled to a Galaxy 2000 data system. The mass spectrometer was operated in electron impact mode (70 eV) with a source temperature of 140°C. A 10 m SE54 silica capillary column (0.25 mm ID) was used with a temperature program set for 50 to 280°C at a rate of 20°C/min and a detector temperature of 280°C. To run samples in the GC/MS, samples (10 ml) of each compound were extracted twice with a half volume of hexane (neutral fraction). After the supernatant was dried over anhydrous Na_2SO_4, the solvent was then evaporated to approximately 100 µl under a stream of N_2. Samples (2 µl) of the hexane solution were then injected into GC/MS. Chemical structures of the metabolites were assigned on the basis of their mass spectra , instrument library searches, and literature data if authentic materials were not available.

Protein concentrations of the cell suspensions were determined by using a Biuret assay (19) after solubilizing the cells in 3 M NaOH (30 min, 60°C).

RESULTS

Inhibitory effects of aryl ethers on nitrite production

Several previous studies with *N. europaea* have reported that alternative substrates on AMO show inhibitory effects on ammonia oxidation.(14, 15, 16). This inhibitory effect occurs because of competitive interactions between ammonia and alternative substrates for oxidation by AMO. The inhibitory effects of four aryl ethers on nitrite production were determined for cells incubated with ammonia (10 mM) with each

ether compound present as a saturated aqueous solution (< 3 mg/l). In the absence of aryl ethers the added ammonia was rapidly oxidized to nitrite within 1.5 hr (Fig. 4-1). The presence of the aryl ethers had very little effect on the time course of nitrite production. Based on our previous experience with alternative substrates this limited effect on nitrite production suggested that these compounds were unlikely to be substrates for AMO.

Transformation of aryl ethers by *N. europaea*

Despite the limited effect of the compounds on nitrite production an analysis of the reaction media of incubations with aryl ethers suggested that these compounds undergo transformations in the presence of *N. europaea*. Our results with DBF are presented here and are representative of our results with all of the ether-bonded compounds examined in this study. Our results (Fig. 4-2) demonstrate that the incubation of cells with NH_4^+ (10 mM) and DBF led to the production of a single product as determined by HPLC analysis (Fig. 4-2B). This product was not generated by cells incubated with C_2H_2 and DBF (Fig. 4-2D). C_2H_2 is a specific inhibitor of AMO activity and this result suggests that AMO activity is required for the generation of this product. This product was subsequently identified as 3-OH DBF by coelution with authentic 3-OH DBF during HPLC analysis of the authentic compounds. Although our analysis suggests DBF underwent hydroxylation by AMO, our results also demonstrated that this hydroxylated product became substantially depleted from our reactions when reaction medium was analyzed after 24 hr (Fig. 4-2C). This result suggests that DBF and other aryl ethers are susceptible to two reactions in the presence of *N. europaea*. The characterization of these two putative reactions is detailed in the remainder of this study.

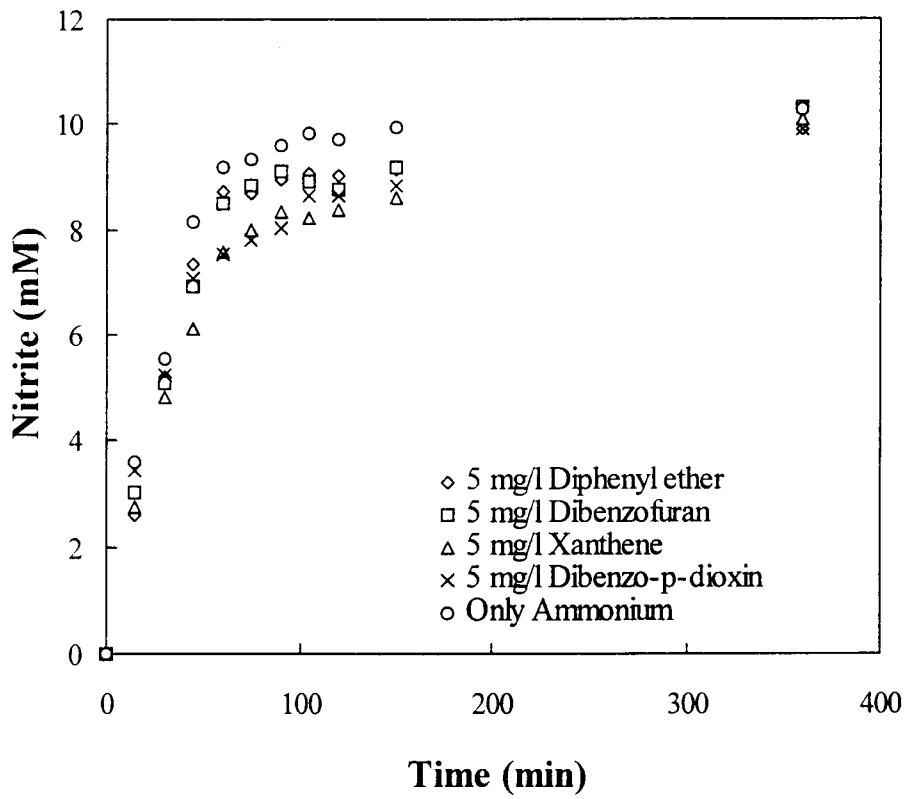


Figure 4-1. Time course of nitrite production by *N. europaea* in the presence of aryl ethers. Cells were incubated in glass serum bottles with 10 mM NH_4^+, cells (100 µl; *ca* 1.5 mg of protein), and 5 mg/l each aryl ether compounds; diphenyl ether, dibenzofuran, xanthene, and dibenzo-*p*-dioxin.

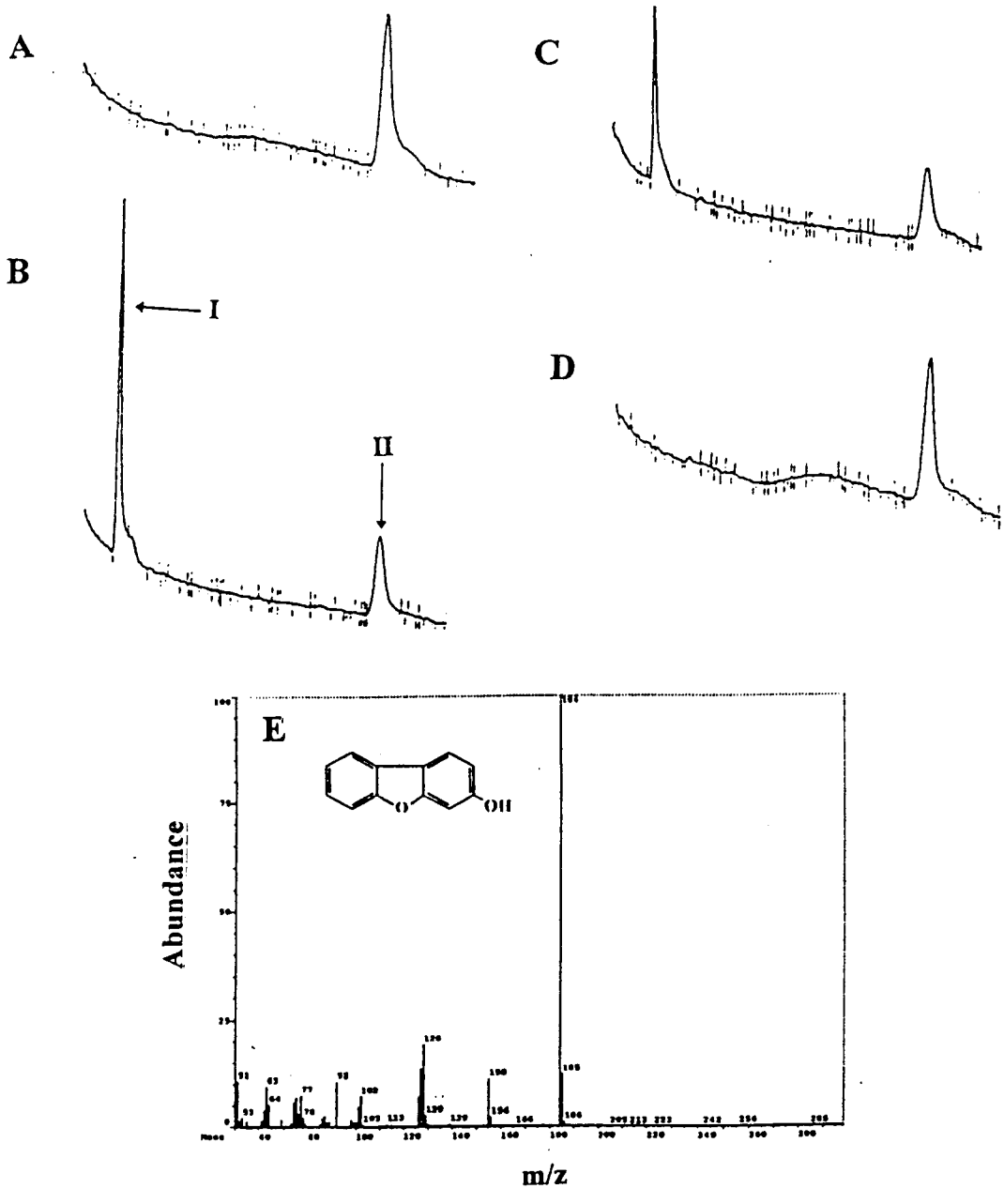


Figure 4-2. HPLC and GC/MS analysis of transformation products obtained from DBF by *N. europaea*. The incubation conditions are as described in the Materials and Methods and each incubation contained DBF (5 mg/l), 10 mM NH_4^+ and cells (100 µl; *ca* 1.2 mg of protein) except (D) addition of C_2H_2 (1 % v/v). Samples were taken at different time; (A) 0 hr (B) 10 hr (C) 24 hr (D) 10 hr. The GC/MS chromatogram (E) represent the peak I produced from oxidation of peak II (DBF).The sample for GC/MS analysis was prepared as described in Materials and Methods.

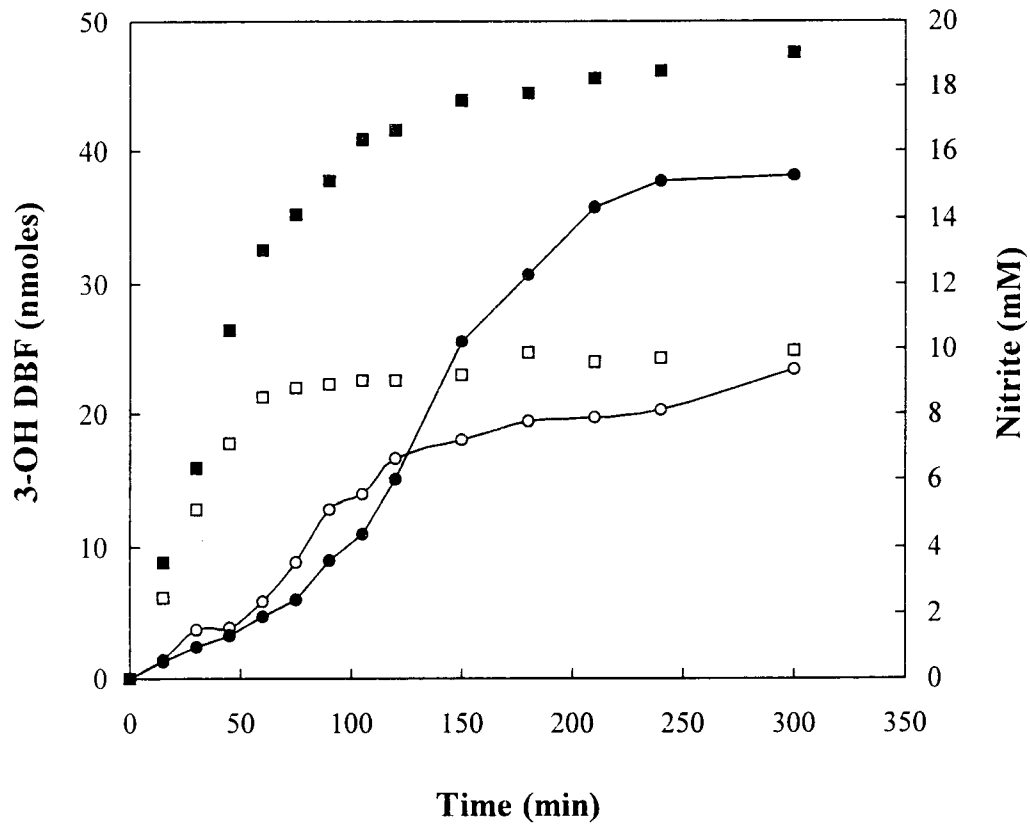


Figure 4-3. Time course of 3-OH DBF and nitrite productions as a function of NH_4^+ concentration. The following symbols represent; nitrite production from cells (500 µl; *ca* 5.5 mg of protein) incubated with (□) 10 and (■) 20 mM NH_4^+, 3-OH DBF production from cells incubated with (○) 10 and (●) 20 mM NH_4^+ . Detailed experimental conditions are described in Materials and Methods.

A time course study following DBF oxidation by AMO was conducted to examine the interaction between ammonia and DBF. Our result (Fig. 4-3) suggests a competitive interaction between ammonia and DBF for oxidation by AMO. For example, the rate of DBF oxidation was initially low in the presence of the high ammonia concentrations present in the early stages of the reaction (< 1 hr). The rate of 3-OH DBF production then progressively increased as the ammonia concentration was further decreased and then the rate of production tended to zero as the ammonia was exhausted. This latter decrease in the rate of production probably reflects the effects of a decreased rate of supply of reductant to AMO. In addition, we also observed that the initial rate of 3-OH DBF production was lower in the incubation with 20 mM NH_4^+ compared to the incubation with 10 mM NH_4^+. However, the incubation conducted with 20 mM NH_4^+ eventually produced 2-fold higher concentrations of 3-OH DBF. This effect is probably due to the greater amount of reductant available to AMO throughout the reaction time course.

Abiotic transformation

As our preliminary experiments (Fig. 4-2) indicated that 3-OH DBF from DBF oxidation progressively disappeared during the reaction time course we were interested to determine the conditions that led to the further transformation of 3-OH DBF. Our first experiment was conducted with incubations that had occurred over a 5 hr period (Fig. 4-3). The reaction medium was subdivided into 3 separate reaction vials and the rates of disappearance of 3-OH DBF were analyzed over the following 12 hr as described in Materials and Methods. These experiments were conducted with reaction media obtained from transformation reactions containing 10 and 20 mM NH_4^+. The final pH of these

reactions were 6.8 and 6.2, respectively. The results (Fig. 4-4) demonstrate that the concentration of 3-OH DBF decreased in all reactions at a constant rate. This result indicates that the 3-OH DBF reaction could be accounted for by an abiotic reaction because the compound was depleted equally rapidly in reactions conducted with and without cells. As shown in Fig. 4-4 the rate of 3-OH DBF removal was approximately 2-fold higher in the reaction medium obtained from the initial incubation conducted with 20 mM NH_4^+ as opposed to 10 mM NH_4^+. This suggests that the rate of 3-OH DBF consumption could potentially be influenced by the pH of the reaction medium and the concentration of nitrite.

As our previous experiment (Fig. 4- 4) suggested 3-OH DBF was abiotically further transformed at different rates depending on the pH and nitrite concentrations, we examined the effects of these two factors, on the abiotic disapperance of 3-OH DBF. In this experiment 27.2 μM 3-OH DBF was incubated with 10 and 20 mM NO_2^- at a various pH values (4.9 to 7.8) in a phosphate-buffered solution. The residual 3-OH DBF was then measured by HPLC after 12 hr. Our results demonstrate that the abiotic removal of 3-OH DBF was strongly influenced by pH whereas there was only a little effect of nitrite concentration. Similar results were observed in the incubations with 2-OH DBF (Data not shown), which suggests the abiotic transformation is not restricted to 3-OH DBF.

Determination of nitro derivatives

As our previous experiments indicated further abiotic transformations of 3-OH DBF we were interested to determine the abiotic products. The capillary GC separation

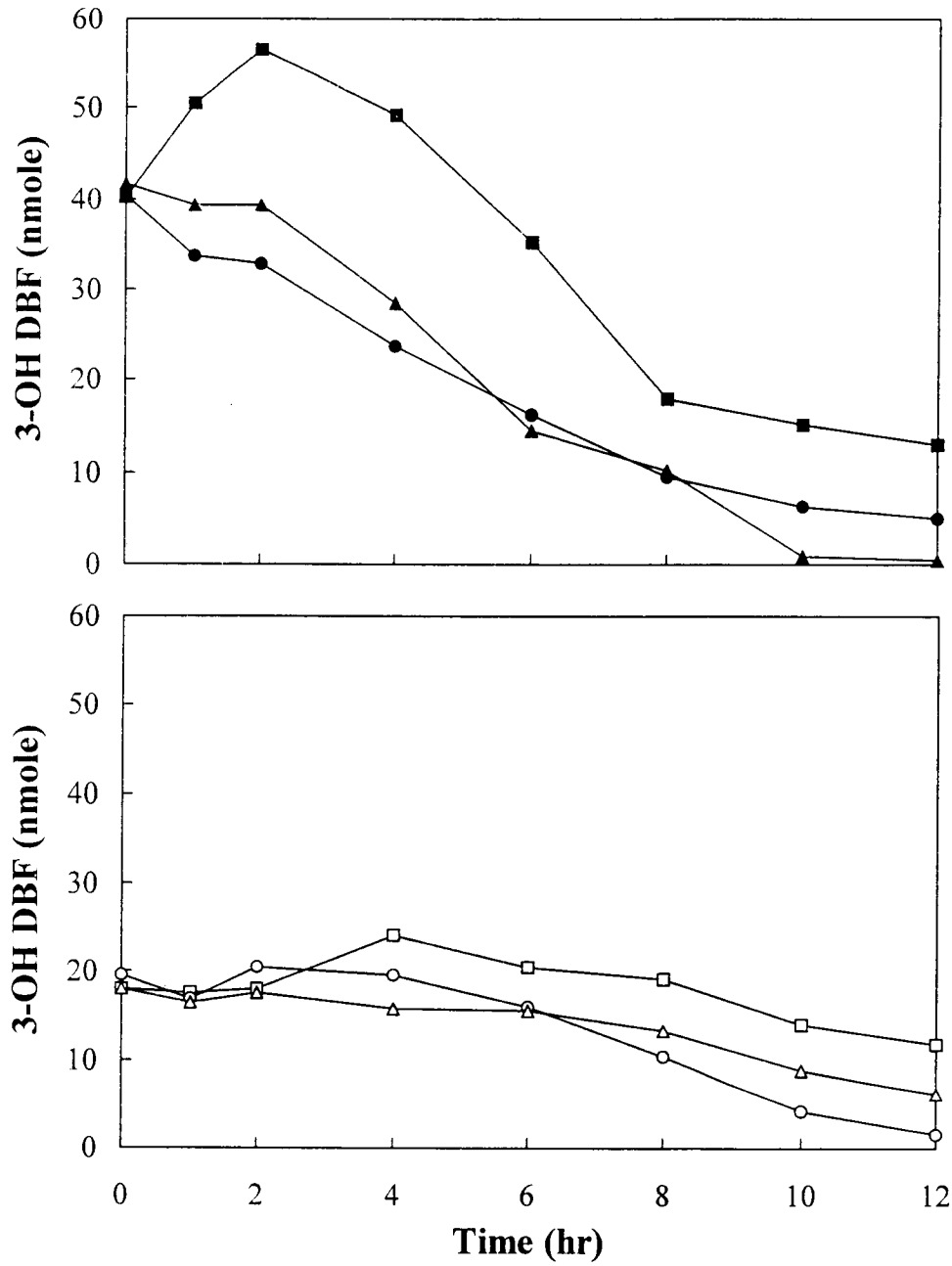


Figure 4-4. Time course for abiotic transformation of 3-OH DBF. These experiments were conducted after a 5 hr incubation of cells with 10 & 20 mM NH_4^+ and 5 mg/l DBF as described in Materials and Methods. The liquid medium of the previous experiment (Fig. 4-3) was subdivided into 3 separate incubation vials under the following conditions: Panel (A) Reaction medium from initial incubation with 20 mM NH_4^+.(▪) unchanged condition with previous experiment, (▲) addition of C_2H_2 (1 % v/v), (●) removal of cells. Panel (B) Reaction medium from initial incubation with 10 mM NH_4^+. (□) unchanged condition with previous experiment, (Δ) addition of C_2H_2 (1 % v/v), (○) removal of cells.

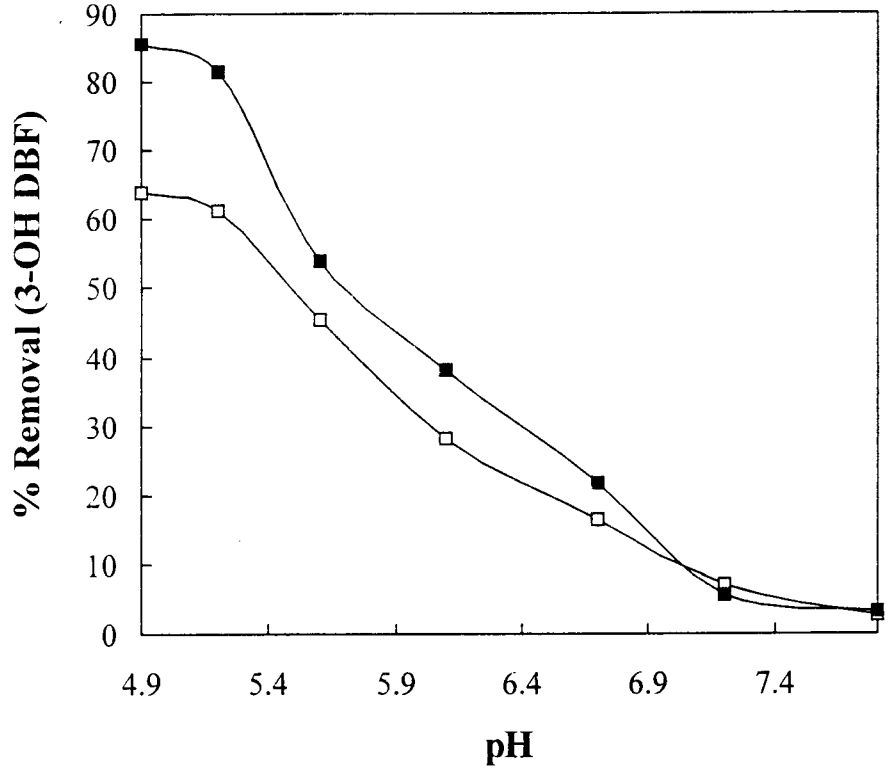
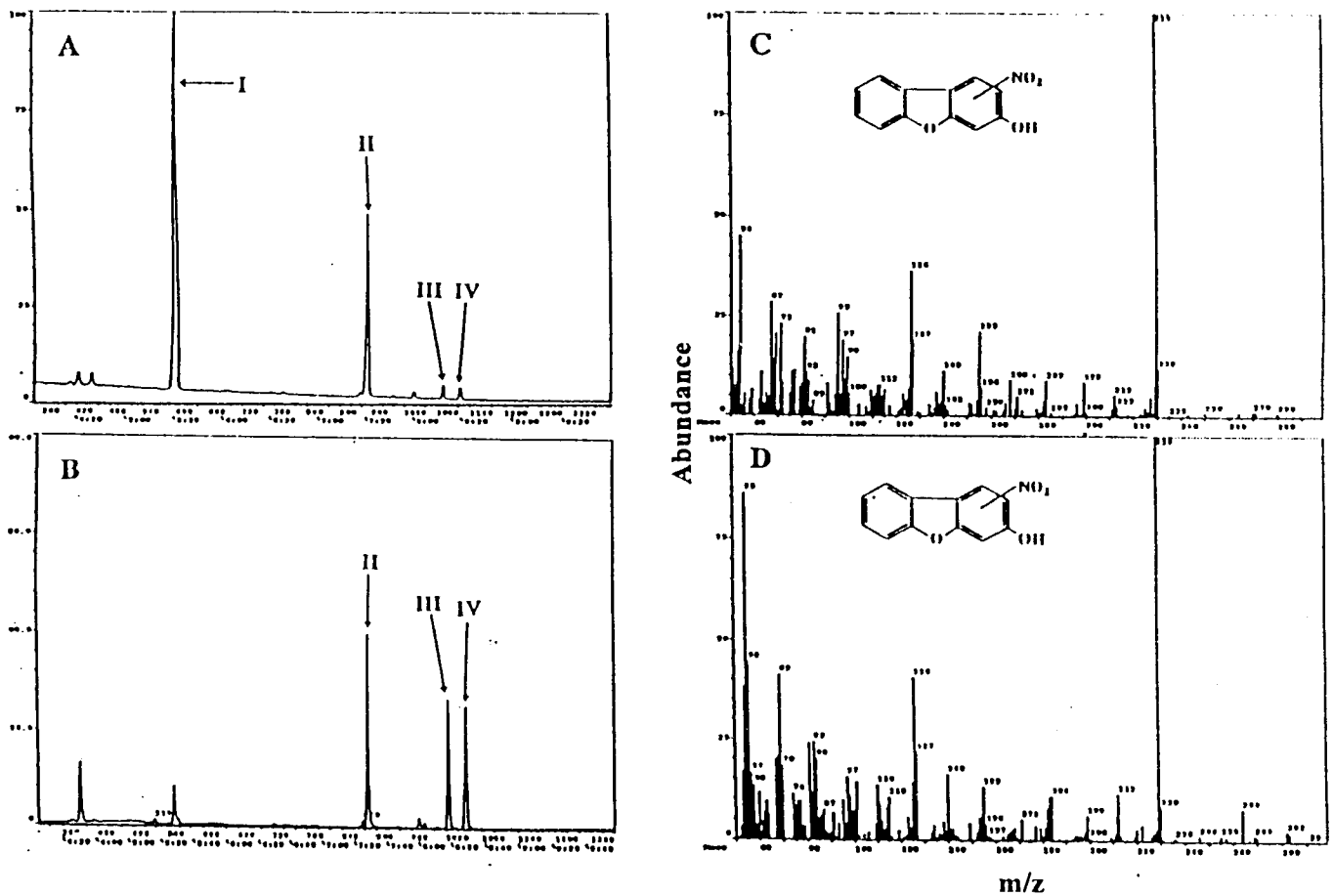


Figure 4-5. Transformation of 3-OH DBF as a function of pH and NO_2^-. The figure shows the mass of 3-OH DBF removed by abiotic reaction after 12 hr incubation at the identical pH values in the presence of (□) 10 mM NO_2^- and (■) 20 mM NO_2^-. Detailed procedures were described in Materials and Methods.

Figure 4- 6. GC separation and identification of abiotic transformation products of 3-OH DBF. Panel (A) shows the GC chromatogram of the sample obtained from a reaction in which cells were incubated in the presence of DBF (5 mg/l) and 10 mM NH_4^+ for 6 hr, and Panel (B) represents the GC chromatogram of the sample obtained from an authentic sample of incubated in 3-OH DBF(5 mg/l) phosphate buffer (50 mM sodium buffer [pH 6.8], 2 mM $MgSO_4$) with 10 mM NO_2^- for 6 hr. Panel (C) and (D) represent the mass spectral analyses of the peaks (III, IV) detected in panel A and B.



of the reaction medium obtained from 6 hr incubation of cells with DBF (5 mg/l) and 10 mM NH_4^+ is shown in Fig. 4-6(A) along with the GC separation of the sample incubated with authentic 3-OH DBF incubated in phosphate buffer solution (pH 6.8) with nitrite (10 mM) in the absence of cells (Fig. 4-6(B)). This analysis indicates the products formed from DBF transformation with cells presented in Fig. 4-6(A) are the same products as those formed when 3-OH DBF was incubated at low pH with nitrite in the absence of cells. The GC peaks III and IV had a retention time 8.22 and 8.31, respectively, and were identified as two isomers of nitro 3-OH DBF (molecular weight, 229) through interpretation of their mass spectral fragmentation pattern. The mass spectra of the 2 isomers of nitro 3-OH DBF corresponding to the GC peaks III and IV, are shown Fig 4-6(C) and 4-6(D), respectively. The mass spectra of peaks III and IV show a molecular ion of m/z 229, which suggests the presence of the nitro compound 3-OH DBF. The fragment at m/z 212 corresponds to the loss of OH. The fragments at m/z 199 and 183 correspond to the loss of NO and NO_2, respectively, based on the presence of a DBF ion (m/z = 168).

Other substrates of AMO

Our experiments using DBF as a model compound demonstrated that DBF is a substrate for AMO and that the oxidation product of DBF is subsequently transformed in a pH- and nitrite- dependent abiotic reaction. In view of these reactions we were interested to examine whether other aryl ethers underwent similar reactions following the oxidation of each aryl ethers. Time course experiments indicated that all the compounds examined in this study are oxidized by AMO and that these products undergo similar

abiotic transformations to those described above. The determination of oxidation products of aryl ethers by AMO and subsequent abiotic transformations are summarized in Table 1.

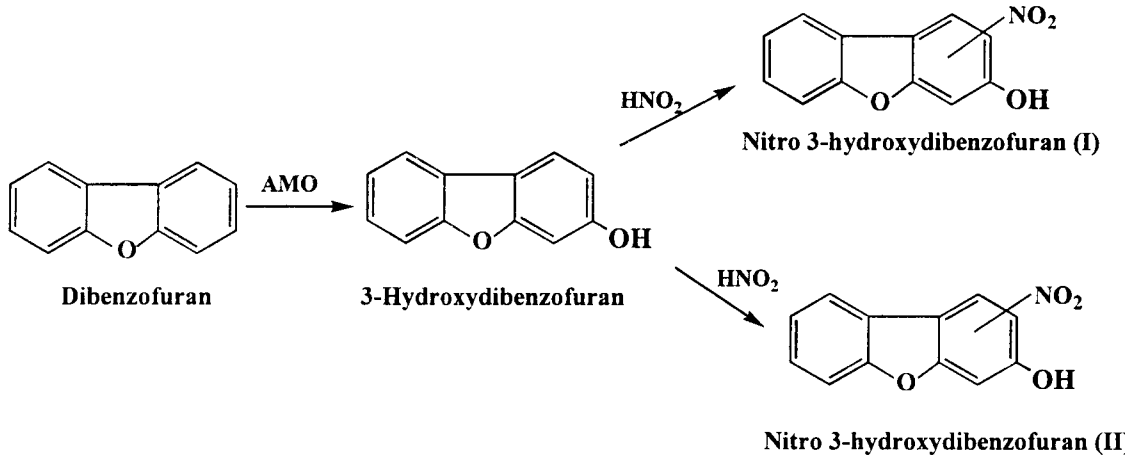


Figure 4-7. Overall pathway of dibenzofuran transformation by *N. europaea*.

Table 4-1. GC retention time (R_t) and electron impact mass spectra of major compounds formed from oxidation of aryl ethers by *N. europaea*

Sbustrate	R_t (min)	m/z of fragment ions (% relative intensity)	Identification
Dibenzofuran	7.36	184(M+, 100), 156(M^+-CO, 2), 155(M^+-CHO, 11), 128 (M^+-CO-CO, 20), 127(M^+-CHO-CO, 13)	3-hydroxy dibenzofuran[a]
	8.22	229(M^+, 100), 212(M^+-OH, 6), 199(M^+-NO, 9), 183 (M^+-NO_2, 9), 168 (9), 155(21), 126(37)	Nitro-3hydroxy dibenzofuran[b]
	8.31	229(M^+, 100), 212(M^+-OH,12), 199(M^+-NO, 6), 183 (M^+-NO_2, 11), 168 (3), 155(13), 126(41)	Nitro-3hydroxy dibenzofuran[b]
Xanthene	7.29	243(M^+, 100), 213(M^+-NO, 8), 197(M^+-NO_2, 7), 168(10), 155(1), 139(20)	Nitro hydroxy xanthene[b]
Dibenzo-*p*-dioxin	7.49	200(M^+, 100), 171(M^+-CHO, 16), 144(M^+-CO-CO, 11), 115(22)	Hydroxy dibenzo-*p*-dioxin[b]
	8.42	245(M^+, 100), 215(M^+-NO, 6), 199(M^+-NO_2, 8), 187(M^+-NO-CO, 11), 171(M^+-NO_2-CO, 38)	Nitro hydroxy diebnzo-*p*-dioxin[b]
Diphenyl ether	6.50	186(M^+, 100), 158(M^+-CO, 6), 157(M^+-CHO, 12), 141(M^+-CO-OH, 2), 129(M^+-CO-CHO,10), 109(17)	Hydroxy diphenyl ether[b]
	7.34	231(M^+, 100), 201(M^+-NO, 1), 185(M^+-NO2, 4), 173(M^+-NO-CO, 1), 128(17)	Nitro hydroxy diphenyl ether[b]
4-chloro dipheyl ether	7.55	220(M^+ , 100), 192(M^+-CO, 2) 185(M^+-Cl, 4), 157(19), 128(11), 109(22)	Hydroxy 4-chloro diphenyl ether[b]
	8.38	265(M^+, 100), 235(M^+-NO, 1), 230(M^+-Cl, 2), 219(M^+-CO-Cl, 3), 207(M^+-NO-CO, 4), 184(6)	Nitro hydroxy 4-chloro diphenyl ether[b]
4-bromo diphenyl ether	8.24	266(M^+, ^{81}Br, 96), 264(M^+, ^{79}Br, 100), 238(M^+-CO, 6)	Hydroxy 4-bromo diphenyl ether[b]
	9.07	311(M^+, ^{81}Br, 96), 309(M^+, ^{79}Br, 100), 283(M^+-CO,4), 281(M^+-NO, 8)	Nitro hydroxy 4-bromo diphenyl ether[b]

extracted with hexane at neutral pH

[a]identified by comparison (of R_t and mass spectra) with authentic material.

[b]suggested structure

DISCUSSION

Several aryl ethers ware recognized by the USEPA as significant environmental contaminants or Priority Pollutants (e.g. 4-chloro diphenyl ether) (27). The report demonstrates that several aryl ethers are degraded to hydroxy derivatives by the soil nitrifying bacterium *N. europaea*. This results of this study also demonstrate that the hydroxylated products undergo further abiotic transformation to yield nitro hydroxy derivatives. The broader implications of our observations are discussed below.

Inhibitory effects of aryl ethers

The result in this study demonstrated that several aryl ethers did not have significant inhibitory effect on ammonia oxidation by *N.europaea* whereas most of the substrates, including aromatic (14, 16) and polyaromatic (15) compounds, of the AMO show inhibitory effects. This result suggests that inhibitory effects on nitrite production is a poor selection criteria for AMO substrates with low aqueous solubilites.

Transformation of aryl ethers

We have previously demonstrated that the oxidation of naphthalene by *N. europaea* results in the generation of 2-naphthol as an unusual dominant oxidation product. While the production of 3-OH DBF from DBF described in the present study is clearly compatible with a monooxygenase-catalyzed reaction, very little is known about the predominant products expected from monooxygenase-catalyzed DBF oxidation because the majority of microbial studies with this compound have concentrated on dioxygenase-catalyzed transformations. For example, the transformation of DBF by the

filamentous fungus *C. elegans* results in the production of 2-OH and 3-OH DBF as 8 % and 6 % of the total transformation products, respectively. This reaction is likely catalyzed by a cytochrome P-450 monooxygenase that has been implicated in the numerous polyaromatic hydrocarbon transformations catalyzed by this organism. In contrast, the well-characterized dioxygenase activity involved in aryl ether oxidations by several *Sphingomonas* species resulted in the production of 3-OH DBF as the dominant initial product of DBF oxidation. Although our study has revealed that all of the compounds examined are oxidized to monohydroxylated products (Table 4-1) we have not resolved the positions of the hydroxy substituents and are therefore unable to comment on the significance of these products. However, it is known that 2-OH DBF is the major product generated from DD oxidation by *Pseudomonas* sp. but initial oxidation product was c*is*-benzo[1,4]dioxane dihydrol which could subsequently undergo dehydration to yield both 1 - and 2-OH DD. (8). *Beijerinckai* produced 2-OH DD (7) although the DBF-degrading bacterium *Pseudomonas* sp. strain HH69 and *Staphyloccus auriculans* DBF63 generated 1-OH DD (27, 30). *Pseudomonas* sp. strain HH69 was also able to generate hydroxyxanthene from xanthene (26). It is interesting to note that our previous studies with PAH oxidation by *N. europaea* have demonstrated that unsubstituted PAHs with more than two aromatic rings such as fluorene and anthracene are not substrates for AMO. In contrast, our present study indicates that structurally similar compounds such as DD are readily oxidized by this enzyme system. The differential reactivity of the aryl ethers and unsubstituted PAHs could reflect the greater reactivity of heterocyclic compounds to electrophiles.

Abiotic transformation of hydroxylated aryl ethers

An unexpected observation in this study was that all of the hydroxylated aryl ethers generated by AMO underwent further transformation to yield nitro hydroxylated compounds. In this study we have concentrated on characterizing this transformation of hydroxylated DBF (Fig. 4-4) but our evidence suggests that the production of these compounds is a common feature of all of the compounds examined in this study (Table 4-1). Our evidence indicates that the production of nitrated derivatives is an abiotic reaction because the consumption of 3-OH DBF occurred at essentially equivalent rates in the presence and absence of cells (Fig. 4-4). Furthermore, our evidence indicates that the same nitrohydroxy DBF isomers generated during prolonged incubations in the presence of cells were also generated in artificial reactions conducted in the complete absence of cells. Nitration reactions between nitrite and phenolic compounds are well known and are thought to require nitrous acid (HNO_2) rather than nitrite (NO_2^-) as the active nitration agent. The pKa for nitrous acid is 3.4 (30 °C) and this suggests that the rate of nitration will increase with decreasing pH. This effect was certainly confirmed in our abiotic reactions (Fig. 4-5) and in our biological experiments (Fig. 4-4) we observed that the rate of 3-OH DBF was faster in incubations containing 20 rather than 10 mM NH_4^+. This latter effect is probably less a result of the higher concentrations of nitrite generated from ammonia oxidation and more a result of the higher degree of acidification of the medium caused by the greater level of ammonia oxidation. In support of this we observed that the oxidation of 10 and 20 mM NH_4^+ reduced the pH of the reaction medium from 7.8 to 6.8 and 6.2, respectively.

The nitration of phenolic compounds has received considerable attention in the past. Nelson and Bremner (35, 36) reported that the rate of nitration of phenolic compounds at pH 5.0 is dependent on the degree of hydroxylation of the target compound. Azhar *et al.* (39) also observed that nitro and nitroso derivatives of 1-naphthol are generated in soils in the presence of nitrite and we have subsequently demonstrated that the oxidation of naphthalene to 1 and 2-naphthol by *N. europaea* generates nitrohydroxy derivatives of naphthalene under the same conditions described in this study (Data not shown).

Significance of potential application

Our observation that a soil nitrifying bacterium can readily transform several aryl ethers suggests that these organisms may be useful to promote the degradation of these compounds. In general bioremediation of dioxin-contaminated soils is a difficult process because the dioxin is usually present at very low concentrations and is highly insoluble because of their hydrophobic characteristics. Although a number of dioxin-degrading bacteria have been isolated (27, 32), the low concentration of these compounds probably limits the growth of these organisms. Biosurfactants have been used to increase the solubility of these hydrophobic substances (6, 39). Our present study suggests that ammonia-oxidizing bacteria could be used as priming catalysts to generate hydroxylated compounds which have greater water solubility and are therefore more available to support the growth of heterotrophic organisms. Future research with *N. europaea* could address the potential transformation of the more toxic chlorinated forms of DD.

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Chapter 5

Cometabolic Degradation of Anisole and Other Aromatic Ethers by Nitrifying Bacteria, *Nitrosomonas europaea*

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ABSTRACT

Our results represent that the oxidation of a variety of aromatic ethers, including anisole, was catalyzed by AMO in whole cells of nitrifying bacterium *Nitrosomonas europaea* through either O-dealkylation or hydroxylation reactions. The highest rate of ether oxidation was observed with anisole and the maximum ratio of O-dealkylation to hydroxylation reactions was 2.5:1. However, the rate of ether oxidation and the ratio of O-dealkylation to hydroxylation reactions decreased with increases in carbon number on ether bond and with increases in the number of methoxy substituents. The accumulation of phenol from the oxidation of aromatic ethers by AMO had a strong inhibitory effect on ammonia oxidation. The O-dealkylating activity of AMO has allowed us to develop a rapid spectrophotometric assay that allows us to follow AMO activity in real time. This assay follows the O-dealkylation of *p*-nitroanisole to 4-nitrophenol.

INTRODUCTION

Aromatic ethers with a variety of substituents are wide spread in nature and are most abundant as polymeric components of lignin (29). Most studies of the microbial metabolism of aromatic ethers have focused on the more toxic compounds, but there is considerable interest in the biodegradation of simple aromatic ethers because these present in the environment in such large quantities. Recently, we reported that are nitrifying bacteria can oxidize simple alkyl ethers through O-dealkylation reaction catalyzed by the enzyme ammonia monooxygenase (AMO) (15). For example, dimethyl ether was oxidized to methanol and formaldehyde by AMO (15). Other prokaryotic organisms have been shown to O-dealkylate aromatic ethers. Methane-oxidizing bacteria are known to oxidize and O-dealkylate several alkyl and aromatic ethers (7, 17, 18). For example, *Methylosinus trichosporium* OB3b transforms anisole and other substituted anisoles, although O-dealkylation is a minor reaction compared to hydroxylation (17, 18). Resnick *et al.* (28) examined the ability of a variety of hydrocarbon-utilizing bacteria to transform anisole and phenetole. They have shown that dioxygenase from different *Pseudomonas* strains oxidized anisole and phenetole to different hydroxylated products by both O-dealkylation and hydroxylation whereas monooxygenase enzymes oxidized aromatic ethers by only hydroxylation. The O-demethylation of phenolic ethers was examined for several aerobic bacteria (3). 4-Methoxybenzoate monooxygenase of *Pseudomonas putida* catalyzed the O-demethylation of methoxybenzoic acids (2). O-Dealkylation of aromatic ethers also has been demonstrated by eukaryotic organisms including several fungal species (3, 9, 20, 30, 31, 33). Smith *et al.* (31) examined a number of organisms but *Aspergillus niger* was the only species which oxidized aromatic

ethers using an O-dealkylation reaction. An O-demethylation of guaiacol has been reported in the case of liver microsome P-450 (30). P-450's have been reported to catalyze the O-dealkylation of *para*-substituted benzoates (3, 20), veratrole (33), guaiacol (30) and *ortho*-substituted alkyphenols (9). Anaerobic O-demethylation was reported by several researchers and they observed that methoxylated lignin monomers are degraded under methanogenic conditions although the biochemical mechanism of these was unclear (2, 6).

In the present study we have examined the O-dealkylating activity of the soil nitrifying bacterium *Nitrosomonas europaea* with respect to aromatic ethers. *Nitrosomonas europaea* is an obligate chemolithotrophic nitrifying bacterium which derives its energy for growth exclusively from the oxidation of ammonia to nitrite. Nitrification in *N. europaea* is initiated by the reductant-dependent oxidation of ammonia to hydroxylamine (NH_2OH) through the action of ammonia monooxygenase (AMO). Reductant for AMO-catalyzed reactions is provided by the further oxidation of hydroxylamine to nitrite by hydroxylamine oxidoreductase (HAO) (37). AMO has been demonstrated as a potent biocatalyst capable of transforming a wide range of non-growth supporting hydrocarbons. For example, n-alkanes (16), n-alkenes (16), and aromatic and polycyclic aromatic hydrocarbons (4, 21), halogenated hydrocarbons (27), and alkyl- (15), thio- (19) and aryl-ethers (5) are all oxidized by *N. europaea*. These studies have extended the substrate range of AMO to aromatic ethers.

In this present study we have examined the pathway of anisole degradation by *N. europaea* and have investigated the structural features of aromatic ethers that influence

the oxidation of these compounds. We have also demonstrated how these activities can be applied.

MATERIALS AND METHODS

Materials

Anisole, phenetole, butyl phenyl ether, dimethoxybenzene, 1,3,5-trimethoxy benzene, 2, 3, 4-methoxyphenol hydroquinone, catechol, formaldehyde, and acetaldehyde were purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). All of these reagents were more than 97% pure by manufactures analysis. Phenol and ethanol were purchased from local sources and were of 97 % + purity.

Growth and preparation of the cells

N. europaea (ATCC 19178) was grown in batch cultures (1.5 liters) in Erlenmeyer flasks (2 liters) at constant temperature (30 °C) on rotary shakers, as described previously (13). The cells were harvested by centrifugation (10,000 × 10 min) after 3 day growth, finally resuspended in assay buffer (1.5 ml, 50 mM sodium phosphate buffer [pH 7.8] containing 2 mM $MgCl_2$), and stored on ice for use within 12 h of harvesting.

Anisole and other aromatic ether degradation studies

Experiments for anisole and aromatic ethers degradation by *N. europaea* were conducted in serum vials (37 ml) sealed with Teflon-lined silicone septa (Sun Brokers™,

Wilmington, NC). The incubation medium (5 - 10 ml) consisted of phosphate buffer (50 mM potassium phosphate [pH 7.8], 2 mg $MgSO_4$). The required concentrations of anisole and other aromatic ethers were added from a stock solution in dimethyl sulfoxide (DMSO). The vials were placed in a sonicator for 5 min to allow dissolution of the compounds in the buffer. The reactions were initiated by the addition of 5 mM $(NH_4)_2SO_4$ and cells (0.25 - 0.5 ml; approximately *ca* 2.5 - 5 mg of protein) to the reaction vials. The vials were then returned to a heated shaking water bath (30 °C). To determine the consumption of each compound and accumulation of oxidation products, a sample of the liquid phase (200 µl) was removed and cells were removed by microcentrifugation; supernants (100 µl) were analyzed by HPLC (See Analytical Procedures).

Colorimetric assay of 4-nitroaniosle

The ability of *N. europaea* to transform 4-nitroanisole was tested by incubating cells with 4-nitroaniosle into 2 ml glass cuvettes. Initially, 1.9 ml phosphate buffer (50 mM potassium phosphate [pH 7.8], 2 mg $MgSO_4$) was added into the cuvette and then 500 µM 4-nitroanisole in dimethyl sulfoxide (DMSO) and 10mM NH_4^+ were added. The reaction was initiated by the addition of 100 µl cells (1.1 mg protein). The 4-nitrophenol production was measured by following the increase in the absorbance at A_{400}. To verify the production of 4-nitrophenol between colorimetric assay and HPLC, liquid samples (0.5 ml) were taken after 5 min and treated with 100 µM allylthiourea (ATU) to prevent any further AMO activity. The samples were centrifuged by microcentrifuge to remove cells, and then 100 µl supernant was injected into HPLC.

Analytical procedures

Aromatic ethers and oxidation products were analyzed by HPLC equipped with a reversed-phase ultramex C_{18} column (150 mm * 4.60 mm; phenomex). The HPLC elution program consisted of isocratic elution for individual aromatic ethers with 30 - 50 % (v/v) acetonitrile mixture in deionized water at the flow rate of 0.8 - 1.0 ml/min. All compounds other than 4-nitrophenol were detected by UV absorbance at 254. Acetaldehyde production was measured by gas chromatograph. Liquid sample (2 µl) were analyzed by using a gas chromatograph (HP 5890 Series II) equipped with a HP-INNOWAX column (30 min × 0.25 mm) and a flame ionization detector. The GC was interfaced to an integrator (HP 3365 Chemstation). The column temperature was initially set at 80°C and, after 4 min, raised to 200°C at a rate 10°C/min. Formaldehyde was measured by a coupled assay with formaldehyde dehydrogenase from *Pseudomonas putida* (Sigma Chemical Co., St. Louis, Mo.), using the modified methods of Ogushi *et al.* (23). After termination of the AMO reaction with ATU, cells were removed by centrifugation (10,000 × 2 min), and 400 µl of supernant was combined with 1.8 ml of 60 mM sodium carbonate (pH 8.9) and 200 µl of 12 mM NAD^+. Formaldehyde dehydrogenase (0.04 U in 10 µl of 10 mM sodium phosphate buffer [pH 6.8]) was added to the mixture, and the change in A340 nm due to the formaldehyde dehydrogenase-catalyzed production of NADH (ε = 6.22 mM^{-1} cm^{-1}) in the presence of formaldehyde was measured and a standard curve was prepared as described previously (26).

The nitrite level was determined colorimetrically as described previously (12). Protein concentrations were determined by the biuret assay (11) after solubilization of cell

protein in aqueous 3N NaOH (30 min at 60°C) and sedimentation of insoluble material by centrifugation (14,000 × g, 5 min).

RESULTS

Time course of anisole degradation

Previous studies with *N. europaea* have demonstrated that simple alkyl ethers are frequently oxidized by O-dealkylation reaction (15). In this study we were interested to determine whether aromatic ethers are substrates for AMO and whether these compounds undergo O-dealkylation reactions. Our initial investigations focused on anisole (methoxybenzene) as a model compound for aromatic ethers. The time course experiment described in Fig. 5-1 demonstrates that anisole was rapidly degraded by *N. europaea* and that phenol, 4-methoxyphenol, catechol and hydroquinone were generated as aromatic oxidation products. These products suggest both O-dealkylation and hydroxylation reactions were involved in anisole degradation. The initial observed ratio of O-dealkylation and hydroxylation reactions after 30 min was 2.3:1, indicated that O-dealkylation was the relatively dominant reaction by AMO at the beginning of the reaction. In the presence of acetylene (1 % v/v) no oxidation products were observed in an identical experiment to that described in Fig. 5-2 (data not shown). This result indicates the AMO is responsible for initiating the oxidation of anisole. In this experiment we also observed that accumulated phenol was depleted after 1 hr although 4-methoxyphenol was still produced up to 2.5 hr into the reaction time course. Concurrent

accumulation of hydroquinone and catechol explain the phenol consumption. And this was confirmed in separate experiments incubating cells with phenol. Hydroquinone and

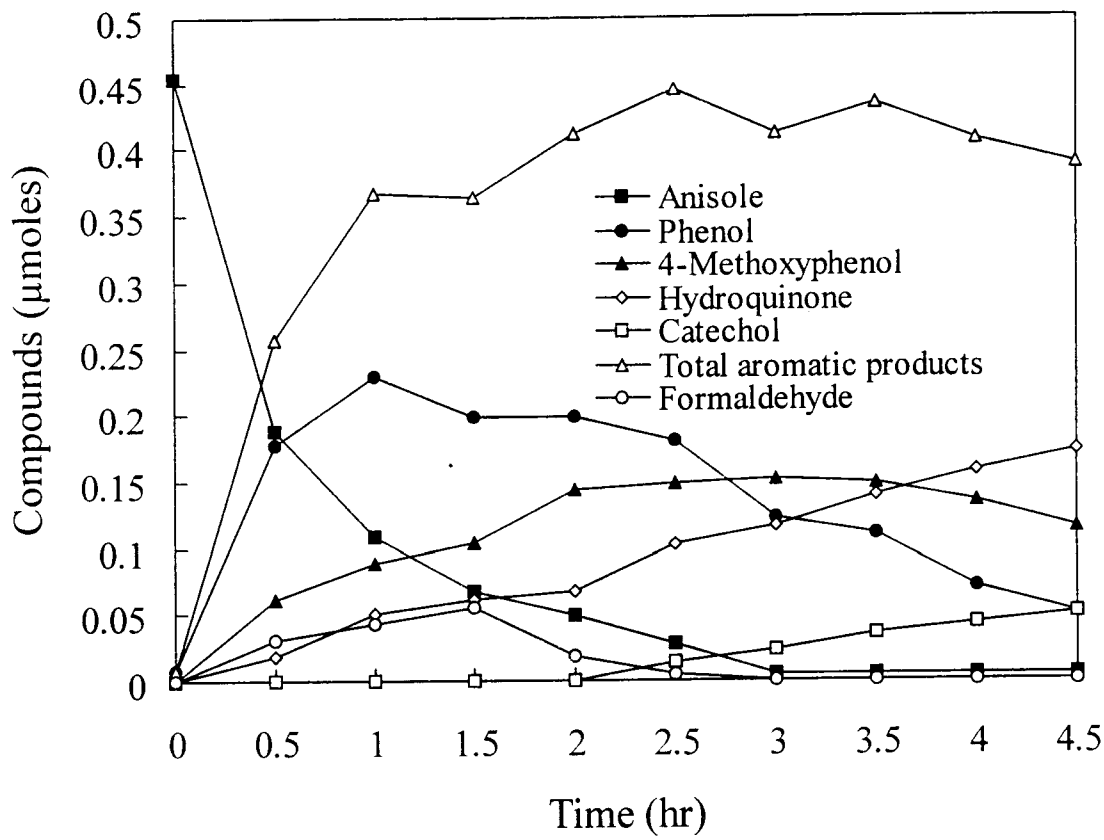

Figure 5-1. Time course for anisole degradation and subsequent accumulation of aromatic products by *N. europaea*. The experiment was conducted as described in Materials and Methods.

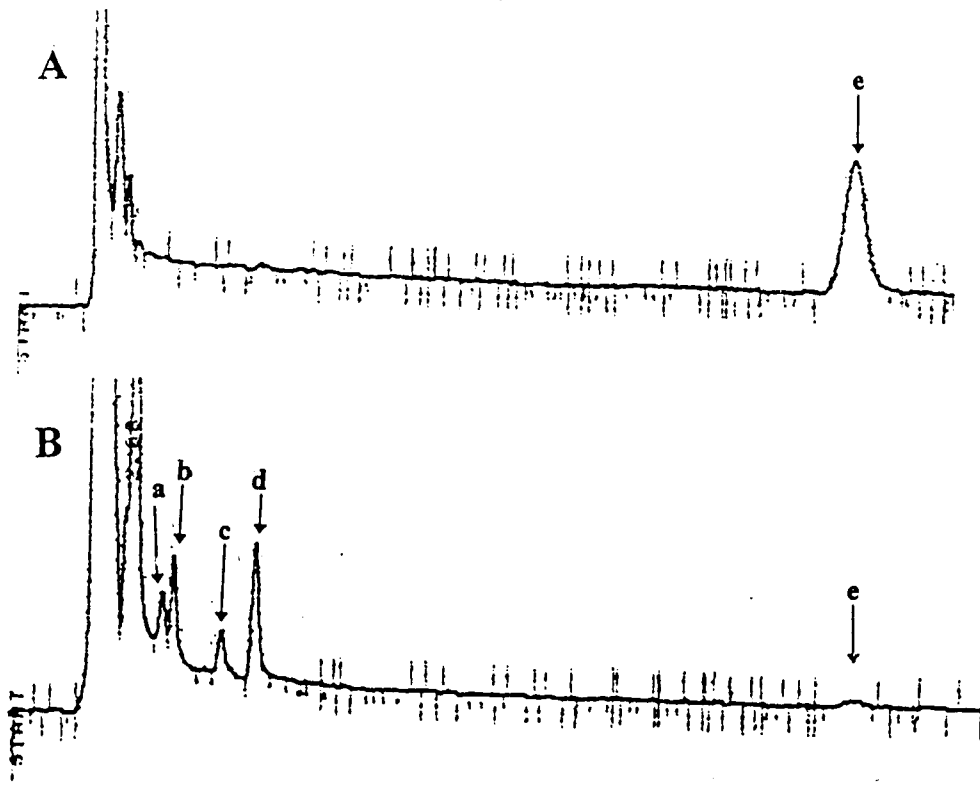

Figure 5-2. HPLC chromatograms showing the oxidation of anisole by *N. europaea.* These chromatograms were analyzed after 2.5 hr incubation from the sample as described in Fig. 5-1 (A) with acetylene (B) without acetylene; (a) catechol, (b) hydroquinone, (c) 4-methoxyphenol, (d) phenol, (e) anisole

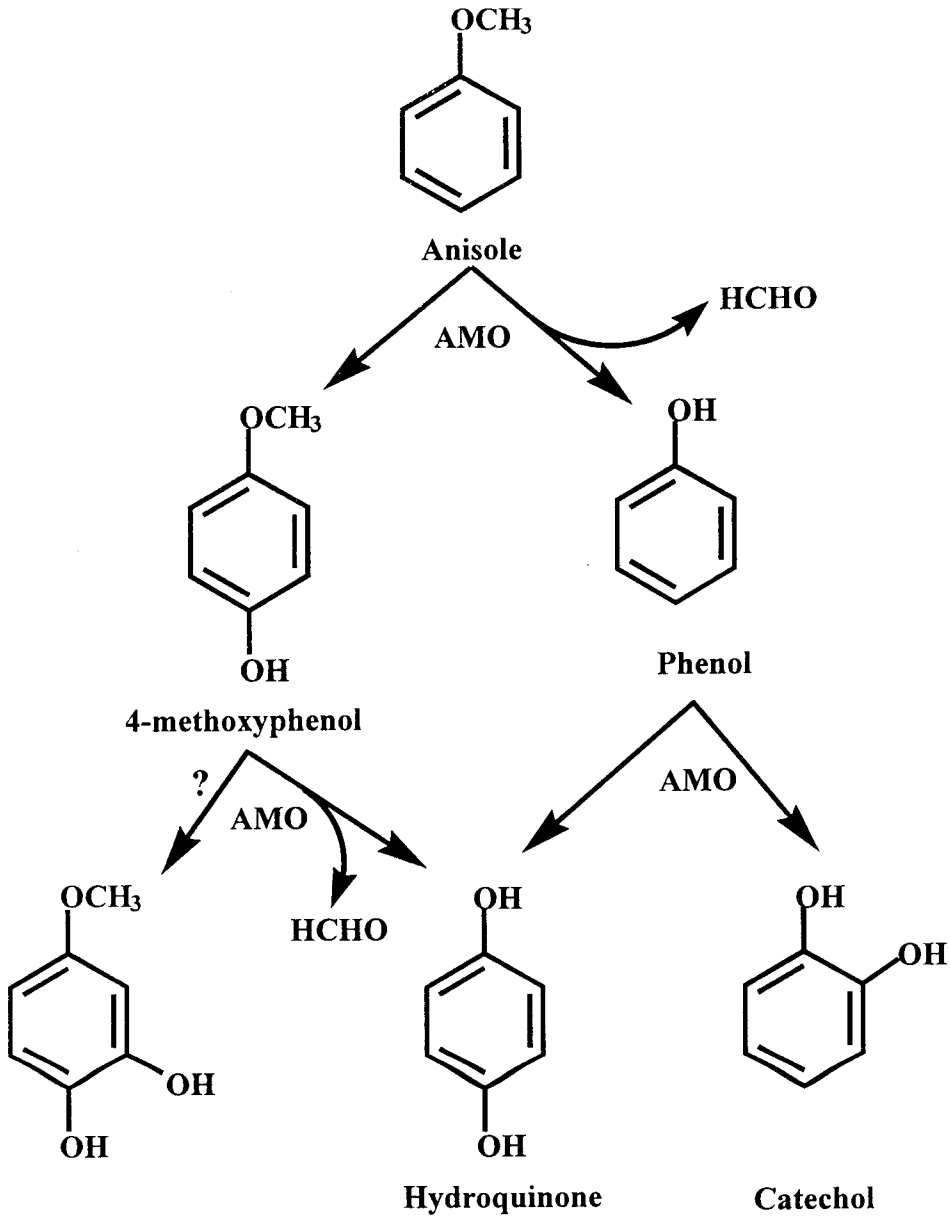

Figure 5-3. Overall pathway of anisole degradation by AMO

catechol were generated as phenol was oxidized, and no oxidation products were observed in the incubation with acetylene, which suggests that AMO is also involved in these further reaction transformations (data not shown). Cells incubating 4-methoxyphenol alone also produced hydroquinone and again no oxidation was observed in the presence of acetylene. Anisole oxidation by O-dealkylation would be expected to produce equimolar concentrations of phenol and formaldehyde. However, our results show considerable deviation from a 1:1 stoichiometry of both oxidation products. The low levels of formaldehyde may be due to further biotic and abiotic reactions which have been described previously (15). The total amount of aromatic products detected accounted for approximately 89 % of anisole depletion over 3 hr of the incubation. The overall pathway of anisole degradation by AMO was summarized in Fig. 5-3.

The effect of carbon number on ether bond oxidation

As our preliminary experiment indicated that anisole was readily degraded by *N. europaea* we were interest to examine to the effect of the size of the alkyl group on the ratio of O-dealkylation to hydroxylation reaction. To examine this, cells were incubated in the presence of equivalent concentrations of anisole, phenetole and butyl phenyl ether in the presence of 10 mM NH_4^+ for 1 hr. The products detected after this reaction indicate that O-dealkylation was the dominant oxidation reaction for both anisole and phenetole. Although no oxidation products were detected in the incubation with butyl phenyl ether formaldehyde was detected as a product of anisole oxidation and both ethanol and acetaldehyde were observed as products of phenetole oxidation. Anisole also produced a greater inhibitory effect on nitrite production as compared to phenetole which indicates it

is a more effective inhibitor of ammonia oxidation. However, this inhibitory effect could be due to both the substrate and the oxidation products. To investigate this we compared the inhibitory effects of both anisole and phenol at concentrations between 10 and 50 μM for the cells incubated with 10 mM NH_4^+. Phenol (50 μM) inhibited nitrite production by 90 % in a 1 hr incubation whereas an equivalent concentration of anisole only inhibited nitrite production by 50 % (data not shown).

Table 5-1. Effect of alkyl substituents on the oxidation of aromatic ethers by *N. europaea*

Substrate (amount)[a]	Product(s) (nmole formed in 60 min)[b]	% of nitrite production compared to control[c]
		10 mM NH_4^+
Anisole (0.17 μmol)	Phenol (87 ± 21) 4-methoy phenol (53 ± 2) Formaldehyde (28 ± 4) Hydroquinone (35 ± 5) Catechol (9 ± 3)	74.5
Phenetole (0.15 μmol)[d]	Phenol (34 ± 7) Acetaldehyde (21 ± 4) Ethanol (18 ± 3) Hydroquinone (19 ± 5) Catechol (6 ± 2)	79.6
Butyl phenyl ether (0.13 μmol)	ND	87.3

[a]Equivalent concentrations of substrate were added initially.
[b]Data represent the average of duplicate determinations ± standard deviation where indicated.
[c]Data represent the average of duplicate determinations.
[d]An additional product was detected by HPLC in substantial quantity but was not identified.
ND: not detected

The effects of the number and position of methoxy substituents

We were also interested to determine the effect of the position and number of methoxy substituents on the products of aromatic ether oxidation. Our results (Table 5-2) demonstrated that lower levels of oxidation product were obtained in 1 hr incubation as the number of methoxy groups was increased. Anisole oxidation generated the largest quantity of products by *N. europaea* whereas no oxidation products were observed in the incubation with 1,3,5-trimethoxy benzene. All three dimethoxy benzene were oxidized only through O-delkylation reactions whereas anisole was oxidized by both O-dealkylation and hydroxylation reactions. In the case of dimethoxy compounds, 1,4-dimethoxy benzene was approximately 4-fold more reactive than other dimethoxy benzene, 1,2- and 1,3-dimethoxy benzene, which suggests the higher activity of AMO on 1,4 configuration. The oxidation products, 2- and 4-methoxyphenol, were further O-dealkylated to diols by AMO whereas 3-methoxyphenol was not further oxidized. No oxidation products were detected from any of the compounds in incubations containing acetylene (1 % v/v). This again indicates that all of the transformations described in Table 5-2 can be attributed to AMO activity.

Colorimetric assay of 4-nitroanisole degradation

Our results to this point indicate that anisole is rapidly degraded by *N. europaea* and that the 1,4 configuration of substituents had a strong directing effect on the activity of AMO. These factors suggested a potential application of this activity in which the O-dealkylating activity of AMO could be used to provide a continuous spectrophotometric assay based on the O-dealkylation of 4-nitroanisole.

Table 5-2. Effect of number and position of methoxy group on the oxidation of methoxybenzene by *N. europaea*

Substrate (amount)[a]	Product(s) (nmole formed in 60 min)[b]	% of nitrite production compared to control[c]
		10 mM NH_4^+
Anisole (0.42 μmol)	Phenol (202 ± 8) 4-methoxyphenol (131 ± 16) Formaldehyde (31 ± 3) Hydroquinone (67 ± 4) Catechol (8 ± 1)	67
1,2 Dimethoxy benzene (0.32 μmol)	2-methoxy phenol (16 ± 1) catechol (6 ± 2)	95.7
1,3 Dimethoxy benzene (0.32 μmol)	3- methoxy phenol (40 ± 4)	96.8
1,4 Dimethoxy benzene (0.32 μmol)	4-methoxy phenol (168 ± 13) Hydroquinone (82 ± 14)	94.6
1,3,5 Trimethoxy benzene (0.26 μmol)	ND	81.8

aAll substrates were added to an equivalent concentrations equal to the aqueous solubility of 1,3,5 trimethoxybenzene.
[b]Data represent the average of duplicate determinations ± standard deviation where indicated.
[c]Data represent the average of duplicate determinations.
ND: Not detected

Our initial experiment (Fig. 5-4) indicated, showed that 4-nitroanisole was oxidized to 4-nitrophenol as the sole aromatic oxidation product. Subsequently we examined this reaction by following the generation of the colored 4-nitrophenol spectrophotometrically, as described in Materials ad Methods. Our results show that after an initial downward deflection there was a constant rate of increase in absorbance (400 nm) when cells were incubated with NH_4^+ (10 mM) and 4-nitroanisole (500 μM). In contrast there was no increase in absorbance when cells were incubated with NH_4^+, 4-nitroanisole and allylthiourea (ATU), a specific inhibitor of AMO activity. Similarly the addition of ATU

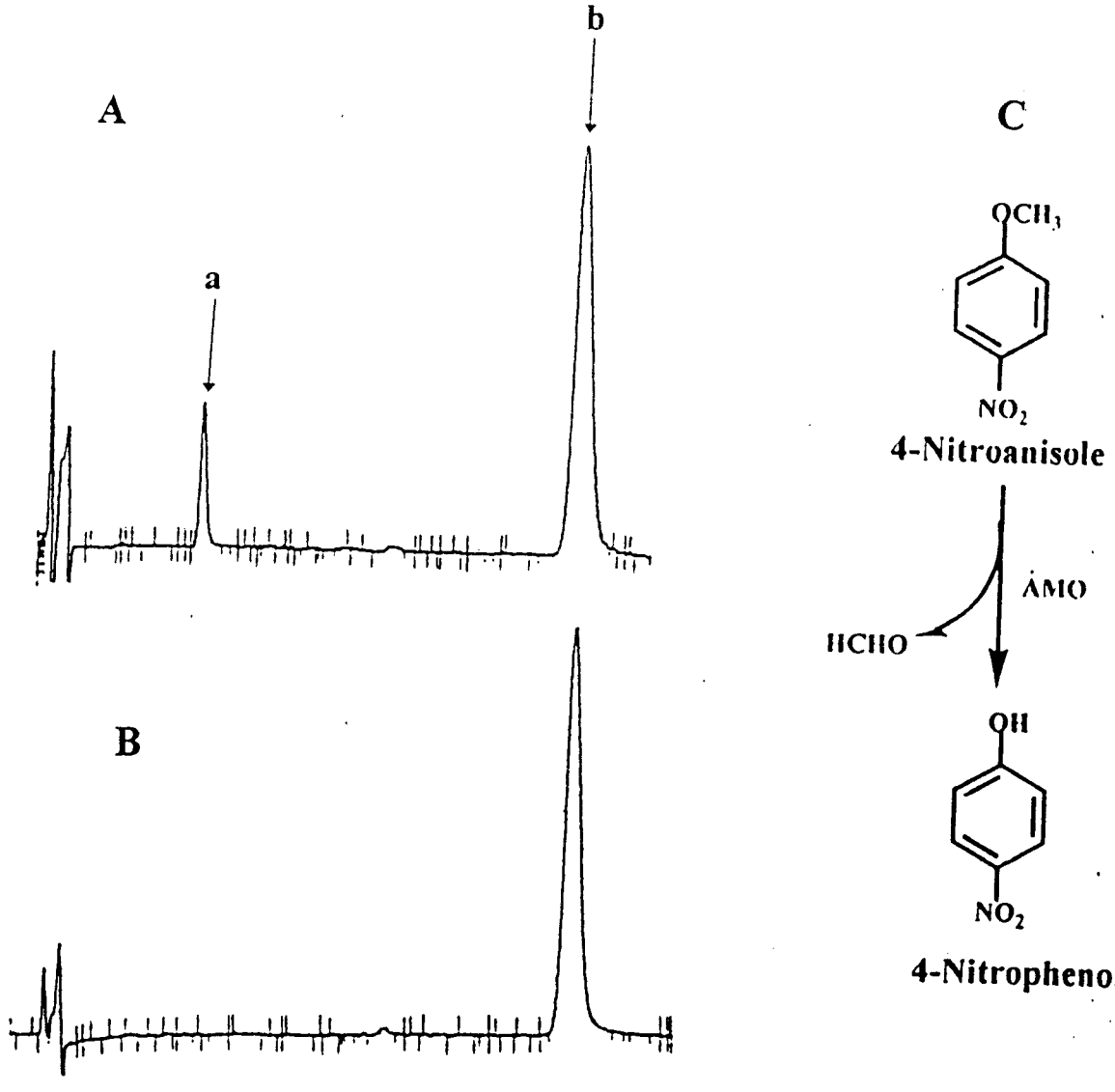

Figure 5-4. HPLC chromatograms of 4-nitroanisole degradation by *N. europaea*. Chromatograms (A) and (B) are analyses of the reaction medium from incubations of cells incubated with 10 mM NH_4^+ and 4-nitroanisole (500 μM). (A) without acetylene and (B) with acetylene (1 % v/v). The peak (a) represent 4-nitrophenol and (b) represent 4-nitroanisole.. (C) represent the pathway of 4-nitroanisole oxidation by AMO.

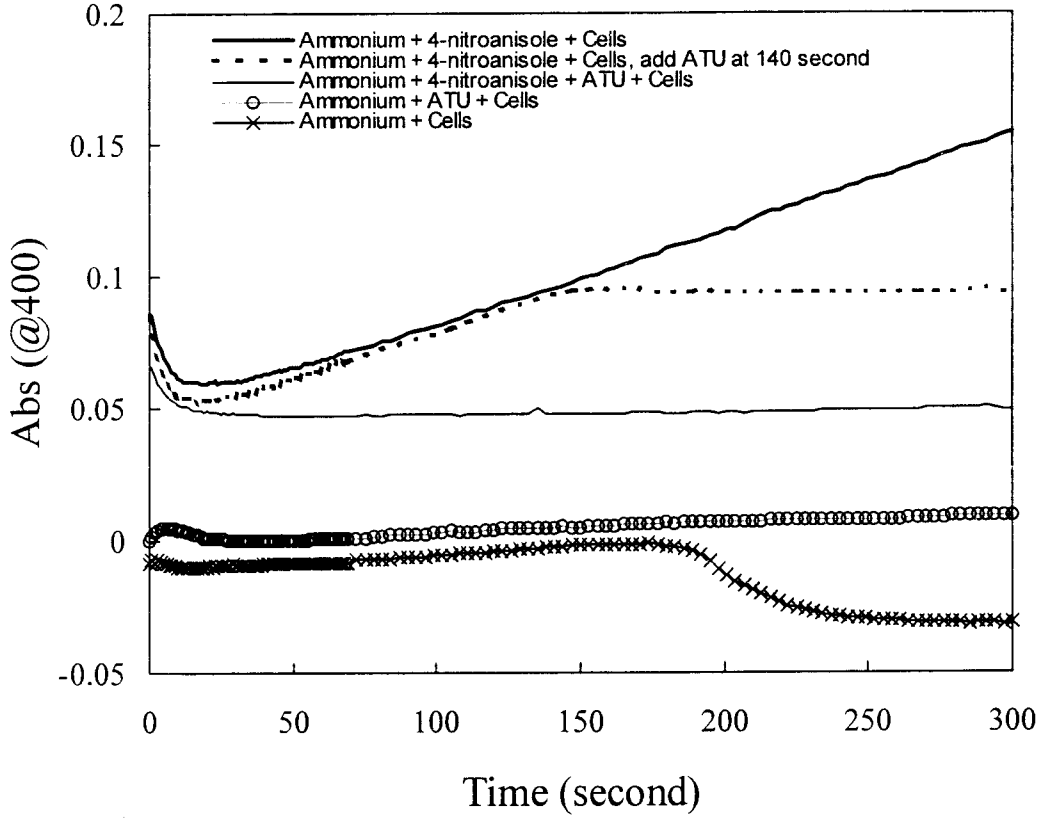

Figure 5-5. Colorimetric assay of 4-nitroanisole degradation by *N. europaea*. The reaction was conducted as described in the Materials and Methods section.

during the reaction time course led to an immediate halt in the increase in absorbance. In control reactions we observed no change in absorbance when cells were incubated with ATU alone. In the case of active cells incubated with NH_4^+ alone we observed no increase in absorbance over the first 3 min of the reaction. However, a subsequent decrease in absorbance after 3 min that plateaued at a lower but stable level was observed. The overall decrease in the absorbance in this instance was very close to the initial drop in absorbance in reactions conducted with 4-nitroanisole. We suspect that both decreases are associated with special changes associated with the use of cell suspensions in these spectrophotometric assay and can be attributed to changes in the oxidation state of the numerous cytochromes present in *N. europaea*.

DISCUSSION

We have examined the ability of soil nitrifying bacterium *N.europaea* to oxidize a series of methoxylated aromatic ethers. These compounds provided an opportunity to determine not only the effect of carbon number but also the position and number of methoxy substituents on the rate of ether oxidation and the products obtained from these reactions. Our results in this study indicate that all of the compounds tested were initially oxidized by AMO through one of two mechanisms; O-dealkylation and hydroxylation. A range of reaction types has been described for AMO including hydroxylation (16, 21), epoxidation (16), dehalogenation (25), and O-dealkylation (15). The O-dealkylation reactions catalyzed by AMO was initially described in the case of dimethyl ethers (15).

An unusual aspect of the AMO catalyzed reactions described in this study is that anisole degradation involves an O-dealkylation as the major reaction whereas

hydroxylations are the dominant reaction in other bacterial system. For example, either 2- and 4-methoxyphenols were observed as the major product from anisole degradation in other bacterial system (31). Anisole oxidation by methane-oxidizing bacteria, *Methylosinus trichosporium* OB 3b generates 4-methoxyphenol production which accounts for 90 % of the total products of aromatic oxidations. Smith *et al.* (31) reported that most organisms which hydroxylated anisole also O-demethylayed this compound but only as a minor reaction. The oxidation of anisole by *Aspergillus ochraceous* (ATCC 1008) produced 2-methoxyphenol, phenol and 4-methoxyphenol in the ratio of 1:3:20 and similar results reported by Ferris *et al.* (10), also indicated that O-dealkylation was the minor reaction. The formations of 2- and 4-methoxyphenol and phenol has been observed for P-450 catalyzed reactions in mammalian systems (31) although methoxyphenol is usually the dominant product (8). However, O-demethylation reaction was observed as the major metabolism in rat microsomes similarly to our results (35). *Aspergillus niger* (ATCC 9142) was the only species they tested in which the O-demethylation reaction was dominant and generated phenol as a only product. Our results demonstrated that initial oxidation products were further transformed and the detection of both hydroquinone and catechol as phenol oxidation has been previously observed by Hyman *et al.* (13) although further oxidation of phenol by other organisms was not been reported. Resnick *et al.* (28) examined ability of a variety of aromatic hydrocarbon-oxidizing bacteria to transform anisole. They have shown that oxygenases from different *Pseudomonas* strains oxidize anisole to different hydroxylated products. However, their results implicated that mono- and di-oxygenase reactions were not predictable in the case of anisole and phenetole degradation, and in certain situations, the distinction between dioxygenase and

monooxygenase activity can be dictated by the substrate rather than the enzyme. However, hydroxylations were the only reaction catalyzed by monooxygenase to oxidize anisole and phenetole whereas dioxygenases transformed these compounds by both hydroxylation and O-dealkylation (28).

Effects of carbon number on ether degradation

Our preliminary results (Fig. 5-1 and Fig. 5-2) showed that both O-dealkylation and hydroxylation were observed during anisole oxidation by AMO. This result addressed to determine the effect of carbon number on the oxidation rate and ratio of both reactions by AMO. Anisole was the most reactive substrate for AMO of the substrates tested (C_1 to C_4), which indicates the methoxy group is more readily oxidized than larger alkyl substituents. The observed ratio of O-dealkylation and hydroxylation reactions was 1.6:1 during 1 hr incubation with anisole. Hydroxylation products were detected by HPLC in substantial quantity but not quantified in the incubation with phenetole. However, O-dealkylation rate were dramatically decreased with increases in carbon number. Rasche *et al.* (25) reported similar results that formation rate of aldehyde indicating O-dealkylation reaction was decreased with increasing *n*-chlorinated C_1 to C_4 alkanes and Hyman *et al.* (16) observed that increases in carbon number for n-alkenes resulted in a decrease in the proportion of epoxidation reactions to hydroxylations. Similar inhibitory effect on ammonia oxidation by the presence of phenol have observed previously and 60 μM phenol inhibited 70 % of ammonia oxidation (32). Less benzene oxidation was observed related to inhibition of AMO activity by accumulated phenol (~ 80 μM) (21).

Effects of the position and number of methoxy substituents

As we have observed that different carbon number effected the rate of ether oxidation and the ratio of O-dealkylation and hydroxylation reactions, our results (Table 5-2) demonstrated that number of methoxy substituents effected the rate of ether oxidation and the ratio of O-dealkylation to hydroxylation reactions. The position of methoxy substituents also affected the oxidation rate. Similar to our results is the observation by Sutherland *et al.*(33) that 1,2-dimethoxy benzene is further transformed to catechol *via* O-dealkylation by P-450 enzymes.

Potential application of colorimetric assay

We have demonstrated the possibility of using the colorimetric assay to detect ammonia monooxygenase (AMO) activity. AMO is a non-specific enzyme like methane-monooxygenases (MMO) and can cometabolically transform a wide range of nongrowth substrates. Therefore, the oxidation of the substrate of AMO is dependent on AMO activity. Our results suggest that the rapid colorimetric assay is potentially applicable to detect AMO activity in whole cell and potentially cell free assays. The O-dealkylation of 4-nitroanisole could also potentially be used as a sample assay for detecting AMO activity in environmental samples.

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Chapter 6

Conclusion and Engineering Significance

This chapter summarizes the main findings of this research, briefly discusses their implications, and presents suggestions for further research.

COMETABOLIC DEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAH) BY A PHENOL-OXIDIZING *PSEUDOMONAS* STRAIN IN BATCH, CONTINUOUSLY STIRRED TANK- AND SEQUENCING BATCH REACTORS

Conclusions: Batch experiments demonstrated that a phenol-oxidizing bacterium identified as a *Pseudomonas* sp. was capable of degrading 2- and 3- ringed polycyclic aromatic hydrocarbons (PAHs) including naphthalene, 2-methyl naphthalene and anthracene cometabolically. Incubating active cells with naphthalene produced 1-naphthol as a major oxidation product, accounting for more than 90 % of the total products similar to the results observed from other bacterial and fungal system. Acetate was the potential reductant of phenol-utilizing organisms to sustain the cell activity. Sequencing batch reactor (SBR) was demonstrated to be a potential alternative technology to treat mixed contaminants containing phenol and lower molecular weight PAHs. Our results indicated that SBR was efficient system to treat PAHs without substrate inhibition. The operation of SBR had advantages compared to CSTR in this study.

Engineering Significance: SBR technology has been widely used to treat conventional activated sludge at the wastewater plant. This technology could easily applied to treat

conventional activated sludge as well as for cometabolic treatment of halogenated compounds (Irvine *et al.* 1983, Shih *et al.* 1997). In this study we have shown the possibility of potential application using SBR to treat the waste stream containing low molecular weigh PAHs. To operate SBR for the pilot scale appropriately several factors should be considered. Determining growth substrate is an important factor in the design and efficient operation of SBRs. Phenol was a excellent growth substrate which is nonvolatile and induces oxygenase activity toward halogenated alkenes as well as the several PAHs observed in this study. Phenol-utilizing organisms offer practical advantages in reactor systems. High growth rates enable more rapid start-up, smaller reactor volumes, and more rapid recovery from possible product toxicity and enzyme inactivation during nongrowth substrate transformation. The results of batch experiments demonstrated competitive interaction between growth substrate and nongrowth substrate. SBR technology has a great advantage to treat contaminants without the inhibitory effects observed in our study with other reactor configuration. Although the toxicity of naphthalene to phenol-oxidizers were not conducted, there way be certain toxic effects depending on the concentrations and exposure time of napthalene. To apply SBR techniques for PAH cometabolism, a recharge stage is needed to regenerate the PAH transformation activity of organism lost during PAH degradation. Such a step enables stable operation and continuous long term biodegradation of PAH.

COMETABOLIC DEGRADATION OF NAPHTHALENE AND OTHER POLYCYCLIC AROMATIC HYDROCARBON BY *NITROSOMONAS EUROPAEA*

Conclusion: Several 2- and 3- ringed PAHs were cometabolically degraded by *N. europaea*. An unusual observation in this study was that 2-naphthol was the major product of naphthalene oxidation by *N. europaea* whereas other procaryotic and eucaryotic organisms generate 1-naphthol. In the case of 2-methyl naphthalene, 2-methyl naphthalenol was produced by AMO and further transformed to 2-methyl naphthaldehyde not by AMO. The maximal rate of naphthol production was 1.65 nmole/mg protein-min incubated with 240 μM naphthalene and 10 mM NH_4^+. The rate of naphthol and nitrite productions was dependent on the concentrations of ammonia naphthalene, indicating the competitive interactions between the substrate and co-substrates. As inhibitory effect of naphthalene on ammonia oxidation, toxicity test was demonstrated. NH_4^+- and N_2H_2-dependent O_2 uptake measurement indicated that naphthalene and oxidation products 1- and 2-naphthols irreversibly inactivate AMO and HAO activity. This effect could limit the application of ammonia-oxidizing bacteria as "priming" catalysts for PAH degradation.

COMETABOLIC DEGRADATION OF DIBENZOFURAN AND OTHER ARYL ETHERS BY THE SOIL NITRIFYING BACTERIUM *NITROSOMONAS EUROPAEA*

Conclusion: The substrate ranges of AMO were extended to aryl ethers including DBF, DD and chloro- and bromo-diphenyl ethers which are USEPA Priority Pollutant. DBF was the most reactive substrate of this class of compounds and generated 3-OH DBF as

the sole biological oxidation product. An important finding was that the hydroxylated products of aryl ether were subsequently transformed in an abiotic nitration nitration reactions. The nitration reactions that occurred following the biological transformation by AMO were highly dependent on pH but less nitrite concentrations. Nitropolyaromatic hydrocarbons are recognized as potent toxins in mammalian systems and their production by *N. europaea* could limit any potential application of ammonia oxidizers to bioremediation of these types of compounds. However, this nitration reaction could be used to decrease the costs involved in conventional aerobic/anerobic processes designed to remove ammonia and from waste waters. Future research should be directed to examining cheap and readily available phenolic compounds that could be used to as nitration substrates.

COMETABOLIC DEGRADATION OF ANISOLE AND OTHER AROMATIC ETHERS BY *NITROSOMONAS EUROPAEA*

Conclusion: In this study, the substrate ranges of AMO was further extended to aromatic ethers. Several aromatic ethers which have methoxy substituent were readily degraded by AMO through two reactions, O-dealkylation and hydroxylation. The maximal rate of ether oxidation was observed in the incubation with anisole, and initial oxidation product was phenol, indicated that O-dealkylation reactions dominated. However, The rate of the oxidation decreased with increases in the size of the alkyl group and the number of methoxy substituents on the benzene ring. Of the dimethoxybenzenes tested, 1,4-dimethoxybenzenz was most rapidly degraded by AMO. The inhibitory effect on ammonia oxidation also decreased with increases in carbon number and number of

methoxy substituents. This results could be explained by the decrease rate of phenol production as less phenol was produced with increases increases in carbon number on ether bond and the number of methoxy substituents. A colorimetric assay was proposed in this study to detect AMO activity. Our findings suggested that rapid colorimetric assay based on the O-dealkylation of 4-nitroanisole could be used to monitor AMO activity in pure culture studies and in environmental samples.

ENGINEERING SIGNIFICANCE AND PROPOSED RESEARCH

The study of microbial transformations of PAHs and related compounds is important for the development of applications for the bioremediation of hazardous waste contamination. In general PAHs and other related compounds are often present in complex mixtures in soil, waste water, and other environments. For the bioremediation processes it is important that these hydrophobic compounds are available to degradative bacteria. Our major observation in this study is that soil nitrifying organisms can readily oxidize several PAHs, aryl ethers, and other aromatic ethers including naphthalene, acenaphthalene, dibenzofuran, dibenzo-*p*-dioxin, bromo-and chloro diphenyl ether, anisole and others. We suggest that nitrifying bacteria could therefore be used as a "priming" catalysts to initiate the degradation of polyaromatic contaminants, generating the products which are more soluble and more readily available to organisms that can fully degrade these compounds.

Another long term aim is that this nitrification process could be potentially coupled to denitrification process. Nitrifying bacteria would act as "priming" catalysts in

this system and ammonia and the target hydrophobic compounds are cometabolically transformed under aerobic conditions to yield nitrite (nitrate) and more soluble and biodegradable compounds, respectively. The products of these reactions could then potentiallly serves the respective electron donor and electron acceptor for further anaerobic transformations catalyzed by denitrifying organism. In general unsubstituted PAH and related compounds are known to be largely resistant to degradation under anaerobic conditions because of the biological stability of these compounds. While ammonia-oxdizing bacteria are not capable of mineralizing PAHs and other related compounds under aerobic conditions they may be able to contribute to the overall degradation of PAHs and other related compounds by catalyzing hydroxylation reaction. Several previous studies supported our hypothesis using coupled aerobic and anaerobic processes. Ruthy *et al.* (1988) observed that naphthalene was largely resistant to degradation under denitrifying conditions whereas 1-naphthol was rapidly consumed by Ruthy *et al.* (1988) and Grabic-Galic *et al.* (1989) observed increased susceptibility of substituted polyaromatics and even heterocyclic polyaromatics relative to unsubstituted compounds. To examine the potential anaerobic degradation of hydroxylated PAHs and other related compounds under denitrifying condition, laboratory experiments are required to isolate denitrifying organisms and determine the substrate range with respect to hydroxylated polyaromatics. These preliminary experiments will influence the design of any possible treatment system. However, bioreactors have proved as most effective system to remediate the soil and potentially wastewater contamination, since conditions for enhanced degradation could be achieved most readily. The feasibility of coupled aerobic/anaerobic processes using bench scale bioreactors could be potentially examined.

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Appendix

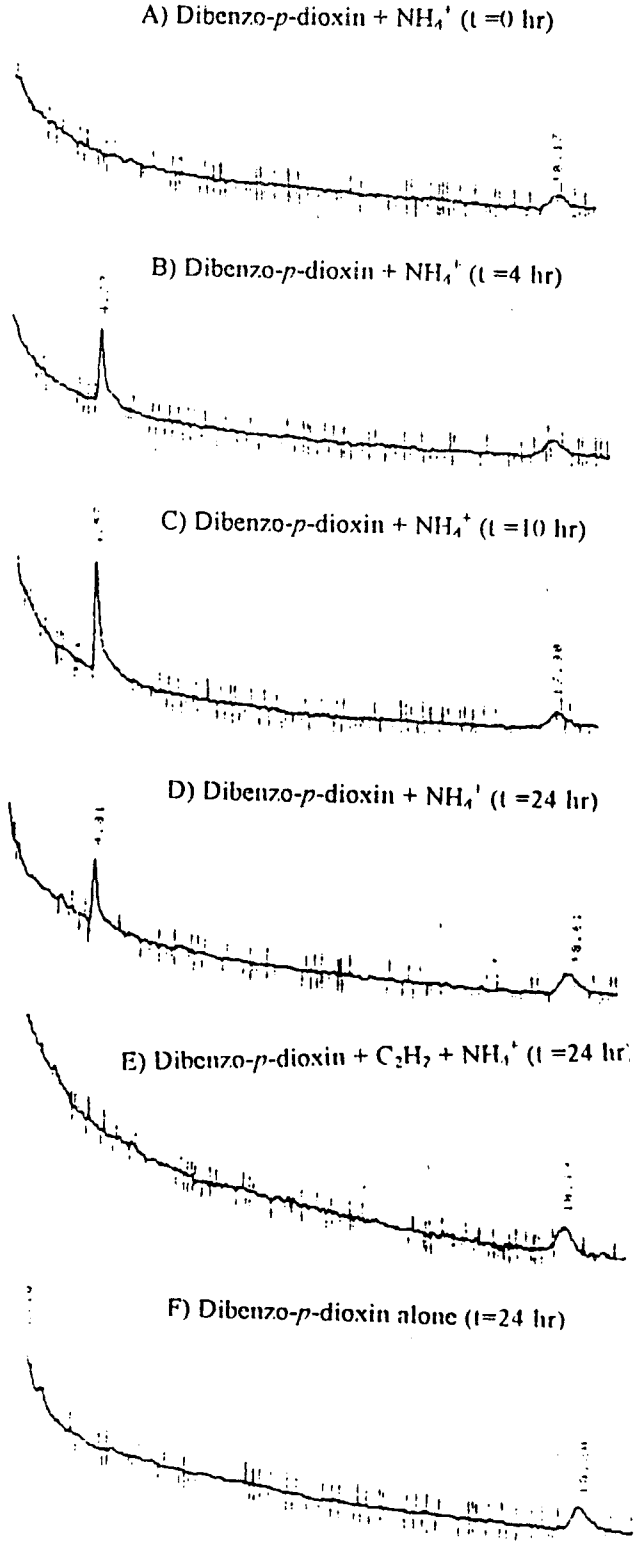


Figure A1. HPLC chromatograms of dibenzo-*p*-dioxin oxidation by *N. europaea.*

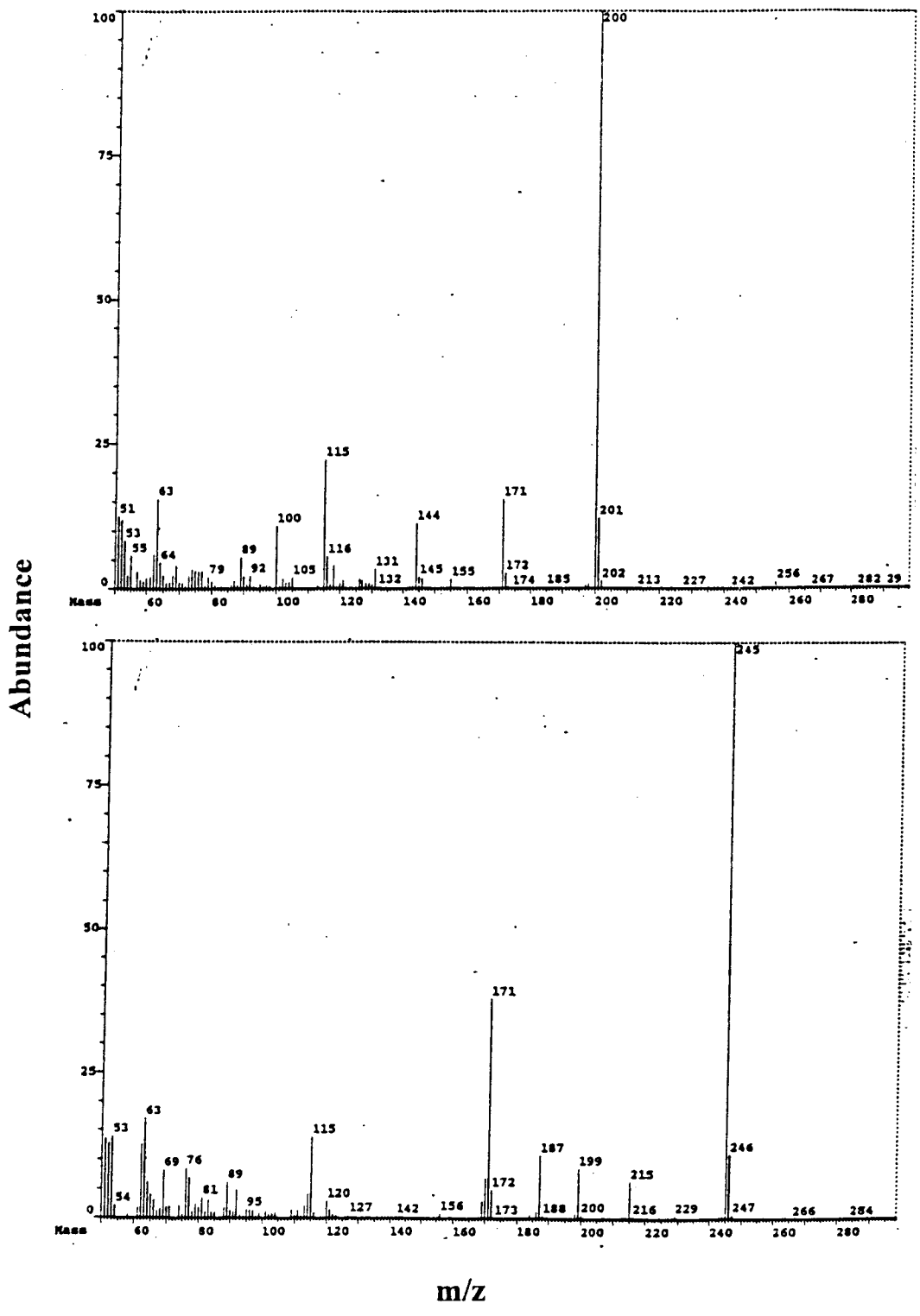


Figure A2. GC/MS chromatograms of biotic and abiotic products of dibenzo-*p*-dioxin.

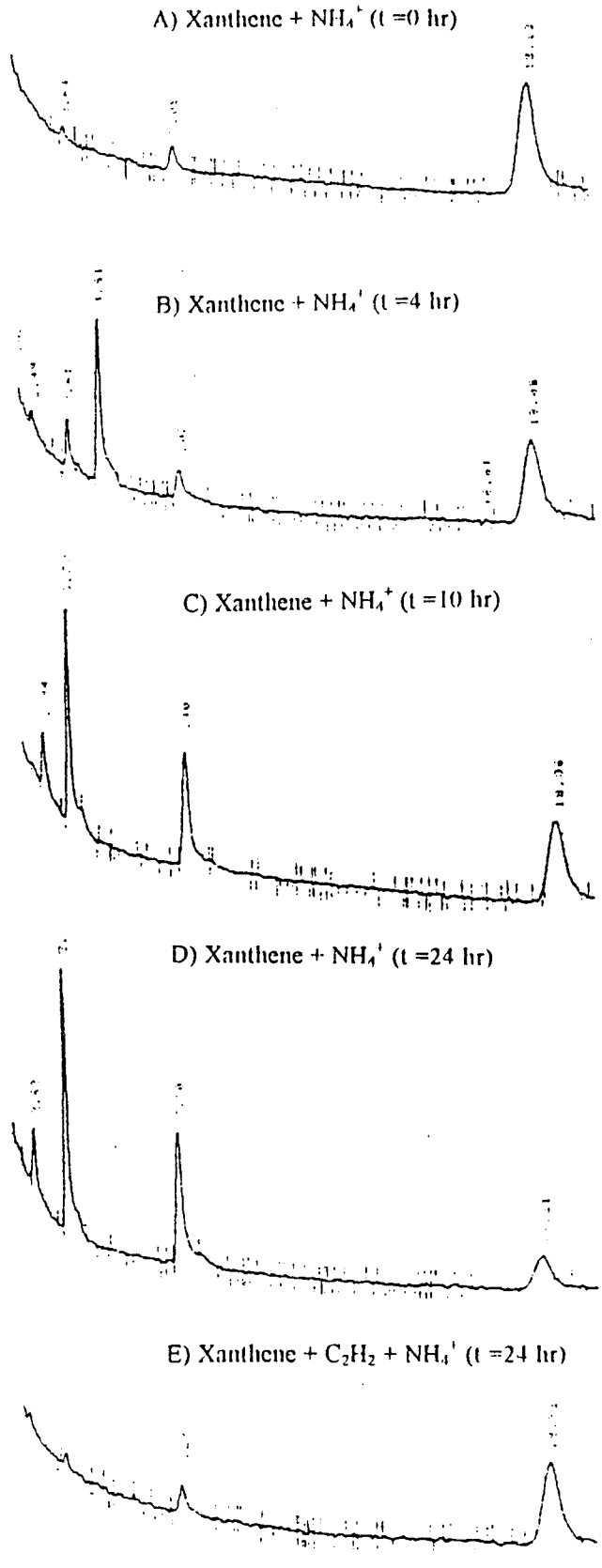


Figure A3. HPLC chromatograms of xanthene oxidation by *N. europaea.*

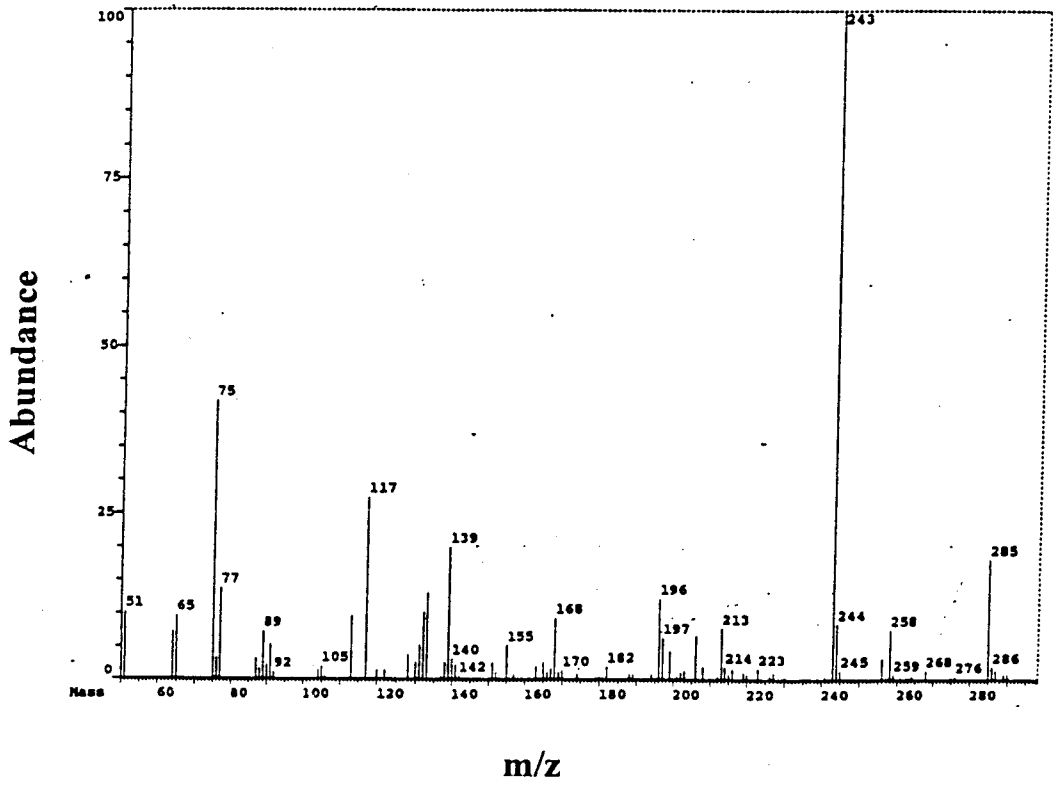


Figure A4. GC/MS chromatograms of biotic and abiotic products of xanthene.

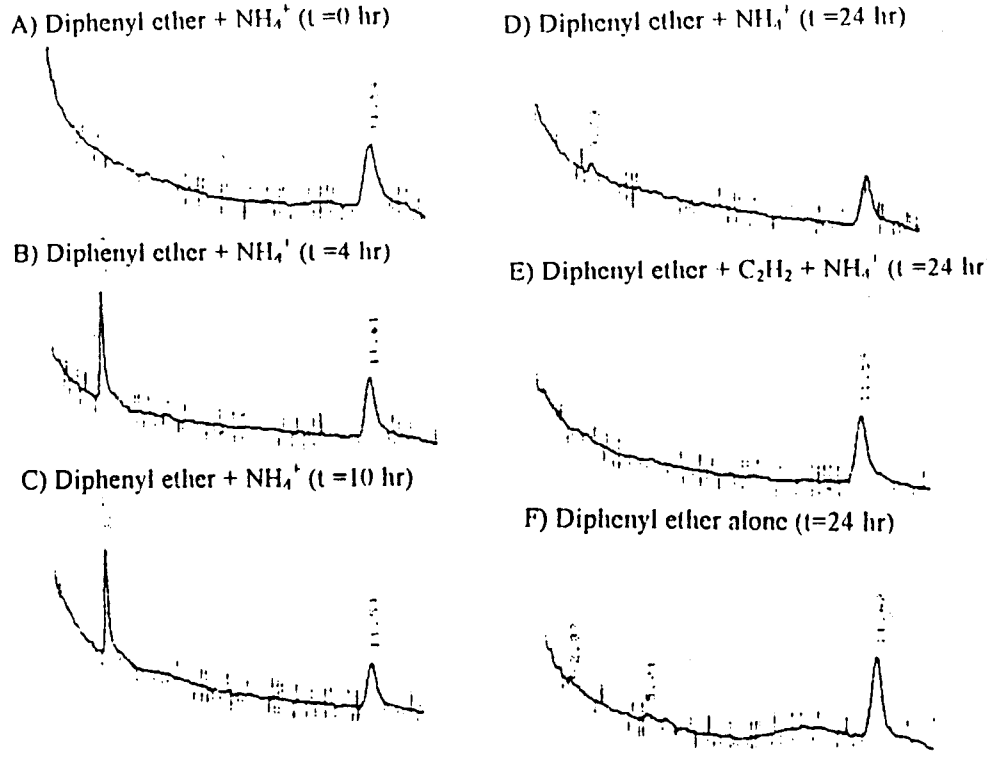


Figure A5. HPLC chromatograms of diphenyl ether oxidation by *N. europaea.*

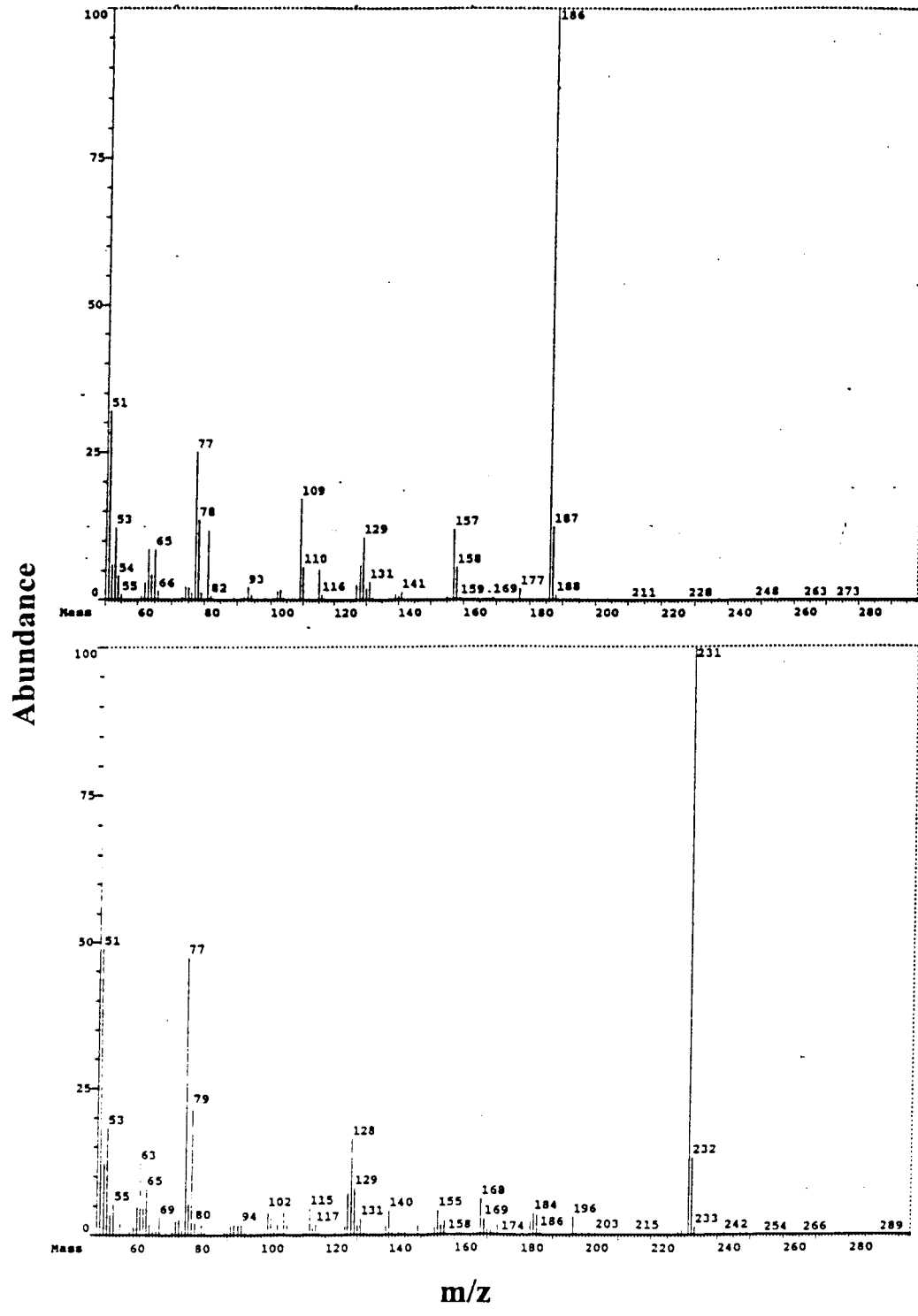


Figure A6. GC/MS chromatograms of biotic and abiotic products of diphenyl ether.

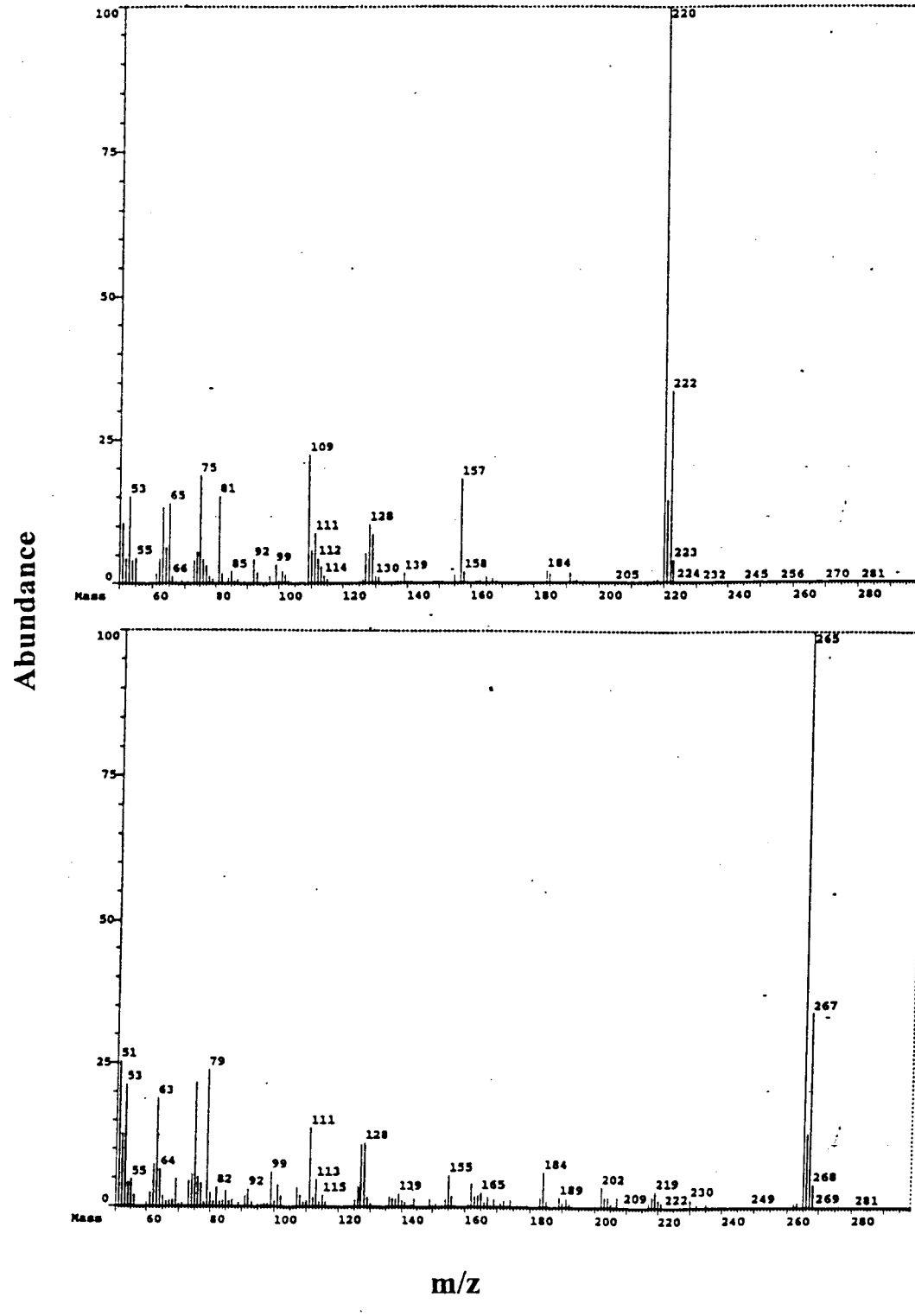
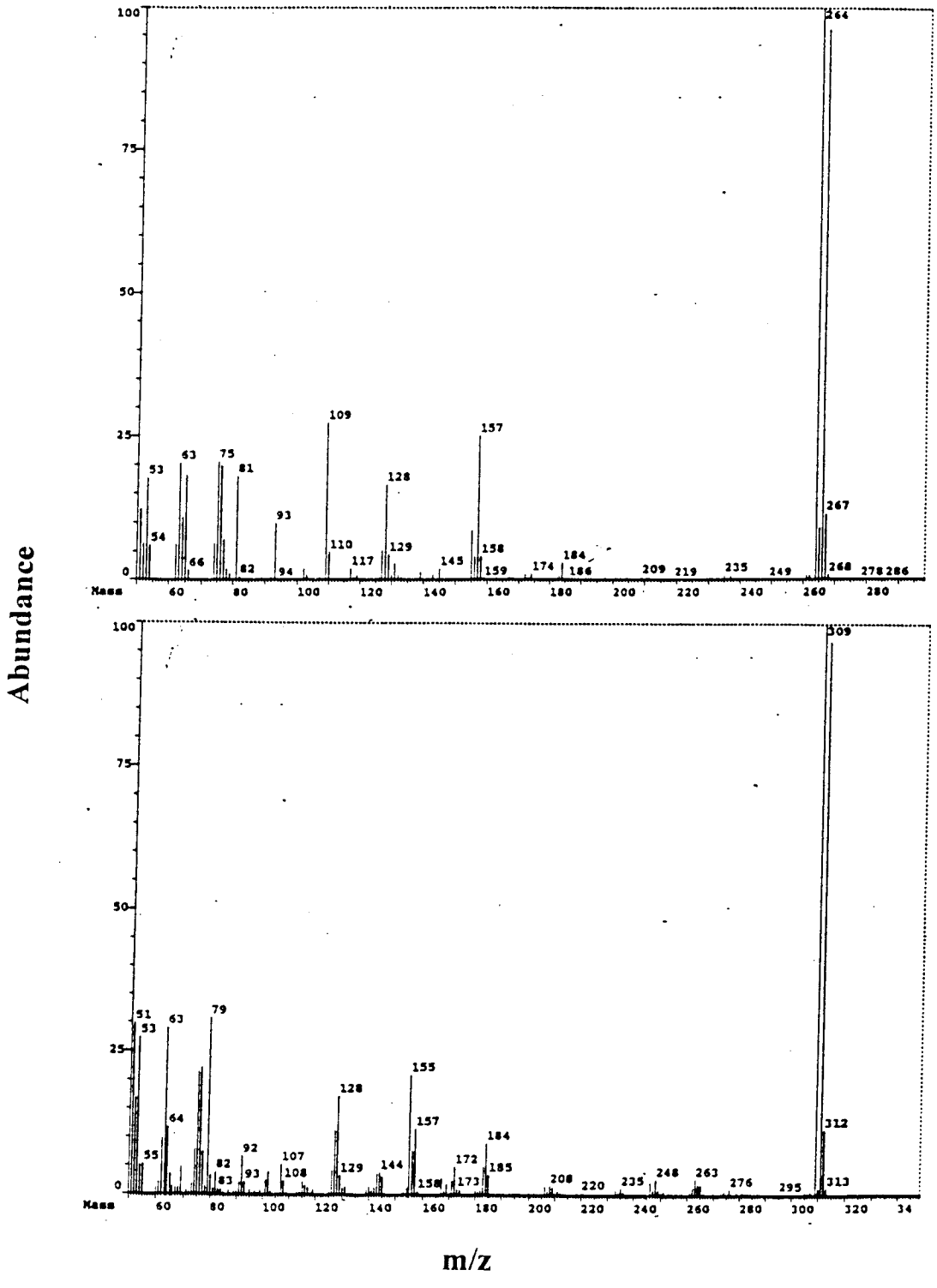


Figure A7. GC/MS chromatograms of biotic and abiotic products of 4-chloro diphenyl ether.



FigureA8. GC/MS chromatograms of biotic and abiotic products of 4-bromo diphenyl ether.

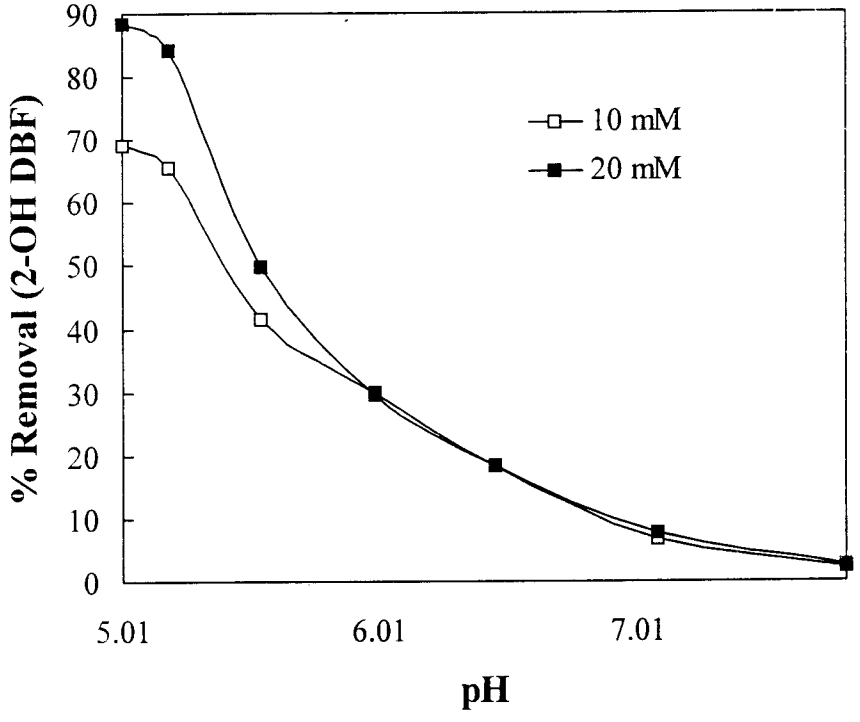


Figure A9. Abiotic transformation of 2-hydroxy dibenzofuran in dependent on pH and nitrite concentrations.

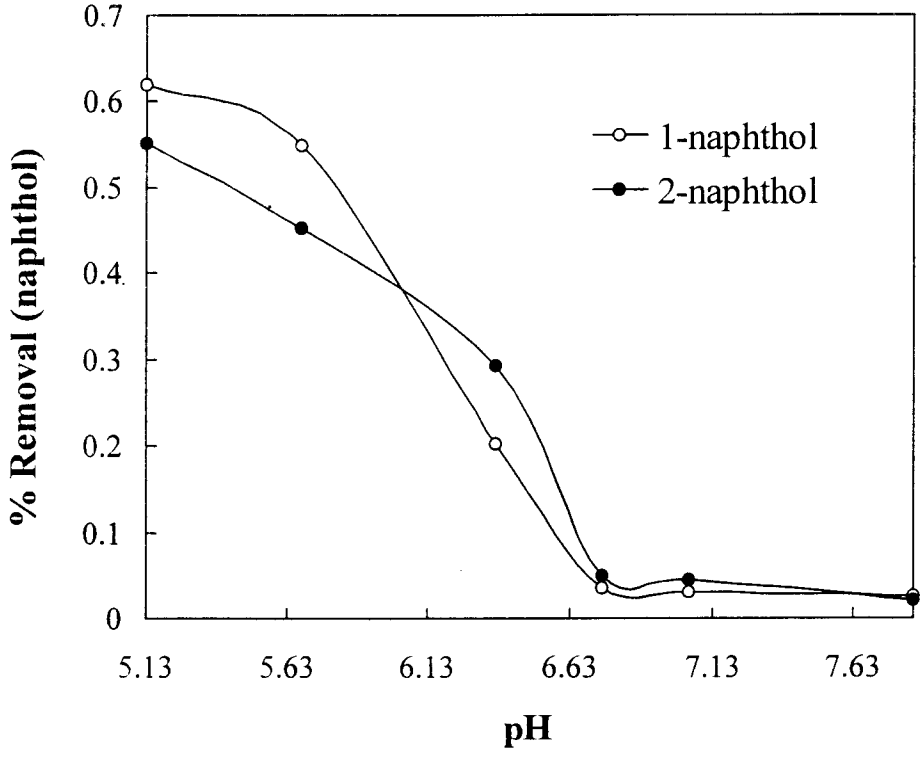


Figure A10. Abiotic transfromation of 1-and 2-naphthol in a dependent on pH and nitrite (10 mM) concentrations.

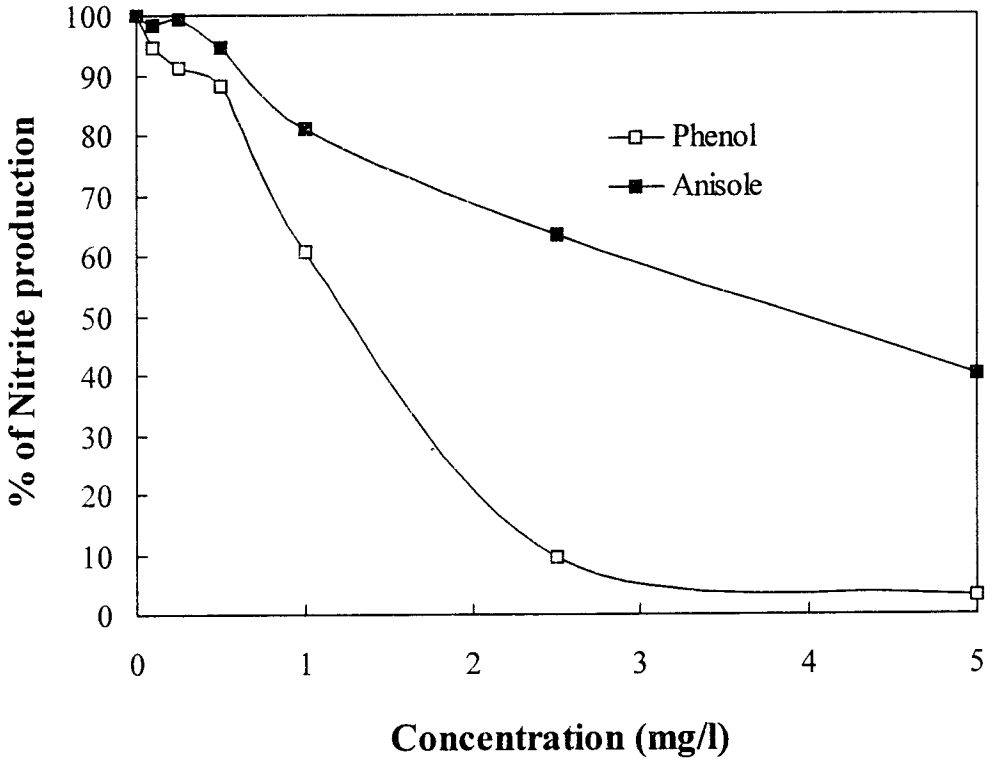


Figure A11. Effects of anisole and phenol on nitrite production during 1 hr incubation