USING STABLE ISOTOPE PROBING TO IDENTIFY SOIL MICROBIAL POPULATIONS INVOLVED IN PHENOL METABOLISM AT AN AGRICULTURAL FIELD SITE

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ABSTRACT

The global distribution and universal toxicity of phenolic compounds make their degradation of great interest. The goal of this field study was to provide insight into three distinct populations of microorganisms involved in *in situ* metabolism of phenol. Our approach measured $^{13}CO_2$ respired from ^{13}C-labeled phenol and stable isotope probing (SIP) of soil DNA at an agricultural field site. Traditionally, SIP-based investigations have been subject to the uncertainties posed by carbon cross-feeding. By altering our field-based, substrate-dosing methodologies, experiments were designed to look beyond primary degraders to detect trophically related populations in the food chain. Using GC/MS, it was shown that ^{13}C-labeled biomass, derived from primary phenol degraders in soil, was a suitable growth substrate for other members of the soil microbial community. Next, three dosing regimes were designed to examine active members of the microbial community involved in phenol metabolism *in situ*: (i) 1 dose of ^{13}C-phenol, (ii) 11 daily doses of unlabeled-phenol followed by 1 dose of ^{13}C-phenol, and (iii) 12 daily doses of ^{13}C-phenol. GC/MS analysis demonstrated that prior exposure to phenol boosted $^{13}CO_2$ evolution by a factor of 10. Furthermore, imaging of ^{13}C-treated soil using Secondary Ion Mass Spectrometry (SIMS) verified that individual bacteria inc orporated ^{13}C into their biomass. PCR amplification and 16S rRNA gene sequencing of ^{13}C-labeled soil DNA from the 3 dosing regimes revealed three distinct clone libraries: (i) unenriched, primary phenol degraders were most diverse, consisting of α-, β-, and γ-proteobacteria, and high G+C Gram-positive bacteria, (ii) enriched primary phenol degraders were dominated by members of the genera *Kocuria* and *Staphylococcus*, and (iii) trophically-related (carbon cross-feeders) were dominated by members of the genus *Pseudomonas*.

Furthermore, fungi-specific PCR amplification and sequencing of the 18S-28S internal transcribed spacer region genes from soil-derived, ^{13}C-DNA revealed a number of fungi involved in phenol degradation at this site. ^{13}C-labeled fungal DNA was only detected in one of our treatments (that representing trophically-related carbon cross-feeders) which suggests that these organisms are potentially secondary consumers in phenol-degradation at this site. These data show that SIP has the potential to document population shifts caused by substrate pre-exposure and to follow the flow of carbon through terrestrial microbial food chains.

BIOGRAPHICAL SKETCH

Christopher Michael DeRito was born on January 12, 1978 in Syracuse, New York and is the youngest child of William and Kathleen DeRito. He, his older brother Billy, and their older sister Dawn all spent their childhood years basically rocking the socks off of the grand suburb of Westvale, on the west-side Syracuse. Chris began playing hockey at the tender age of 5. Organized hockey lured both him and his brother (and their two biggest fans, Mom and Dad) all over the great state of New York…and sometimes into Canada. They were hooked. One might have thought the average five to six days a week of hockey might have been sufficient. It was not. So they did the only thing they could think of…they convinced their father to build them an ice rink in their backyard for *in between* playing organized hockey.

Each spring, however, after the ice thawed, Chris began to focus his attention on other things. Much of his time was spent outdoors…camping, hiking, and fishing. He became fascinated by nature and everything that makes it tick. Like hockey, he couldn't get enough. So he did the only thing he could think of…he co-founded his high school's first after-school Science Club. After graduating from Westhill High School in 1996, Chris decided to continue education at Boston College where he received his B.S. degree in Chemistry. He worked as an undergraduate lab assistant in the laboratory of Dr. Rudolph Hon of the Department of Geology and Geophysics studying Equilibria and Pathways of Fe/Mn in Naturally Contaminated Groundwater in Norwell, MA. During this time, Chris also waited tables and tended bar in order to help fund his education. After graduating from BC in 2000, he accepted a chemist position at SciLabs Inc., in Weymouth, MA.

Throughout Chris's time in Boston, one constant in his life was his relationship with his high school sweetheart, Amy Katherine Parry. Amy was then a resource

teacher and swim coach at Owego Free Academy in Owego, NY. After deciding it was time to join her, Chris wrote a letter to Dr. Eugene Madsen at Cornell University and in May of 2001, he arrived in Ithaca, NY where he accepted a technician position in Dr. Madsen's laboratory. In September of 2003, Chris joined Cornell's Employee Degree Program in pursuit of a Masters Degree in Environmental Toxicology. On September 3, 2004, Chris and Amy were married in Geneva, NY.

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Thanks to Cathy, Patti, Doreen, Shirley and John for making Wing Hall the well oiled machine that it is.

Finally, a very special thanks to all of my family: Mom, Dad, Dawn, and Billy thank you for all of your love, support and encouragement. And to my wife Amy, thank you for being everything you are.

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CHAPTER ONE

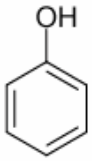
Introduction

1.1 What is Phenol and why is it a contaminant?

Phenol is a colorless to white, crystalline solid with a sickeningly sweet and tarry odor. Phenol consists of a benzene ring substituted with one hydroxyl group. The term "phenols", however, is used to describe a family of compounds containing a hydoxylated benzene ring in their structure. Phenolic compounds are ubiquitous in the environment due to both natural and anthropogenic sources. Phenol is corrosive, poisonous and very reactive. Table 1.1 and Table 1.2 provide a list of many of the chemical and physical properties of phenol.

Phenol enters the environment naturally by decomposition of organic matter and by burning wood (Scow *et al.*, 1981). It is a constituent of coal tar and is also one of the volatile components of liquid manure (Jenkins, 1994). Most contamination by phenol, however, is anthropogenic. In 2004, the U.S. alone produced over 6 billion pounds of phenol for various uses (CMR, 2005). The majority enters the environment during the production of phenolic resins (adhesives and coatings), bisphenol-A (polycarbonate plastics and epoxy resins) and caprolactam (nylon-6) (ATSDR). Phenol is found in exhaust gases, cigarette smoke, smoked foods, and treated lumber (Scow *et al.*, 1981; Potthast, 1976; Toth, 1982; Goerlitz *et al.*, 1985). Phenol is used as a slimicide and as a general disinfectant (Budavari, 1989; ATSDR). Other sources of phenol contamination include the commercial use of phenol-containing ointments, ear and nose drops, mouthwashes, toothache drops, analgesic rubs, throat lozenges, and antiseptic lotions (ATSDR).

Table 1.1 Chemical Identity of Phenol (Agency for Toxic Substances and Disease Registry).

Characteristic	Information	Reference
Chemical name	Phenol	Lide 1993
Synonym(s)	Benzenol, hydroxylbenzene, monophenol, oxybenzene, phenyl alcohol, phenyl hydrate, phenyl hydroxide, phenylic acid, phenylic alcohol	Lewis 1996
Registered trade name(s)	Carbolic acid, phenic acid, phenic alcohol	Gardner et al. 1978
Chemical formula	C_6H_6O	Lide 1993
Chemical structure		Budavari et al. 1989
Identification numbers:		
CAS registry	108-95-2	HSDB 2006; OHM/TADS 1998
NIOSH RTECS	SJ3325000	RTECS 2006
EPA hazardous waste	U188	EPA 1998; HSDB 2006
OHM-TADS	7216849	OHM/TADS 1998
DOT/UN/NA/IMCO shipping	UN 1671 (solid) UN 2312 (molten) UN 2821 (solution)	HSDB 2006
HSDB	113	HSDB 2006
NCI	C50124	Lewis 1996

CAS = Chemical Abstracts Services; DOT/UN/NA/IMCO = Department of Transportation/United Nations/North America/International Maritime Dangerous Goods Code; EPA = Environmental Protection Agency; HSDB = Hazardous Substances Data Bank; NCI = National Cancer Institute; NIOSH = National Institute for Occupational Safety and Health; OHM/TADS = Oil and Hazardous Materials/Technical Assistance Data System; RTECS=Registry of Toxic Effects of Chemical Substances

Table 1.2 Physical and Chemical Properties of Phenol (Agency for Toxic Substances and Disease Registry).

Property	Information	Reference
Molecular weight	94.11	Lide 1993
Color	Colorless to light pink	HSDB 2006
Physical state	Crystalline solid liquid (w/ 8% H_2O)	
Melting point	43 °C	Lide 1993
Boiling point	181.8 °C	Lide 1993
Density at 20 °C/4 °C	1.0545 at 45 °C/4 °C	Lide 1993
Vapor density	3.24	Lewis 1996
Odor	Distinct aromatic, somewhat sickening, sweet and acrid odor	HSDB 2006
Odor threshold:		
Water	7.9 ppm (w/v)	Amoore and Hautala 1983
	1 ppm (w/v)	Baker et al. 1978
Air	0.040 ppm (v/v)	Amoore and Hautala 1983
Solubility:		
Water at 20 °C	87 g/L	Lide 1993
Organic solvent(s)	Very soluble in alcohol, chloroform, ether benzene, acetone, water	Lide 1993
Partition coefficients:		
Log K_{ow}	1.46	HSDB 2006
Log K_{oc}	1.21–1.96	Artiola-Fortuny and Fuller 1982; Boyd 1982; Briggs 1981; Sacan and Balcioglu 1996; Scott et al. 1983
Vapor pressure at 25 °C	0.35	HSDB 2006
Henry's law constant	4.0×10^{-7} m^3/mol	Lide 1993
Autoignition temperature	715 °C	Lewis 1996
Flashpoint, open cup	85 °C	HSDB 2006
Flashpoint, closed cup	79 °C	NIOSH 1997
Flammability limits (in air, by % v)	1.7–8.6%	NIOSH 1997
Conversion factors:		
ppm (v/v) to mg/m^3 in air (25 °C)	ppm (v/v)x3.92=mg/m^3	
mg/m^3 to ppm (v/v) in air (25 °C)	mg/m^3x0.225=ppm (v/v)	

atm = atmosphere; v = volume; w = weight

1.2 Phenol Toxicity

Phenol is a universally toxic chemical. Documented studies of phenol toxicity have spanned a broad range of taxonomic biota, including (but definitely not limited to) bacteria, algae, protozoa, worms, fish, rodents, and humans (ATSDR; Jenkins, 1994). As with any environmental toxicant, the degree of phenol toxicity is determined by the dose received. In humans, varying degrees of toxicity, ranging from minor skin rashes to death, have been linked to phenol exposure (ATSDR). Cases resulting in death to humans tend to involve oral exposure (phenol was once a popular suicide agent (Lister, 1867)) and dermal exposure. Bruce *et al.* (1987) reported a minimal lethal oral dose of ~140 mg/kg while death resulting from accidental dermal exposure to as low as 25% phenol solutions have been reported (Griffiths, 1973; Lewin and Cleary, 1982). Studies directly involving humans also link phenol exposure to a variety of systemic, immunological and neurological disorders (ATSDR). Toxicity studies with laboratory animals suggest that exposure to phenol may have potentially negative reproductive and developmental effects as well (ATSDR). Phenol is also a potential carcinogen (Kauppinin *et al.*, 1986; ATSDR).

Very little is known about the biochemical mechanism of phenol toxicity. In high doses, phenol is very corrosive and can produce coagulation necrosis by denaturing and precipitating proteins (ATSDR). It has also been suggested that phenol can block cardiac sodium channels resulting in cardiovascular toxicity (Zamponi *et al.*, 1994). It is unclear, however, whether the systemic toxicity associated with phenol exposure is directly linked to the parent compound itself or to one of its metabolites (i.e. benzoquinone, hydroquinone, catechol). Chapman *et al.* (1994) demonstrated that rats exposed to both phenol and hydroquinone resulted in a

"more than additive" (synergistic) embroyotoxicity. The authors suggest that peroxidative activity may be important to the mechanism of phenol toxicity.

Phenol is readily absorbed by all routes of exposure and once absorbed is rapidly distributed to all tissues of the body. The metabolism of phenol occurs mainly in the liver, lungs, kidneys, intestine and gastrointestinal mucosa (ATSDR). It is detoxified by both oxidation and conjugation reactions. Cytochrome P450 2E1 catalyzes the hydroxylation of phenol producing catechol and hydroquinone. Hydroquinone can then be further oxidized by peroxidase enzymes producing benzoquninone. Phenol can also be directly oxidized by peroxidase enzymes producing biphenol and diphenylquinone. Conjugation of phenol and its metabolites is catalyzed by phenol sulfotransferase, UDP-glucuronosyl transferase, and glutathione-S-transferase producing various sulfate, glucuronide and glutathione conjugates (Figure 1.1) (Powell *et al.*, 1974; Cassidy and Houston, 1984; Campbell *et al.*, 1987). Phenol and its metabolites are excreted primarily in the urine (Pitrowski, 1971).

1.3 Microbial Degradation of Phenol

Phenolic compounds enter the environment from both natural and anthropogenic sources. The universal toxicity of phenol limits its own degradation by microorganisms (Sparling *et al.*, 1981). Although phenol, in its unsubstituted form, is not likely to persist in the environment (Howard *et al.*, 1991), the presence of substituents on the phenol ring can inhibit its biodegradation (Powlowski and Shingler, 1994; Sarand *et al.*, 2001). The magnitude of phenol pollution by industry coupled with its potential adverse health effects on humans makes the degradation of phenol of great interest.

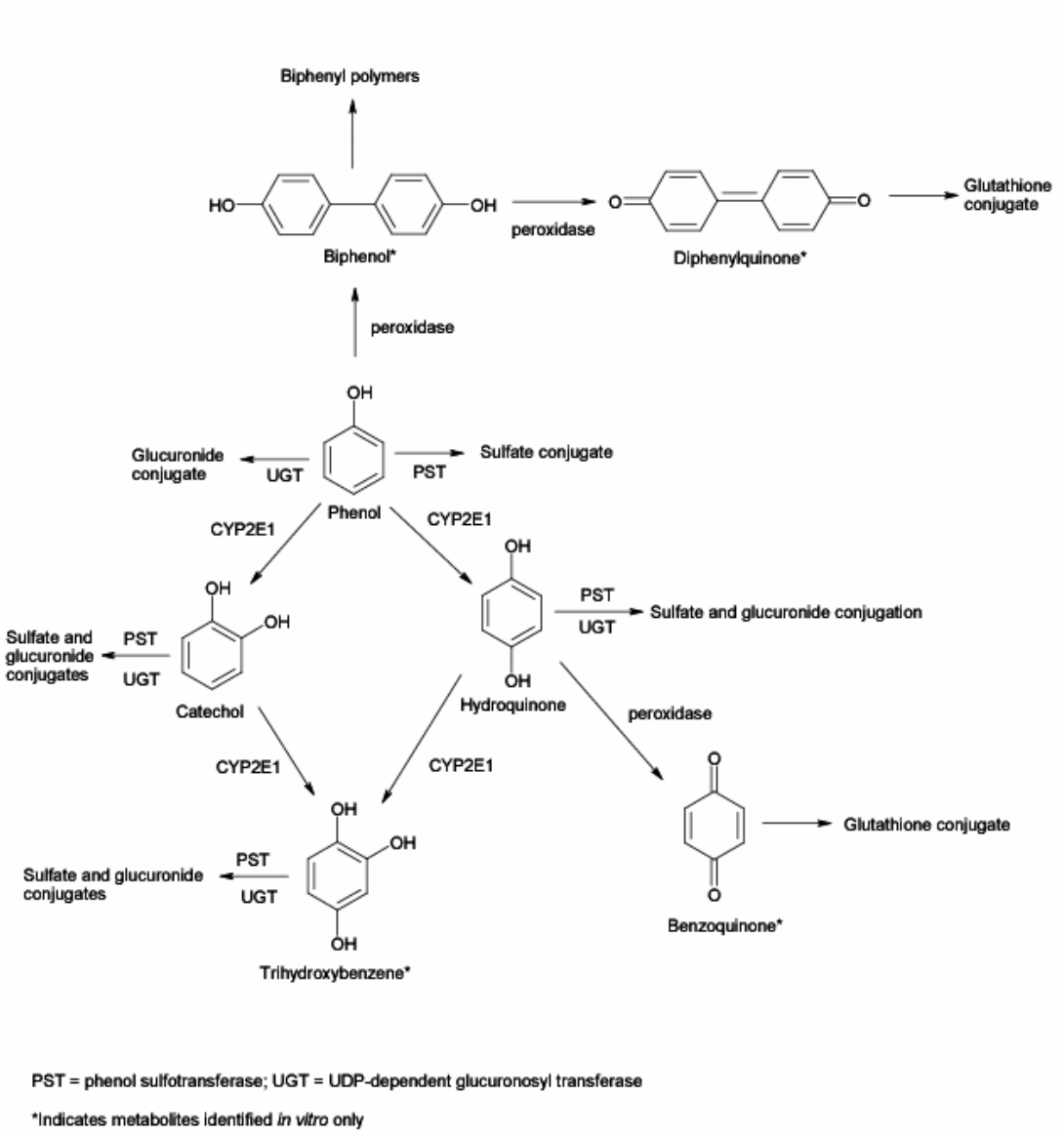


Figure 1.1 General metabolic pathways of phenol transformation in various tissues of mammals prior to urinary excretion. Conjugation pathways are driven by three enzymes: phenol sulfotransferase, uridine diphosphate (UDP) glucuronosyltransferase, and cytochrome P4502E1 (Agency for Toxic Substances and Disease Registry).

The microbial degradation of phenol has been studied since at least the 1930s (Happold and Key, 1932) and since then, a number of phenol-degrading microbes have been isolated. Cultured organisms that use phenol as a sole carbon and energy source include (but are not limited to) members of the genera *Pseudomonas*, *Bacillus*, *Ralstonia*, *Thauera* and *Trichosporon* (Shingler *et al.*, 1989; Gurujeyalakshmi and Oriel, 1989; Hino *et al.*, 1998; Manefield *et al.*, 2002; Neujahr and Gaal, 1973). The most extensively studied of phenol-degraders, however, belong to the genus *Pseudomonas* (Powlowski, 1994).

Pseudomonads are known to be metabolically versatile in utilizing natural and synthetic compounds as carbon and energy sources (Dagley, 1984). Classic examples of phenol degrading pseudomonads include *Pseudomonas putida* U and *Pseudomonas. sp.* strain CF600 (Bayly and Barbour, 1984; Shingler *et al.*, 1989). Studies involving these two strains are responsible for much of our current understanding of aerobic phenol catabolism by microbes.

meta- and ortho- ring cleavage

Work with the archetypal phenol-degrader, *Pseudomonas putida* U, is responsible for much of our current understanding of the *meta*-cleavage pathway of aromatic degradation (Bayly and Barbour, 1984). *Pseudomonas putida* U possesses the enzymes for both the *meta*- and *ortho*-cleavage pathways (Feist and Hegeman, 1969). Both pathways of phenol degradation involve the initial hydroxylation of phenol to catechol followed by cleavage of the aromatic ring. In the *meta*-cleavage pathway, the catechol ring is cleaved adjacent to the two hydroxyl groups. This differs from *ortho*-cleavage (common to marine phenol-degraders) where ring cleavage occurs between the two aromatic hydroxyls (Powlowski and Shingler, 1994).

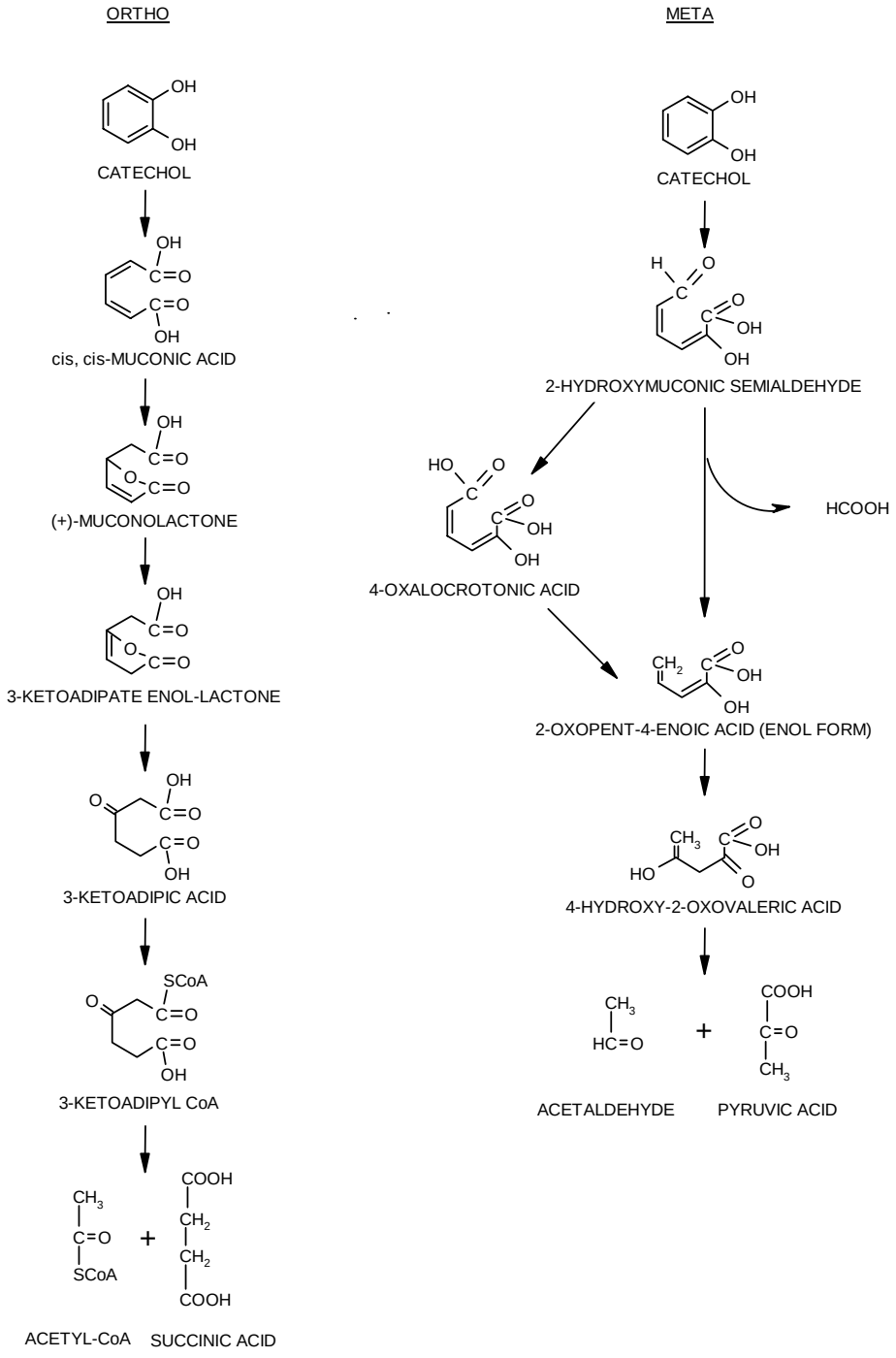


Figure 1.2 *Ortho*- and *meta*-cleavage pathways of catechol metabolism by bacteria (Environmental Protection Agency, 1986).

In both pathways (Fig. 1.2), ring-cleavage is followed by subsequent reactions leading to intermediates of central metabolism: acetaldehyde and pyruvate for *meta*-fission, and acetyl-CoA and succinate for *ortho*-fission (Bayly and Dagley, 1969; Dagley and Gibson, 1965; Ornston and Stanier, 1966; Stanier and Ornston, 1973).

Pseudomonas CF600

Work with *P. putida* U led to other studies of microbial phenol catabolism. Perhaps the most influential work involves the phenol-degrading strain, *Pseudomonas* sp. strain CF600. Through a series of studies, the catabolic pathway for phenol degradation by *Pseudomonas* CF600 was dissected and it was shown that phenol metabolism by this organism is by virtue of the plasmid-borne, multi-component phenol hydroxylase and a *meta*-cleavage pathway (Shingler *et al.*, 1989).
By measuring the activity of five enzymes [phenol hydroxylase (PH), catechol 1,2-dioxygenase (C12O), catechol 2,3-dioxygenase (C23O), hydroxymuconic semialdehyde dehydrogenase (HMSD), and hydroxymuconic semialdehyde hydrolase (HMSH)] of *Pseudomonas* CF600 cells grown in the presence and absence of phenol, Shingler *et al.* (1989) demonstrated that phenol catabolism by this organism involves initial conversion of phenol to catechol by a multi-component hydroxylase followed by *meta*-cleavage of the aromatic ring (Fig. 1.3). The authors showed that there was minimal induction of catechol 1,2-dioxygenase (the first enzyme of the *ortho*-pathway) in the presence of phenol compared to that of catechol 2,3-dioxygenase (the first enzyme of the *meta*-pathway). They also showed that phenol induced activity of both HMSD and HMSH, confirming that CF600 possesses both branches of the *meta*-cleavage pathway. The catabolic pathways for many aromatic compounds are plasmid encoded. By showing that the ability to degrade phenol could be transferred from

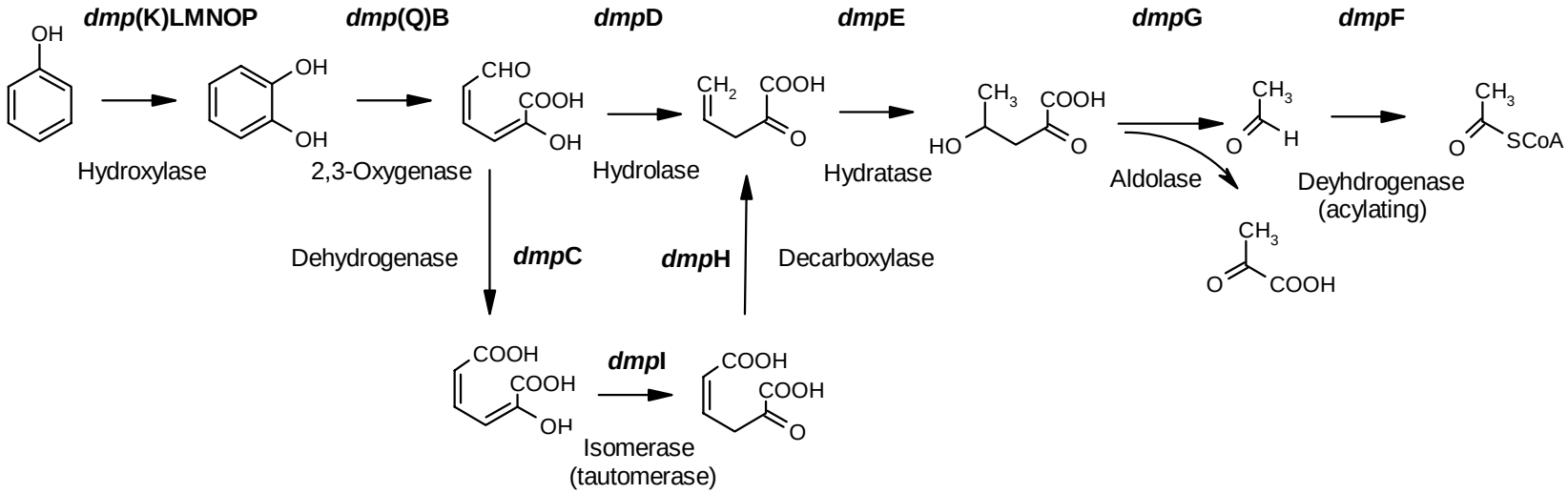


Figure 1.3 Phenol catabolic pathway of *Pseudomonas sp.* CF600 by a meta-cleavage pathway. Indicated are the genes of the *dmp* operon that encode for the enzymes that catalyze each step of the pathway (Shingler *et al.*, 1989; Powlowski and Shingler, 1994).

CF600 to other strains, Shingler *et al*. (1989) confirmed that the genes for phenol metabolism in CF600 are plasmid encoded as well. The nine enzymes responsible for phenol catabolism in *Pseudomonas* CF600 are encoded by fifteen genes of the *dmp* (dimethylphenol) operon (Powlowski and Shingler, 1994). These genes are located on the relatively large (>200 kb) pVI150 plasmid (Shingler *et al.*, 1989). Table 1.3 summarizes the genes and gene products of the *dmp* operon, as described by Powlowski and Shingler (1994). Nordlund *et al.* (1990) showed that the genes encoding the multi-component phenol hydroxylase are located within a 5.5 kb fragment of the pVI150 plasmid. They demonstrated that this 5.5 kb region consists of six open reading frames (ORFs) which produce 6 polypeptides (P0-P5) required for phenol metabolism (Table 1.3). Powlowski *et al.* (1990) showed that five of the six PH enzymes (P1-P5) in CF600 are required for oxygen uptake and require NAD(P)H as an electron donor for oxygenating activity.

The *dmp* system is tightly regulated by the regulatory protein, DmpR (Shingler *et al.*, 1994; Shingler and Moore, 1994; Sarand *et al.*, 2001). DmpR shares sequence homology with many other regulatory proteins of aromatic degradation, including XylR (Abril *et al.*, 1989), AphR (Arai *et al.*, 1998), BphR (Romine *et al.*, 1999), HbpR (Jaspers *et al.*, 2000), PhhR (Ng *et al.*, 1998), PhlR (Muller *et al.*, 1996), PhnR (Laurie and Lloyd-Jones, 1999), PoxR (Hino *et al.*, 1998), and MopR (Schirmer *et al.*, 1997). Aromatic degradation by many microorganisms often involves a two-component regulatory system in which the transfer of a phosphate group from a sensory protein activates the regulatory protein (Stock *et al.*, 1989). In the *dmp* system, however, there is no sensory protein involved. Using a *lux* gene reporter system, in which the DmpR-regulated operon promoter determines expression of luciferase activity, Shingler and Moore (1994) demonstrated that a direct interaction occurs between the substrate, phenol, and the regulatory protein, DmpR. They

Table 1.3 Summary of the genes and gene products of the *dmp* operon (Powlowski and Shingler, 1994).

Gene	Amino acid residues	Molecular mass (kDa) predicted/estimated	Product	Function	Reference
*dmp*K	92	10.6/12.5	P0	Unknown	Nordlund *et al.*, 1990
dmpL	331	38.2/34.0	P1	Phenol hydroxylase component	Nordlund *et al.*, 1990
dmpM	90	10.5/10.0	P2	Phenol hydroxylase component	Nordlund *et al.*, 1990
dmpN	517	60.5/58.0	P3	Phenol hydroxylase component	Nordlund *et al.*, 1990
dmpO	119	13.2/13.0	P4	Phenol hydroxylase component	Nordlund *et al.*, 1990
dmpP	353	38.5/39.0	P5	Phenol hydroxylase component	Nordlund *et al.*, 1990
dmpQ	112	12.2/12.0	DmpQ	Ferredoxin-like protein	Shingler *et al.*, 1992
dmpB	307	35.2/32.0	C23O	Catechol 2,3-dioxygenase	Bartilson & Shingler, 1989
dmpC	486	51.7/50.0	HMSD	2-Hydroxymuconic semialdehyde dehydrogenase	Nordlund & Shingler, 1990
dmpD	283	31.0/30.0	HMSH	2-Hydroxymuconic semialdehyde hydrolase	Nordlund & Shingler, 1990
dmpE	261	27.9/28.0	OEH	2-Oxopent-4-dienoate hydratase	Shingler *et al.*, 1992
dmpF	312	32.7/35.0	ADA	Aldehyde dehydrogenase (acylating)	Shingler *et al.*, 1992
dmpG	345	37.5/39.0	HOA	4-Hydroxy-2-oxovalerate aldolase	Shingler *et al.*, 1992
dmpH	264	28.4/28.5	4OD	4-Oxalocrotonate decarboxylase	Shingler *et al.*, 1992
dmpI	63	7.1/6.7	4OI	4-Oxalocrotonate isomerase	Shingler *et al.*, 1992

confirmed that this interaction is responsible for regulating transcription of the *dmp* system in *Pseudomonas* sp. strain CF600.

As mentioned earlier, the presence of substituents on the phenol ring can limit its biodegradation. Although CF600 has been shown to utilize both phenol and methylsubstituted phenols as a sole carbon and energy source, growth of CF600 on *para*-substituted methylphenols is relatively poor (Shingler and Moore, 1994). Pavel *et al.* (1994) demonstrated that a single point mutation in DmpR resulted in enhanced recognition of both 4-methylphenol and 3,4-dimethyl phenol by the regulatory protein. This mutation, when introduced to the wild-type CF600, conferred enhanced utilization of these substrates. The authors concluded that aromatic effector activation of DmpR by *para*-substituted methylphenols is a major factor limiting the degradation of these compounds.

These landmark studies involving *Pseudomonas* sp. strain CF600 are responsible for much of our current understanding of the biochemistry involved in microbial phenol catabolism. Since then, a number of other genetic systems for phenol metabolism have been investigated. A few of these systems are homologous to the *dmp* system of CF600 (ie, the *pox* system of *Ralstonia eutropha* strain E2 and the *mop* system of *Acinetobacter calcoaceticus* NCIB 8250); (Hino *et al.*, 1998; Ehrt *et al.*, 1995). However, some organisms degrade phenol using distinctly different catabolic enzymes. Table 1.4 compares the genetic systems of various phenol degraders.

Metabolism of phenol by anaerobic bacteria and fungi

Although the scope of this thesis focuses primarily on aerobic metabolism of phenol by bacteria, it is important that the reader is aware of other systems of microbial phenol degradation. A number of sulfate-reducing, denitrifying, and iron-

Table 1.4 Microorganisms used in biochemical and genetic investigations of phenol metabolism.

Organism	Information	Reference
Pseudomonas sp. strain CF600	NAD(P)H dependent, multi-component hydoxylase, encoded by *dmp*KLMNOP (located on pVI150 plasmid)	Nordlund *et al.*, 1990; Shingler et al., 1992
Pseudomonas putida P35X	multi-component hydroxylase, chromosomally encoded by *phh*KLMNOP	Ng *et al.*, 1994
Pseudomonas putida BH	multi-component hydroxylase encoded by *phe*A1A2A3A4A5A6	Takeo *et al.*, 1995
Pseudomonas putida H	multi-component hydroxylase encoded by *phl*ABCDEF (located on pPGH1 plasmid)	Herrmann *et al.*, 1995
Acinetobacter calcoaceticus NCIB 8250	multi-component hydroxylase encoded by *mop*KLMNOP	Ehrt *et al.*, 1995
Pseudomonas sp. strain EST1001	NADPH dependent, single-component monooxygenase encoded by *phe*A	Kivisaar *et al.*, 1989
Burkholderia (Pseudomonas) pickettii PKO1	NADPH dependent, single-component monooxygenase encoded by *thu*D	Kukor & Olsen, 1992
Bacillus stearothermophilus BR219	NADH dependent, single-component monooxygenase encoded by *phe*A	Kim & Oriel, 1995
Ralstonia eutropha E2	multi-component hydroxylase encoded by *pox*RABCDEFG	Hino *et al.*, 1998
Comamonas testosteroni P1	multi-component hydroxylase encoded by *aph*KLMNOPQB	Arai *et al.*, 1998
Klebsiella oxytoca KH 1	single-component monooxygenase encoded by genes on a TOL-like plasmid	Heesche-Wagner *et al.*, 1999
Pseudomonas putida PaW85	single-component monooxygenase encoded by *phe*A	Kasak *et al.*, 1993
Variovorax paradoxis	multi-component hydroxylase encoded by LmPH genes	Watanabe *et al.*, 1998
Alcaligenes sp.	multi-component hydroxylase encoded by LmPH genes	Zhang *et al.*, 2004
Trichosporon cutaneum	NADPH dependent, single-component hydroxylase encoded by *phy*A	Kalin *et al.*, 1992; Sejlitz *et al.*,1990

reducing bacteria have been shown to grow anaerobically on phenol (Bak and Widdel, 1986; Shinoda *et al.*, 2000; Lovley and Lonergan, 1990). These organisms metabolize phenol using a completely different set of enzymes compared to those of aerobic bacteria. Work with the denitrifying beta-proteobacterium, *Thauera aromatica* (originally isolated by Tschech and Fuchs, 1987) provides most of our current understanding of the enzymology of anaerobic phenol metabolism. The initial steps of anaerobic phenol degradation by *T. aromatica* proceeds via three distinct reactions: (i) ATP-dependent carboxylation to 4-hydroxybenzoate via a phenylphosphate intermediate (Lack *et al.*, 1991; Lack and Fuchs, 1992; Lack and Fuchs, 1994; Schmeling *et al.*, 2004; Schühle and Fuchs, 2004), (ii) reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA (Biegert *et al.*, 1993; Brackman and Fuchs, 1993), and (iii) ATP-dependent reductive dearomatization of benzoyl-CoA (Boll and Fuchs, 1995; Boll *et al.*, 1997; Boll *et al.*, 2000). Figure 1.4 illustrates the enzymatic reactions involved in anaerobic phenol degradation by *Thauera aromatica* (Boll and Fuchs, 2005). Furthermore, using RNA-based stable isotope probing, Manefield *et al.* (2002) identified a member of the genus *Thauera* that was responsible for phenol degradation within an industrial bioreactor.

A number of fungi have also been studied for their ability to degrade phenol. *Phanerochaete chrysosporium,* which is known for its ability to degrade lignin, has also been shown to degrade phenol and a number of other environmental pollutants (Aust, 1990; Higson, 1991; Chung and Aust, 1995). Phenols, which are byproducts of lignin biodegradation (Higuchi, 1986), can be oxidized by the extracellular isozyme, lignin peroxidase (LiP), excreted by fungi (Paszczynski *et al.*, 1986). Chung and Aust, (1995), studied the kinetics of the oxidation of various phenolic compounds by LiP (produced by *P. chrysosporium*) and demonstrated that the inactivation of LiP

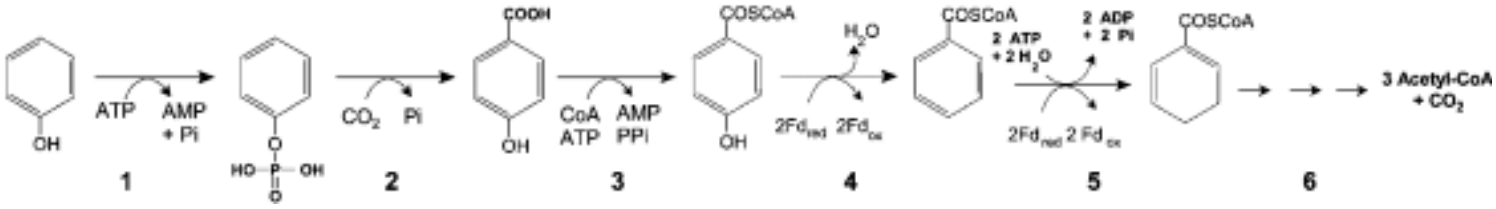


Figure 1.4 Enzymatic reactions involved in anaerobic phenol metabolism.

Reactions catalyzed by (1) phenylphosphate synthase, (2) phenylphosphate carboxylase, (3) 4-hydroxybenzoate CoA ligase, (4) 4-hydroxybenzoyl-CoA reductase, (5) benzoyl-CoA reductase and (6) enzymes involved in modified b-oxidation reactions (Boll and Fuchs, 2005).

during the oxidation of phenol was due to the formation and accumulation of LiP compound III (an inactive form of the enzyme).

Research involving the basidiomycete yeast, *Trichosporon cutaneum,* indicates that this organism can use phenol as a sole carbon and energy source (Neujahr and Gaal, 1973; Gaal and Kjellen, 1978; Gaal and Neujahr, 1979; Neujahr and Kjellen, 1980; Shoda and Udaka, 1980; Gaal and Neujahr, 1981; Powlowski *et al.*, 1985; Spanning and Neujahr, 1987; Aleksieva *et al.*, 2002) Studies have shown that this organism possesses two distinct, energy-dependent systems for phenol uptake (Mörtberg and Neujahr, 1985; Mörtberg *et al.*, 1988). By measuring both ^{14}C-phenol uptake rates and the induction of phenol hydroxylase in *T. cutaneum* cells grown in both the presence and absence of phenol, Mörtberg *et al.*, (1988) showed that the high affinity transport system of phenol-grown cells is induced simultaneously with phenol hydroxylase while the low-affinity uptake system of glycerol-grown cells is constitutive. Further studies with *T. cutaneum* have shown that phenol degradation by this organism occurs via a single-component, phenol hydroxylase encoded by the gene, *phy*A (Kälin *et. al.*, 1992; Sejlitz *et al.*, 1990).

1.4 Bioremediation Efforts

There have been a handful of cases involving applied phenol bioremediation strategies. These efforts have greatly advanced our current understanding of the microbial ecology of phenol degradation. One example of such a case involves the bioremediation efforts at an oil shale mine in Estonia in 1989, studied by Peters *et al*. (1997). A major subterranean fire, which burned for nearly four months, resulted in high levels of phenol contamination in the subsurface waters. This contamination

leached into local waterways and eventually into the Baltic Sea. Two derivatives of the phenol-degrading strain, *Pseudomonas putida* PaW85, were introduced to the site and phenol concentrations were monitored. Peters *et al.* (1997) investigated the potential for altering native genotypes after the release of this non-pathogenic pollutant-degrader to the contaminated waters. Using DNA probes designed to target specific fragments of the *phe*BA operon, the authors were able to document the horizontal gene transfer of genetic material from *P. putida* PaW85 into the native microflora six years after the release of the phenol degrading strains (Peters *et al.*, 1997).

Another situation in which phenol bioremediation strategies have been implemented involves an industrial facility north of England whose effluent wastewater contained high levels of phenol. A mixed microbial community within Vitox® bioreactors was responsible for phenol removal (~95%) from the plant's effluent before it was released into a public waterway (Whiteley and Bailey, 2000). These efforts paved the way for studies conducted by Manefield *et al.* (2002). It was originally assumed that phenol degradation in the reactors was dominated by the easily culturable *Pseudomonas putida* BS564. However, using RNA-based stable isotope probing (SIP), Manefield and his colleagues were able to identify a member of the genus *Thauera* that was crucial to phenol removal efficiency within the reactors. These two studies encourage further investigation of phenol remediation technologies.

1.5 Stable Isotope Probing

Stable isotope probing (SIP) provides a culture-independent way of identifying microbial populations responsible for important environmental processes. The process

involves the introduction of an isotopically enriched (^{13}C, ^{15}N) substrate to an environment, followed by isolation and analysis of labeled biomarkers (DNA, RNA, PLFA (polar lipid fatty acids)). This allows for the identification of organisms involved in the catabolism of that substrate.

In 1958, a study conducted by Meselson and Stahl paved the way for SIP. Their work verified that a labeled atom would increase the buoyant of DNA (Meselson and Stahl, 1958). Forty years later, Boshker *et al.* (1998) were the first to directly link microbial populations to the environmental processes they catalyze. By analyzing ^{13}C-labeled polar lipid fatty acids (PLFAs), they concluded that ^{13}C-acetate oxidation in estuarine and brackish sediments was dominated by the sulphate-reducing, Gram-positive *Desulfotomaculum acetoxidans*. They also successfully coupled ^{13}C-methane oxidation in freshwater sediments to members of the genera *Methylobacter* and *Methylomicrobium* (Boschker, 1998).

The above experiments opened the doors to many new and exciting SIP-based studies of microbial ecology. Radajewski *et al.* (2000) were the first to use DNA-based stable isotope probing to study methylotrophs and Manefield *et al.* (2002) established that RNA could also be used as a highly sensitive biomarker in SIP studies. In 2003, Padmanabhan *et al.* were the first to directly apply field-based, DNA-SIP to study glucose, phenol, caffeine, and naphthalene metabolism by microbes in agricultural soil. Jeon *et al.* (2003) further explored these field-based methodologies, which led to the discovery and isolation of a novel bacterium, *Polaromonas naphthaleneivorans.* This Gram-negative, coccoid bacterium was found to have a distinctive dioxygenase and is responsible for the naphthalene catabolism at an industrially polluted site in Upstate New York (Jeon *et al.*, 2003). The methods pioneered in the studies cited above provide the foundation for the experiment discussed in Chapter 2 of this thesis.

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CHAPTER TWO

Using Field-based Stable Isotope Probing To Identify Adapted Populations and To Track Carbon Flow Through a Phenol-degrading Soil Microbial Community

2.1 Introduction

Documentation of *in situ* biogeochemical processes and discovery of microbial populations responsible for such processes are long-standing challenges for microbial ecologists (27). Stable isotope probing (SIP) is a procedure that has led to recent progress in this area. The approach incorporates a stable isotope (e.g., ^{13}C) into cellular biomarkers of organisms actively involved in metabolism of a ^{13}C-labeled substrate (39). Biomarkers that have been used in these types of studies include PLFAs, DNA, and RNA (6, 28, 29, 36, 37). Following ^{13}C enrichment in nucleic acid-based studies, density gradient ultracentrifugation, and a series of molecular methods (PCR, molecular cloning, T-RFLP, and sequencing) are employed to separate and analyze the isotopically labeled biomarker; thus providing insight into the microbial populations actively involved in substrate-specific metabolism.

One of the limitations to SIP has been the ambiguities posed by carbon cross-feeding effects (37, 38, 39, 45). In addition to biomarker labeling of targeted primary degraders, the stable isotope can be incorporated indirectly into the biomass of trophically-related microorganisms (28). This can occur either by uptake of labeled metabolites released by primary degraders or by microbial scavenging of labeled biomass. The uncertainties associated with carbon cross-feeding increase as incubation time with the labeled-substrate increases. In some cases, SIP studies have involved incubation times of >40 days (33, 37). Longer incubations increase the potential for

the isotopic label to be passed down the food chain into the biomass of non-target organisms.

The investigative strategy developed in this study sought to differentiate between enriched and unenriched primary degraders of phenol and trophically related populations. Phenol was selected as a model organic chemical pollutant due to its ubiquitous distribution in the environment and its documented biodegradability (28, 35, 41, 44). By modifying the field-based DNA-SIP methodologies developed by Padmanabhan *et al.* (35), with key variables being substrate isotope and prior exposure to the substrate, we were able to gain insight into three distinct populations involved in phenol degradation *in situ*: (i) unenriched, primary degraders, (ii) enriched, primary degraders, and (iii) trophically related organisms (carbon cross-feeders).

2.2 Materials and Methods

Chemicals and standards.

Uniformly labeled $^{13}C_6$-Phenol was purchased from Isotec (Miamisburg, OH). Phenol was purchased from Fisher Scientific (Fair Lawn, NJ). Carbon dioxide standard (1.01%) was purchased from Scott Specialty Gases (Plumsteadville, PA). High purity helium was supplied by Airgas (Elmira, NY).

Field Study Site.

This study was conducted at the Cornell University Agricultural Experiment Station, Ithaca, NY. The soil plot (Collamer Silt loam) was level and free of vegetation. A table was placed over the plot (0.8 m high) to protect the experiment from rain and direct exposure to sunlight.

Carbon Cross Feeding Field Assay.

One gram of Collamer Silt loam was added to 5 ml phosphate buffered solution, vortexed and allowed to settle for ~0.5 min. The upper suspension (1 ml) was added as innoculum to 1 L minimal salts media [MSB; (38)] with 2.5 mM $^{13}C_6$-phenol and shaken at room temperature for 14 days. A similar culture was prepared using unlabeled phenol. HPLC analysis was used to confirm phenol metabolism in the cell cultures (see below). The cells were pelleted, washed 3 times with PBS, resuspended in 1 ml deionized water, and autoclaved. The resulting ^{13}C- and ^{12}C-cell preparations were mixed vigorously and 100 µl were added dropwise to triplicate soil plots in the field. Septa-fitted stainless steel chambers (18) were inserted in the soil, enclosing 5.2 cm^2 of the dosed surface. Using gas-tight syringes (Hamilton, Reno, NV) 100 µl of the 14.6 cm^3 headspace was removed, shuttled immediately by car to the laboratory (~0.5 km) and analyzed by GC/MS for both $^{13}CO_2$ and $^{12}CO_2$, as previously described (35). Total carbon in the cell preparations was determined using a ThermoQuest Italia S.p.A. EA/NA 1110 Automated Elemental Analyzer (Milan, Italy) operated by the Analytical Laboratory, Cornell University, Dept. of Crop and Soil Science.

HPLC Analysis of Phenol.

Phenol was analyzed by high-performance liquid chromatography (HPLC). Samples (1.0 ml) of culture medium were collected at various time-points, immediately diluted with an equal volume of methanol, sealed and stored at 4°C until analyzed. Samples were filtered through nylon acrodisc filters (0.2 µm; Acrodisc 25 mm Syringe filter; Gelman, Ann Arbor, MI). Phenol was separated using a Varian Microsorb-MV 100-5 C18 HPLC column (250x4.6 mm). A Waters model 590 HPLC pump was used to pump a mobile phase of methanol:40mM acetic acid (60:40) at a

flow rate of 1.0 ml/min. Eluents were monitored by UV-VIS detection (ABI Analytical Absorbance Detector, Spectroflow 757) at a wavelength of 270 nm and quantified using external standard calibration curves.

Soil Field Treatments.

Five soil treatments (Table 2.1) were designed to probe three distinct communities of phenol degraders *in situ*. Key variables for each treatment were carbon isotope of phenol (unlabeled [^{12}C] or ^{13}C) and the number of daily doses (0 or 11) prior to a final dose of phenol. Each 20-µl dose contained 200 µg of phenol. Immediately after the final dose of ^{13}C-phenol, the plots were covered with septa-fitted chambers and headspace analysis followed, as described above.

GC/MS Analysis of CO_2.

A Hewlett-Packard HP5890 gas chromatograph (Wilmington, DE) equipped with an HP5971A mass selective detector was used for the respiration analyses. With high-purity helium as the carrier gas, a Hewlett-Packard Pora Plot Q column (25 m x 0.32 mm, 10-um film thickness) was used to separate carbon dioxide from other gaseous components. The detector was operated at an electron energy of 70 eV and a detector voltage of 2000. The ion source pressure was maintained at $1x10^{-5}$ torr. A splitless injection was used and the GC oven was isothermal at 60ºC. CO_2 eluted at 2.5 min. Single ion monitoring mode allowed simultaneous quantification of both $^{12}CO_2$ (m/z = 44) and $^{13}CO_2$ (m/z = 45). The concentration of $^{13}CO_2$ was quantified using calibration curves prepared using external standards (Scott Specialty Gases). Net $^{13}CO_2$ produced from metabolism of the ^{13}C-phenol was calculated by subtracting background $^{13}CO_2$ produced by the native microbial community from soil organic matter. Background $^{13}CO_2$ was inferred from direct measurement of $^{12}CO_2$ adjusted to

Table 2.1. Soil treatments providing insight into three distinct microbial populations involved in *in situ* phenol degradation.

Treatment	**# Prior Doses**	**Isotope** $_{\text{prior doses}}$	**Isotope** $_{\text{final dose}}$	**Probed Microbial Population**
N/12	0	NA	^{12}C	
N/13	0	NA	^{13}C	unenriched 1° phenol degraders
12/12	11	^{12}C	^{12}C	
12/13	11	^{12}C	^{13}C	enriched 1° phenol degraders
13/13	11	^{13}C	^{13}C	mixed, trophically related (^{13}C cross-feeders)

the known fixed ratio of ^{12}C to ^{13}C in naturally occurring carbon [1.11% (17, 35)]. This ratio was confirmed analytically. Net $^{13}CO_2$ values from replicate chambers were averaged at each time point and compared with Student's t tests.

Secondary Ion Mass Spectrometric (SIMS) Imaging of Soil

One-tenth gram of surface soil was aseptically collected from the field treatments receiving 12 doses of ^{13}C-phenol and unlabeled phenol. The soil was fixed in 4% formalin (1 ml) and stored in screw-cap glass vials. Soil smears were prepared after dilution (1:50) in filter-sterilized deionized water by spreading 1 µl onto a sterile, clean high-purity silicon wafers (Silicon Quest Interntional, Inc.; ~1cm^2) supported by a glass microscope slide. After air drying and heat fixation by passing rapidly over a flame, SIMS analysis was perfomed. A CAMECA IMS-3f SIMS ion microscope (Paris, France) operated with a positive oxygen beam was used and negative secondary masses were monitored in the imaging mode for the detection of ^{12}C, ^{13}C, ^{26}CN, and ^{27}CN signals as previously described (10). SIMS images were recorded on a CCD camera and digitized to 14 bits per pixel (Photomatrix, Tucson, AZ). Images were processed using a McIntosh computer and DIP Station image-processing software (Hayden Image Processing, Inc.).

DNA Extraction and Isopycnic Fractionation of 13C DNA.

After headspace sampling had been completed (~30 h), the chambers were removed from the soil, keeping a 2 cm thick, intact soil core in the bottom. These were immediately transported to the laboratory. Using a sterile spatula, approximately 0.125 g was removed from the upper 1 mm layer of soil. Four replicates from each of

the five treatments were pooled to a final weight of 0.5 g. DNA extraction was carried out using the Fast DNASPIN kit with a bead-beating procedure (Qbiogene, Carlsbad, CA).

As positive controls for ^{13}C and ^{12}C DNA, *Pseudomonas putida* strain G7 and *Bacillus subtilis* were grown in two mineral salts media: one with 0.4% $^{13}C_6$-glucose and one with 0.4% unlabeled glucose. DNA was extracted as above. One hundred microliters of both the heavy (^{13}C) and light (^{12}C) DNA solutions from both *P. putida* and *B. subtilis* were combined and brought to a final volume of 1 ml with TE buffer (10 mM Tris/1 mM EDTA, pH 8). To examine the influence of G+C content of DNA on its migration during ultracentrifugation, we compared band locations of the pseudomonad (62% G+C) with *Bacillus subtilis* (43% G+C).

One milliliter of the DNA solution from the standard and each field treatment was diluted to 4.5 ml with TE buffer, and 4.5 g CsCl was added and shaken gently until dissolved. Ethidium bromide (100 µl: 10mg/ml) was added to each ultracentrifuge tube, which was then sealed. Tubes were centrifuged at 140,000 x g (Vti 80 rotor; 41,900 rpm) for 66 h at 20˚C (20, 35). Resultant bands in the standard were clearly separated. The ^{13}C band in field treatments was not visible; we used the standard to guide DNA removal. Using an 18-g needle to puncture ~2 mm below each band, 0.5 ml of CsCl solution containing DNA was withdrawn. Ethidium bromide was extracted from the DNA by the addition of 10 volumes of TE-saturated 1-butanol and gently mixing. The organic layer was discarded and the extraction repeated five times. The DNA was brought to a final volume of 3 ml in TE. DNA precipitation occurred overnight at –20˚C by addition of 300µl of 3 M sodium acetate (pH 4.6) and 2x volume of ethanol. After pelleting at 13,000-15,000 x g for 30 min, the DNA was washed twice with 70% ethanol, centrifuged at the same speed for 10 min, resuspended in 100 µl of distilled water, and stored at –20˚C.

PCR cloning, restriction digestion and sequencing.

PCR amplification of 16S rRNA genes in the ^{13}C-DNA fraction from soil used universal eubacterial primers (27f and 1492r) by methods described previously (4, 20, 35). Cloning from the ^{13}C-DNA derived from ^{13}C-phenol treated soil only proceeded when the corresponding band from ^{12}C-phenol treated soil failed to yield a PCR amplicon. The product was ligated into the vector pCR2.1 (TA cloning, Invitrogen) following the manufacturer's recommended protocol. Following transformation of plasmids into host cells and blue/white screening, colonies with inserts were verified by PCR with vector-specific primers (5'-GTAACGGCCGCCAGTGTGCT and 5'-CAGTGTGATGGATATCTGCA) that flanked the cloning region. The amplicons were digested with *Hae*III and *Hha*I. Restriction fragment length polymorphism (RFLP) patterns were analyzed on 3% MetaPhore agarose gels (BioWhittaker; Molecular Applications, Rockland, Maine) with a 100-bp ladder (Promega) as a marker. Clones containing unique RFLP patterns were selected for sequencing, grown overnight in 5 ml of Luria-Bertani broth with kanamycin (50µg/µl), and pelleted, and plasmids were purified (QiaPrep spin miniprep kit; Qiagen, Santa Clarita, Calif.). Sequencing (Cornell University DNA Sequencing Facility) was conducted with 6 primers: M13 forward [5'-TGTAAAACGACGGCCAGT-3'], M13 reverse [5'-AACAGCTATGACCATG-3'], 1114 forward [5'-GCAACGAGCGCAACCC-3'], 907 reverse [5'-CCGTCAATTCATTTGAGTTT-3'], 531 reverse [5'-TACCGCGGCTGCTGGCAC-3'], and 533 forward [5'-GTGCCAGCMGCCGCGG-3']. Raw sequence data from both strands were assembled into full length sequences with at least 2x coverage using the program SEQMAN II (DNASTAR, Inc.). After assembly, the consensus sequence was verified manually by referring to the corresponding ABI chromatograms of the sequencing reactions. The computational

tools of the Ribosomal Database II project (http://rdp.cme.msu.edu/html) were used to check chimeras and to calculate the similarity values for individual rDNA sequences by using the sequence_match program. A BLAST search (http://ncbi.nlm.nih.gov/BLAST) was also used to identify the additional related sequences. The closest relatives identified from both searches were included in dendrograms.

Nucleotide sequence accession numbers.

The nucleotide sequence data reported here have been submitted to GenBank under accession no. DQ158099 to 158132.

2.3 Results

We set out to demonstrate that ^{13}C-labeled biomass, derived from primary phenol degraders in soil, is a suitable growth substrate for other members of the soil microbial community. To accomplish this, a site-derived soil innoculum was used to prepare two mixed cultures of phenol degraders. These were grown in the laboratory in MSB supplemented with either ^{13}C- or unlabeled ("^{12}C") phenol as the sole carbon source. After a 14-day incubation, HPLC analysis showed that all initial phenol was below detection (data not shown). The resulting labeled and unlabeled cells were harvested, washed 3 times, autoclaved, and the resulting cell preparations were used as the substrate in a field respiration assay. Net $^{13}CO_2$ over background was measured by GC/MS over the course of 24 h (Fig. 2.1). Treatments receiving ^{13}C-labeled biomass

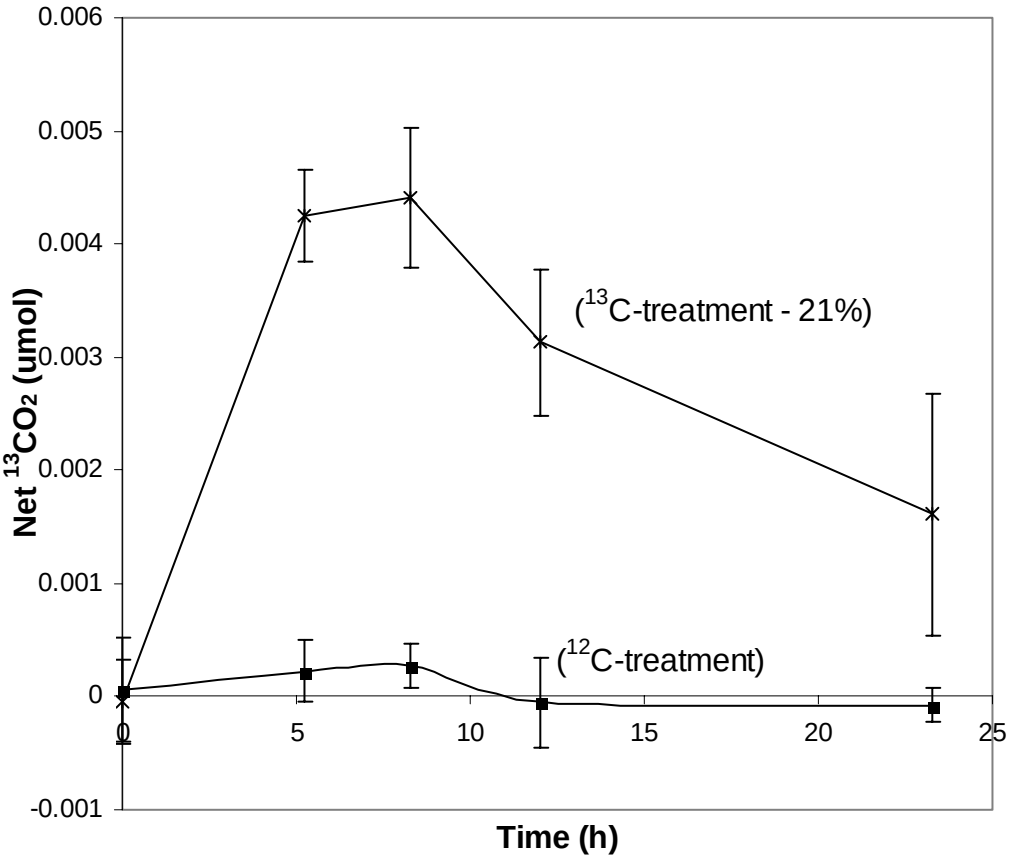

Figure 2.1 Evolution of $^{13}CO_2$ from unlabeled (^{12}C) and ^{13}C-labeled biomass added to field soil plots. GC/MS analysis monitored both $^{12}CO_2$ and $^{13}CO_2$ concentrations. Net $^{13}CO_2$ reflects total $^{13}CO_2$ minus inferred background $^{13}CO_2$. The percentage in parentheses shows the proportion of the total added ^{13}C-labeled carbon recovered as $^{13}CO_2$. Data points are the average of three replicate treatments. Error bars indicate standard deviation. The field chambers are not sealed beneath the soil surface. The late drop in 13CO2 concentration is caused by diffusion of gas into the soil.

as the added carbon source produced a significant increase in net $^{13}CO_2$ while treatments receiving unlabeled cell substrate displayed no detectable increase in net $^{13}CO_2$ production. The mass of labeled carbon recovered as $^{13}CO_2$ amounted to 21% of the added labeled substrate within 10 h (Fig. 2.1). This confirmed that microorganisms native to our field site can rapidly metabolize labeled biomass derived from primary degraders of phenol. Thus, carbon in added phenol can be expected to flow through the microbial community.

In our prior field-based SIP studies (20, 35), our goal was to identify a single population, those members of the soil community responsible for initial catabolism of the added ^{13}C-labeled substrate. Thus in prior studies, we intentionally minimized the time interval between substrate dosing, $^{13}CO_2$ determination, and DNA extraction and analysis. In the experimental design implemented here, the three treatments sought to identify three distinct microbial populations involved in *in situ* metabolism of phenol (Table 2.1). In one of these treatments (N/13; Table 2.1), soil microorganisms in the field plots received no prior exposure to the substrate, but simply received one dose of ^{13}C-labeled phenol on day 12. A net increase in $^{13}CO_2$ production was observed in the headspace of the chamber covering the soil. This amounted to 1.3% of the added labeled carbon within 24h (Fig. 2.2). Respiration in soil plots in control treatments receiving a single dose of unlabeled (^{12}C) phenol produced no net increase in $^{13}CO_2$ over background, as expected. The second key treatment (12/13; Table 2.1) received 11 doses of ^{12}C-phenol prior to a final ^{13}C-phenol dose. This dosing regime undoubtedly caused an *in situ* enrichment of phenol degraders before the ^{13}C label was introduced to the system. The resulting respiration data (Fig. 2.2) showed that prior exposure to the substrate boosted $^{13}CO_2$ production by a factor of ten, as 18% of the added ^{13}C label was recovered as $^{13}CO_2$. The third key treatment (13/13; Table 2.1) received 11 prior doses of ^{13}C-phenol prior to a final dose of the labeled substrate.

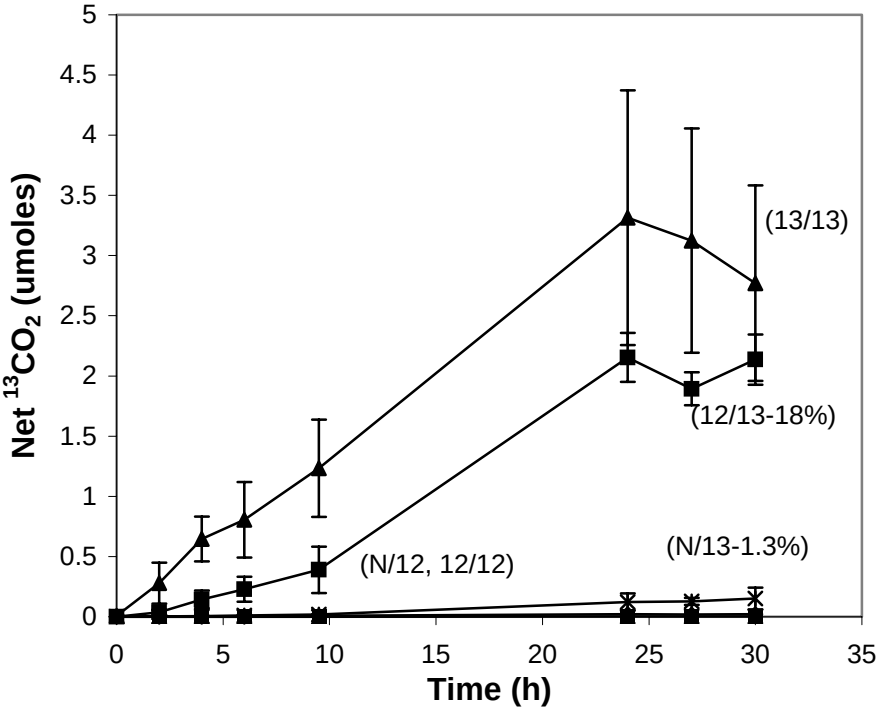

Figure 2.2 Evolution of $^{13}CO_2$ from ^{13}C-phenol added in the field in 5 experimental treatments ([N/12], [N/13], [12/12], [12/13] and [13/13]; Table 1). [N/12] and [12/12] were control treatments receiving only unlabeled (^{12}C) phenol. Net $^{13}CO_2$ reflects total $^{13}CO_2$ minus inferred background $^{13}CO_2$. The percentage in parentheses in the [N/13] and [12/13] treatments shows the proportion of the total added ^{13}C-labeled carbon recovered as $^{13}CO_2$. Data points are the average of three replicate treatments. Error bars indicate standard deviation.

While this treatment allowed for enrichment of phenol degraders, it also delivered (in repeated pulses) a substantial mass of ^{13}C-phenol to the microbial community. We hypothesize that this delivery regimen allowed the ^{13}C-labeled carbon to pass through the food chain into the biomass of non-phenol degraders. As expected, the net $^{13}CO_2$ produced was greatest in the 13/13 treatment because of the cumulative dose of ^{13}C. The respective control treatment 12/12, which received multiple doses of unlabeled phenol, showed no net increase in $^{13}CO_2$ over background.

To microscopically verify that the soil microbial community grew in situ in the treatment receiving ^{13}C phenol, samples were fixed in formalin, diluted, and then the soil smear was analyzed using Secondary Ion Mass Spectrometry (SIMS). The instrument was adjusted to focus on 4 key masses: ^{12}C and ^{13}C (whose emissivity, hence sensitivity and resolution are relatively low) and ^{26}CN and ^{27}CN (whose emissivity, hence sensitivity and resolution are relatively high). The four images on the left of Figs. 2.3 (a-d) were derived from the treatment that received twelve doses of ^{12}C-phenol in the field plots. Only the mass 26 signal, indicative of ^{12}C-labeled bacteria growing on native soil carbon or ^{12}C-phenol, showed strong signals from individual cells above background levels of ~ 1% ^{13}C. However, when the same analyses were performed on the soil smear from the treatment receiving twelve doses of ^{13}C-phenol, very strong signals were detected from mass 27, indicative of ^{13}C in combination with ^{14}N (Figure 2.3h). Clearly, the ^{13}C-treated cells showed enrichment of ^{13}C and brighter signals for ^{27}CN (see arrows in Fig. 2.3 comparing ^{13}C and ^{27}CN images of the same cells). Digital image analysis of ratios of ^{13}C and ^{12}C signals in individual cells revealed 10-40 fold enhancement of ^{13}C signals in the ^{13}C-treatment. As expected, signals from unlabeled cells (mass 26) were also detected in the ^{13}C-phenol-treatment and the unlabeled cells were both more numerous and largely distinctive from those found in ^{12}C-phenol treated soil.

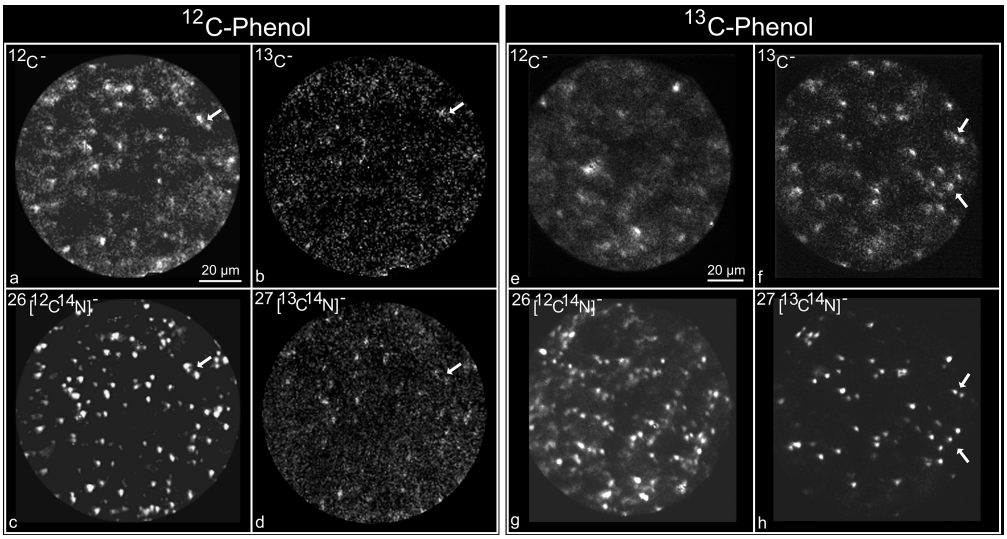

Figure 2.3 Dynamic SIMS ion microscopic images of soil bacteria from 12/12 and 13/13 field treatments receiving unlabeled (Fig. 3a-d) and ^{13}C-labeled (Fig. 3e-h) phenol, respectively. Upper row of images shows signals from ^{12}C and ^{13}C. Lower row shows higher resolution, high emissivity images combining signals from ^{12}C and ^{13}C with ^{14}N (see text).

Following the field respiration and SIMS assays, total DNA was extracted from each of the five soil treatments (N/12, N/13, 12/12, 12/13, 13/13; Table 2.1) and density gradient ultracentrifugation was performed to separate the heavy (^{13}C enriched) from light (unenriched or "^{12}C") DNA. Although only the [^{12}C] DNA band was visible, the [^{13}C] DNA band's location was determined using known ^{13}C-DNA standards (35). In our positive controls, the difference in G+C content (19%; *B. subtilis* versus *P. putida*) caused a splitting of ^{12}C- and ^{13}C-DNA bands by 7mm. Furthermore, the high %G+C ^{12}C-DNA from *P. putida* (lower member of the light pair) was separated from the low %G+C ^{13}C-DNA from *B. subtilis* (upper member of the heavy pair) by 5mm. Thus, despite potential complications posed by DNA composition, we feel that separation of heavy and light DNA fractions was adequate. As found in previous studies (13, 20, 35, 40), when PCR primers designed to amplify the 16S rRNA gene were applied to DNA recovered from the location in the CsCl gradient that corresponded with a ^{13}C-DNA standard, a robust amplicon was obtained from the ^{13}C-phenol-treated soils but not from the corresponding dilution from the ^{12}C-phenol treated soils (Fig. 2.4). These results provided confidence that 16S rDNA sequences amplified and cloned from heavy DNA fractions reveal the identity of populations involved in ^{13}C-phenol metabolism. Following cloning of the 16S rDNA amplicons, 100 white colonies from each ^{13}C-phenol-dosed treatment were screened for the proper sized (1500 bp) insert. Transformants potentially containing the 16S rDNA insert were screened by RFLP and those with unique RFLP patterns were sequenced (Table 2.2). After chimeras and other sequences of questionable quality were discarded, a dendrogram was constructed from 34 soil-derived sequences and 11 reference sequences (Fig. 2.5). Sequences from the N/13 treatment (those which received no prior exposure to phenol) were scattered throughout the dendrogram. These were diverse--containing representatives from the α, β, and γ-Proteobacteria and

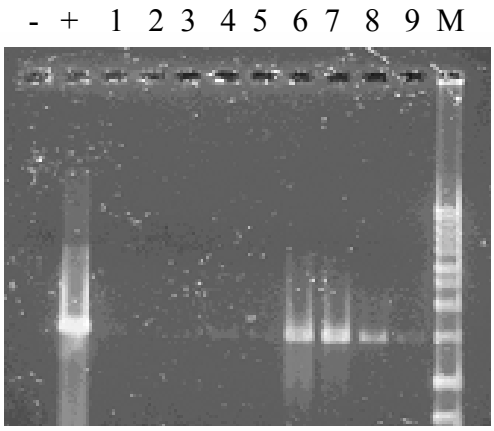

Figure 2.4 16S rDNA PCR amplification of ^{13}C-DNA fractions. Lane "-" is a negative PCR control using sterile water. Lane "+" is a positive PCR control using DNA obtained from the ^{12}C band of the [12/12] treatment. Key treatments amplified at 3 different dilutions: (10^{-2}, 10^{-3}, 10^{-4}). Lanes 1-3 (12/12), lanes 4-6 (12/13), lanes 7-9 (13/13). Lane "M" is from a 1 kb ladder. Note that amplicons were only obtained from treatments receiving ^{13}C-phenol. Lack of amplification at low dilutions can be attributed to soil-derived inhibitors of the PCR reaction.

Table 2.2. Results of molecular cloning of ^{13}C-DNA from three key soil treatments.

Soil Treatment	N/13	12/13	13/13
# clones containing ~1500bp insert	38	86	100
# unique RFLPs	34	11	9
# sequenced	31	29	24
# chimeras/questionable sequences	24	9	10
# sequences entered in dendrogram	7	13	14

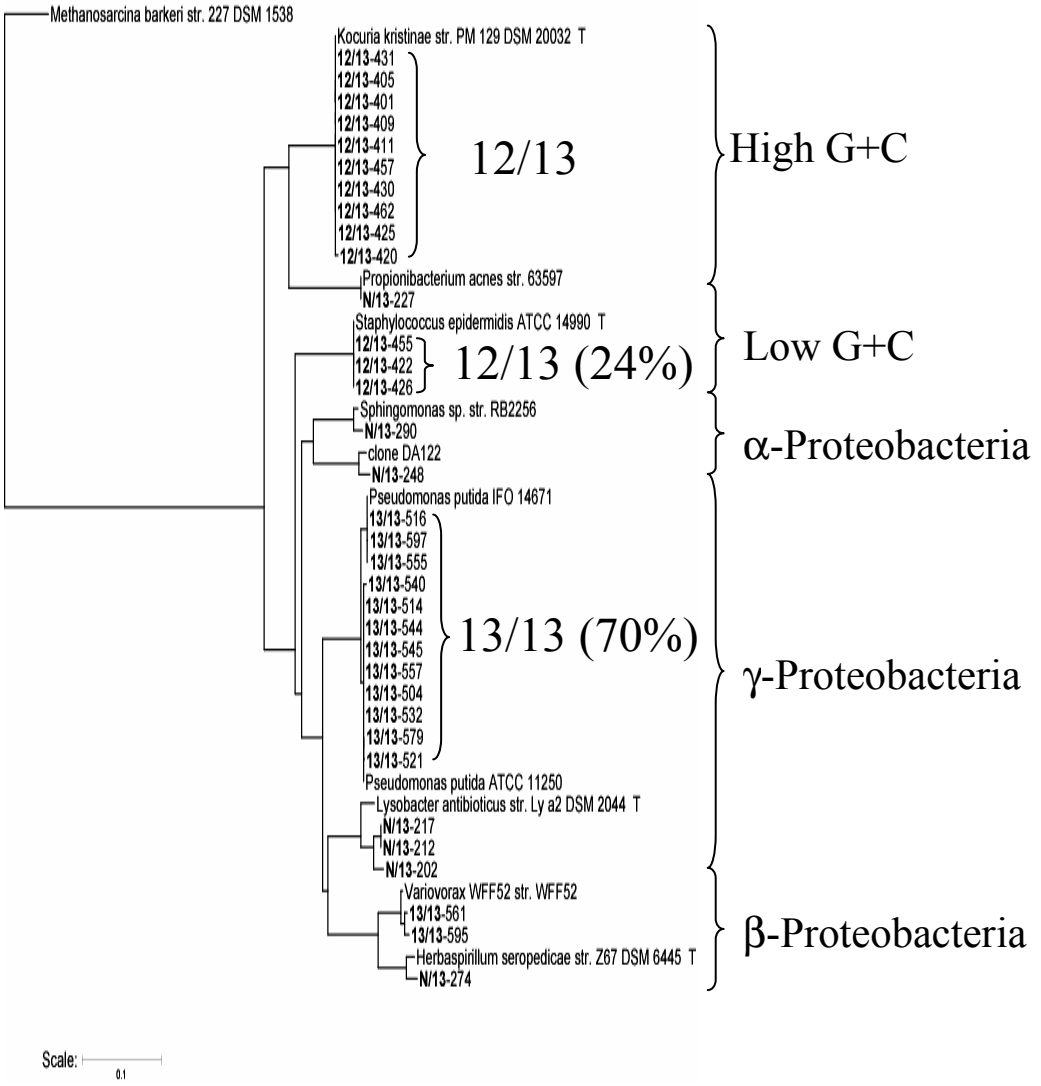

Figure 2.5 Phylogenetic analysis of cloned bacterial 16S rRNA genes from the sediment-derived ^{13}C-DNA fraction. Potential clones were screened by RFLP and 84 were sequenced. After chimeras and other sequences of questionable quality were discarded, 34 sequences were aligned and phylogenetic relationships were completed using the computational tools of the Ribosomal Database II Project (http://rdp.cme.msu.edu/html). Numbers in parentheses indicate percentage of clones analyzed with identical RFLPs for a given treatment (eg. 70% of the [12/13] clones had identical RFLPs and are most closely identified with the genus *Kocuria*).

high G+C Gram (+) bacteria. These findings support the RFLP data which showed that 34 out of the 38 clones analyzed produced unique RFLP patterns. Interestingly, sequences from the 12/13 and 13/13 treatments (which received multiple doses of phenol) were far less diverse than those found from the N/13 treatment. Furthermore, the clones from 12/13 and 13/13 treatments clustered in distinct clades on the dendrogram (Fig. 2.5). Sixty out of the 86 clones from the 12/13 treatment yielded identical RFLP patterns; these had high sequence similarity (99.5%) with *Kocuria kristinae*. Another 21 clones from the same treatment also produced a common RFLP pattern and had high sequence similarity (99.8%) with a member of the genus *Staphylococcus*. The 13/13 treatment yielded sequences that grouped in a distinct cluster on the dendrogram, one with high similarity values (99.9%) to the genus *Pseudomonas*. Low diversity and distinctiveness in the 16S rDNA sequences found in the 13/13 treatment was expected, as RFLP analysis prior to cloning found that 70 out of the 100 clones were virtually identical.

2.4 Discussion

SIP is a means of ecological inquiry that has been applied to both model systems (7, 15, 16, 19, 28, 32, 37, 38, 40) and to field sites (5, 20, 23, 31, 35, 36). The goal of SIP is to identify which microbial populations within complex communities are responsible for a probed process. Like all measurement procedures in microbial ecology, data must be interpreted with an awareness of potential pitfalls and artifacts. Results of SIP studies may suffer from ambiguities posed by: (i) the addition of substrates at unrealistic concentrations, (ii) transfer of the labeled atom(s) through food chains and (iii) isotopic fractionation during metabolism. The first two points were addressed experimentally in the present study and are discussed below. We feel

the latter point is a negligible concern when highly isotopically enriched substrates are utilized. Carbon isotope ratios in bacterial biomass have been found to generally match the isotopic ratios of their food sources (1, 2, 5, 8, 11, 12, 43).

This investigation sought to identify three distinct groups of microorganisms involved in phenol degradation at an agricultural field site: (i) unenriched, primary degraders, (ii) enriched primary degraders, and (iii) trophically-related (carbon cross-feeders). The field-based assay, established by Padmanabhan *et al.* (35), was employed in order to minimize experimental artifacts that may develop during laboratory incubations. The first group (unenriched, primary degraders (N/13); Table 2.1) received only one dose of labeled phenol. The brief exposure (30 hours) to the labeled substrate was designed to minimize carbon cross-feeding and reveal the 16S rDNA sequences of organisms directly involved in phenol metabolism prior to any enrichment. This treatment identified a relatively diverse group of microorganisms (Fig. 2.5), containing representatives from the α-, β-, and γ-proteobacteria, and high G+C Gram (+) bacteria. Given reduced cloning efficiency of the DNA recovered from the N/13 treatment (Table 2.2), the diversity of sequences representing the unenriched, primary phenol degraders was probably conservative. To our knowledge, two other studies to date have used SIP to identify microorganisms actively involved in phenol degradation. Using DNA-SIP, Padmanabhan *et al.* identified *Pseudomonas*, *Acinetobacter*, and *Variovorax spp*. as active phenol degraders at the same field site as the current study (35). Manefield *et al.* used RNA-SIP to show that a member of the genus *Thauera* dominated phenol degradation within a bioreactor (28).

The second group of populations (enriched, primary degraders (12/13); Table 2.1) probed in this study received the same amount of labeled substrate as the N/13 treatment. Multiple daily-doses of unlabeled phenol, prior to the respiration assay, resulted in an *in situ* enrichment of phenol metabolizing organisms. The 10-fold

increase in net $^{13}CO_2$ over background (Fig. 2.1), when compared to the N/13 treatment, confirmed that soil populations had been enhanced because substrate metabolism occurred at a faster rate. Because the exposure time to the single dose of ^{13}C-substrate was identical to that of the N/13 treatment, we again are reasonably confident that minimal carbon cross-feeding of ^{13}C atoms occurred. The ^{13}C-enriched 16S rDNA obtained from this treatment represented a much less diverse group of bacteria compared to the N/13 treatment. Low sequence diversity was illustrated by small variability in RFLPs (Table 2.2) and by the localized clusters in the 16S rDNA dendrogram (Fig. 2.5). The two dominant genera in the 12/13 treatment, *Kocuria and Staphylococcus,* have routinely been isolated from soil habitats (3, 22, 30). One might expect some common sequences to be revealed in the N/13 and 12/13 treatments. We hypothesize that the absence of commonalities between the two retrieved populations can be explained by the relatively small number of clones analyzed (100 from each treatment), by dilution effects associated with microbial enrichment in the 12/13 treatment, and/or by small-scale heterogeneity between soil samples.

The third group of populations (trophically-related, carbon cross-feeders (13/13); Table 2.1) received the same total mass of phenol as the 12/13 treatment prior to the respiration assay. However, unlike the 12/13 treatment, the 13/13 treatment uniformly received only the ^{13}C-labeled substrate over the 14-day dosing period. The resulting net $^{13}CO_2$ produced in this treatment was greater than the other two key treatments (Fig. 2.1). This rapid rate of $^{13}CO_2$ production can be attributed to microbial enrichment (as in the 12/13 treatment) in combination with the larger mass of labeled carbon administered to this treatment. We validated our notion that ^{13}C-labeled, phenol-derived biomass is a suitable substrate for the soil microbial community (Fig.2.1). The addition of phenol-free, sterile biomass was essential for data interpretation. Because autoclaved cell preparations are almost surely a more

readily utilizable carbon source than intact cells grown *in situ*, rates of ^{13}C transfer shown in Fig. 2.1 are probably unrealistically high. Interestingly, sequencing data indicate that the 12/13 and 13/13 treatments were dominated by completely different organisms (Fig. 2.5). While the 12/13 treatment featured representatives of the genera *Kocuria and Staphylococcus*, the 13/13 treatment was dominated by members of the genus *Pseudomonas*. We hypothesize that the ^{13}C label was incorporated into the biomass of the pseudomonads as a direct result of microbial scavenging of labeled biomass and metabolites derived from primary phenol degraders. This theory is supported by the fact that pseudomonads can use a wide range of carbon sources for energy (14). Although ^{13}C enrichment of *Pseudomonas* DNA could also have resulted from direct metabolism of phenol, the contrast in clone libraries retrieved from the 12/13 and 13/13 treatments suggests that labeling of the pseudomonads was more likely the result of carbon cross-feeding.

The sequences retrieved via SIP provide clues about the ecological role of active microbial populations. Our data show that enriched, *Kocuria*-related, phenol-degrading soil populations (repeatedly exposed to high concentrations) are distinctive and less diverse than unenriched populations. Such changes in community composition in response to long-term exposure to pollutants has been documented previously (21, 24). Our data also show that, contrary to expectation, pseudomonads in the agricultural study site are indirectly involved in *in situ* phenol metabolism. This implies that the long-recognized metabolic versatility of pseudomonads may be a manifestation of their role in catalyzing carbon flow within the microbial community at large.

Other recent SIP-based studies have aimed to document carbon flow in microbial communities. Using a mixed culture (containing a phenol-degrading- and a non-phenol-degrading-pseudomonad) grown on ^{13}C-phenol, Manefield *et al.* showed

that labeling of non-degrader RNA occurred later in the incubation period (28). The authors attribute this finding to ^{13}C cross-feeding between species. Other groups have used $^{13}CO_2$ in an attempt to trace ^{13}C-labeled plant photosynthate into the biomass of microbial populations in the rhizosphere (7, 9, 16, 25). Similarly, Middelburg *et al.* used ^{13}C-bicarbonate to study the flow of carbon from microphytobenthos to benthic consumers (31). We feel that the potential is enormous for the combination of SIP and SIMS (as supported by other analytical, molecular, and microscopic procedures) to probe detailed complexities of carbon flow through microbial communities. To our knowledge, microscopic images documenting the isotopic composition of bacteria in environmental samples have appeared in only two prior reports (18, 34).

Without a doubt, the weakest methodological step in our procedures is choosing the ^{13}C-labeled DNA that represents the active members of the microbial community. In the procedures described here, we were careful to be sure that identically-processed ^{12}C-treated negative controls were implemented. Only when our negative controls (invisible bands from the location where the ^{13}C-DNA is expected) were successful did we trust that the amplicons from the ^{13}C-treatments represented the sought microorganisms. But clearly, resolving labeled (^{13}C) from unlabeled (^{12}C) DNA can be confounded by %G+C of microorganisms (see Results), as well as by heterotrophs or predators whose biomass is derived from mixtures of labeled and unlabeled carbon. In following the flow of ^{13}C from the added substrate to primary degraders, to secondary and tertiary consumers, the likelihood of mixotrophic feeding increases. Eventually, the added ^{13}C atoms are lost in a blur of trophic interactions and DNA composition. To improve resolution of labeled from unlabeled populations, molecular fingerprinting (e.g., T-RFLP, DGGE, etc.) procedures have been implemented (13, 20, 26, 28). This and related quality-control procedures are likely to ensure continued application of SIP to microbial ecological inquiries.

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Future Work

I would like to continue analysis on the ^{13}C fractions from the field-based phenol SIP study. I would like to investigate the array of phenol catabolic genes present in each of the enriched fractions. I would also like to continue to use SIP to dissect microbial food chains in different systems. Fungi-specific PCR amplification of the ^{13}C-enriched DNA fractions (obtained from the phenol study) proved successful in identifying representatives of the genera *Trichosporon*, *Rhodotorula*, and others. The samples obtained from this study could also be used to investigate other potential organisms in this specific food chain, such as algae and protozoa. I also hope to expand my field-based methodologies to investigate the degradation of other important environmental contaminants. Current work focuses on the degradation of benzene and toluene in soil and groundwater at Site 24 in South Glens Falls, New York (the same site where *Polaromonas naphthaleneivorans* was discovered).

APPENDIX A

Tracking Carbon Flow Through Biota

Introduction

Stable Isotope Probing (SIP) is a means of ecological inquiry. SIP has proven to be a critical tool in identifying many microorganisms actively degrading chemical pollutants in many real world, contaminated sites. Traditional molecular methods were limited in that they lacked the ability to distinguish between primary degraders of a chemical and trophically-related organisms (carbon cross-feeders). Recent developments in our field-based, DNA-SIP methodologies have allowed us to potentially look beyond the primary degraders of organic material and further into the food chain. The potential is enormous, for it allows us to examine specific relationships among microorganisms living in a very complex, microbial world.

Recent work focused on the *bacterial* ecology of phenol-degradation at an agricultural field site. Our modified methods allowed us to identify a number of primary degraders of phenol as well as potential secondary consumers directly involved in this food chain. The current study explores beyond the bacterial populations involved in phenol remediation and into trophically related fungi. Fungi-specific PCR amplification of the 18S-28S internal transcribed spacer (ITS) region in the ^{13}C-DNA fractions from the previous study (DeRito *et al.*, 2005), followed by molecular cloning and sequence analysis, allowed us to identify a number of fungi directly involved in this phenol-based food web. A member of the genus *Trichosporon* dominated the RFLP profile of the 13/13 clone library. We suggest that

Trichosporon and the other identified organisms are *potential* secondary consumers in this food chain since their nucleic acids were detected in only our 13/13 treatment.

Materials and Methods:

Fungi-specific PCR

Five ^{13}C-DNA fractions obtained from our previous phenol study (DeRito *et al.*, 2005), were PCR amplified using the fungi-specific primers 1406f (TGYACACACCGCCCGT) and 3126r (ATATGCTTAAGTTCAGCGGGT) by methods previsouly described (Fisher and Triplett, 1999; Ranjard *et al.,* 2001, Hansgate *et al.*, 2005). These primers target the last 120 bp of 18S rRNA gene, internal transcribed spacer region 1 (ITS1), 5.8S rRNA gene, and ITS2, with the 3126r primer matching the 5' end of the 28S rRNA gene. Each 50 ul PCR reaction contained 10 µl of template DNA, 400 nM of each primer, and 1× *Taq* PCR Master Mix (ABGene, Rochester, NY).

Cloning, restriction digestion and sequencing

Molecular cloning of the resulting fungal amplicon (from the 50x dilution) was carried out using the TOPO Cloning Kit (TA cloning, Invitrogen) following the manufacturer's recommended protocol. Following transformation of plasmids into host cells and blue/white screening, colonies with inserts were verified by PCR with vector-specific primers (5'-GTAACGGCCGCCAGTGTGCT and 5'-CAGTGTGATGGATATCTGCA) that flanked the cloning region. Amplicons were digested with *Hae*III and *Hha*I and RFLP patterns were analyzed on 3% MetaPhore agarose gels (BioWhittaker; Molecular Applications, Rockland, Maine) with a 100-bp

ladder (Promega) as a marker. Clones containing unique RFLP patterns were selected for sequencing, were grown overnight in 5 ml of Luria-Bertani broth with kanamycin (50µg/µl), and pelleted, and plasmids were purified (QiaPrep spin miniprep kit; Qiagen, Santa Clarita, Calif.). Sequencing (Cornell University DNA Sequencing Facility) was conducted using the vector-specific M13 forward [5'-TGTAAAACGACGGCCAGT-3'] and M13 reverse [5'-AACAGCTATGACCATG-3'] primers. Raw sequence data were assembled into full-length contigs using the program SEQMAN II (DNASTAR, Inc.). After assembly, the consensus sequence was verified manually by referring to the corresponding ABI chromatograms of the sequencing reactions. The computational tools of the Ribosomal Database II project (http://rdp.cme.msu.edu/html) were used to check chimeras and a BLAST search (http://ncbi.nlm.nih.gov/BLAST) was used to identify the closest relatives for each clone. Sequences were aligned using the Clustal V option in the program MEGALIGN (DNASTAR, Inc.) and phylogenetic analysis was conducted using ClustalX version1.83.

Results and Discussion:

The methodologies established in our previous study (DeRito *et al.,* 2005) sought to identify distinct populations of soil bacteria involved in phenol degradation at an agricultural field site. These populations include (i) unenriched, primary degraders, (ii) enriched, primary degraders, and (iii) trophically related organisms (carbon cross-feeders). The same DNA extracts from that study were probed for fungal populations. PCR primers, originally designed for qualitative analysis of fungal communities by F-ARISA (fungal-automated rRNA intergenic spacer analysis)

(Fisher and Triplett, 1999; Ranjard *et al.,* 2001, Hansgate *et al.*, 2005) but lacking the fluorescence label, were used to amplify sediment-derived ^{13}C-DNA fractions from five key soil treatments (DeRito *et al.*, 2005; Table 2.1). Fungi-specific PCR amplification was only successful in the 13/13 treatment (Fig 3.1). The absence of an amplicon from both the N/13 and 12/13 treatments suggests that fungi may not be directly involved in phenol degradation at this site. However, slow fungal growth rates and/or low fungal biomass may also explain why fungi that have been directly growing on ^{13}C-phenol were below detection in the N/13 and 12/13 treatments.

The methodology we used in this study was developed by Fisher and Triplett (1999). Their goal was to obtain an indication of bacterial diversity using a molecular fingerprint of freshwater bacterial communities based on a size distribution of the 16S-23S internal transcribed spacer (ITS) region genes. In the original application of the method, a typical ARISA assay revealed ~ 50 ITS fragments whose sizes ranged from ~400 to 1,200 bp. The authors confirm this range of sizes by examining over 300 16S-23S ITS sequences from the GenBank database, where the reported range of ITS fragment lengths is between ~150 and 1,500 bp. Furthermore, these authors reported the ITS lengths and closest database matches for seven clones that were fully sequenced between the 16S and 23S rRNA genes.

Ranjard *et al.* (2001) extended the use of ARISA to study the diversity of fungal communities from five different soil types. Their modified method uses fungal-specific, PCR primers that target the 18S-28S ITS region. Their typical F-ARISA profile revealed approximately 200 ITS fragment sizes ranging between 300 and 1,100 bp. Hansgate *et al.* (2005) used F-ARISA to evaluate the shift in fungal diversity within a composting reactor during its initial stages of operation. Their results clearly identified 27 distinct ITS fragments (sizes ranging from 300 to 1,000

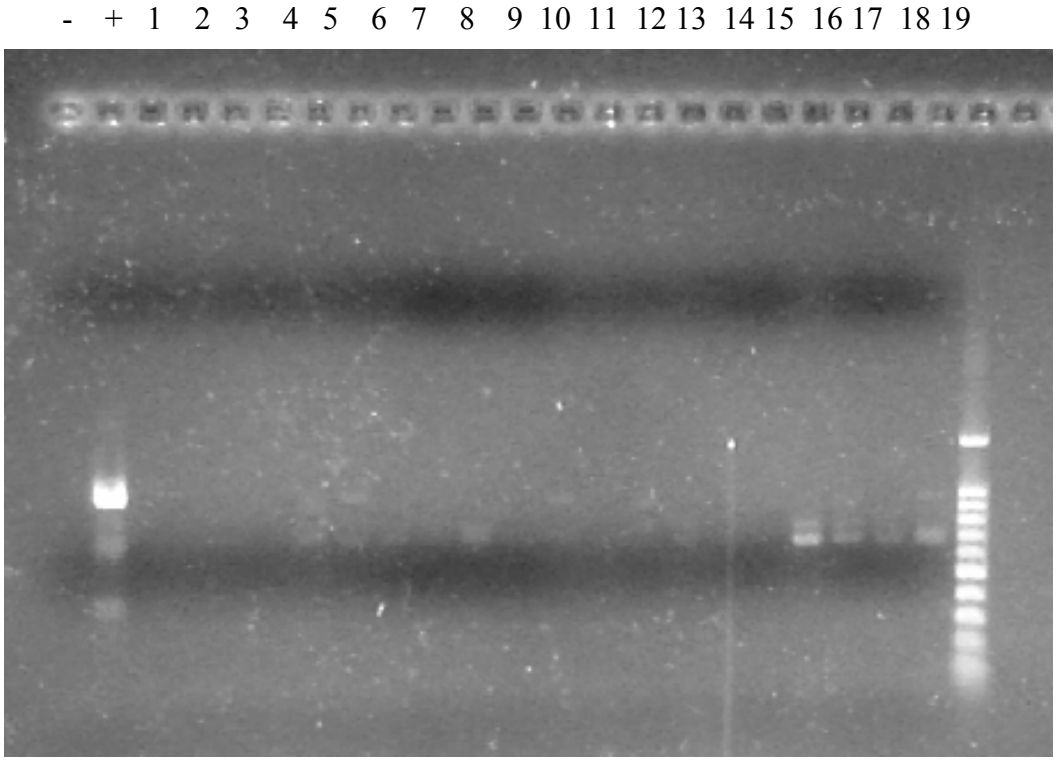

Figure 3.1 PCR amplification of fungal genes (partial 18S rRNA, complete ITS1, 5.8S rRNA, and ITS2, and partial 28S rRNA) from sediment-derived ^{13}C-DNA fractions. Lane "-" is a negative PCR control using sterile water. Lane "+" is a positive PCR control using DNA obtained from a strain of *Saccharomyces cerevisiae*. Key treatments amplified at 4 different dilutions: (5×10^{-1}, 10^{-1}, 10^{-2}, 5×10^{-2}). Lanes 1-4 (N/12), lanes 5-8 (N/13), lanes 9-12 (12/12), lanes 13-16 (12/13), and lanes 17-20 (13/13). Lane "M" is from a 100-bp ladder. Note that significant amplification was only successful in the 13/13 treatment.

bp). The authors were able to document an emergence of three populations (with distinct F-ARISA fragment sizes), which coincided with a rise in pH and temperature within the reactor.

In this study, we did not use a fluorescently-tagged PCR primer; thus we did not have the option to use an ABI sequencing instrument to obtain a high resolution profile of the range of sizes of the ITS spacer region for the ^{13}C-DNA. Nonetheless, we were able to discern from our electrophoresis gels, cloning, RFLP assays, and sequencing procedures that the fungal community contained at least twelve different sized amplicons, ranging from 516 to 629 bp.

The clone library (a total of 80 white colonies) from the 13/13 field treatment was subjected to RFLP analysis which revealed fifteen patterns. Representatives of these were sequenced and compared. The resulting dendrogram, which includes amplicon sizes for each clone, is shown in Figure 3.2. DNA sequences retreived from the clone library were dominated by those matching a member of the fungal genus *Trichosporon*-->40% of the clones analyzed shared identical RFLPs. By applying the same logic set forth by DeRito *et al.* (2005), we infer that these fungi may catalyze processes later in the food chain. This interpetation is not fully consistent with several studies that have found *Trichosporon* and other fungi to have phenol-degrading capacities (Neujahr and Gaal, 1973; Rubin and Schmidt, 1985; Margesin *et al.*, 2005; Alexievaa *et al.*, 2004). Therefore, we must consider the possibility that the organisms identified in the 13/13 treatment may be primary degraders.

The experimental approach described in this appendix sought to find members of the soil fungal community involved in overall metabolism of phenol. Although the results left an ambiguity to be reconciled (was *Trichosporon* a primary phenol degrader or a consumer of primary degrader?), we successfully identified

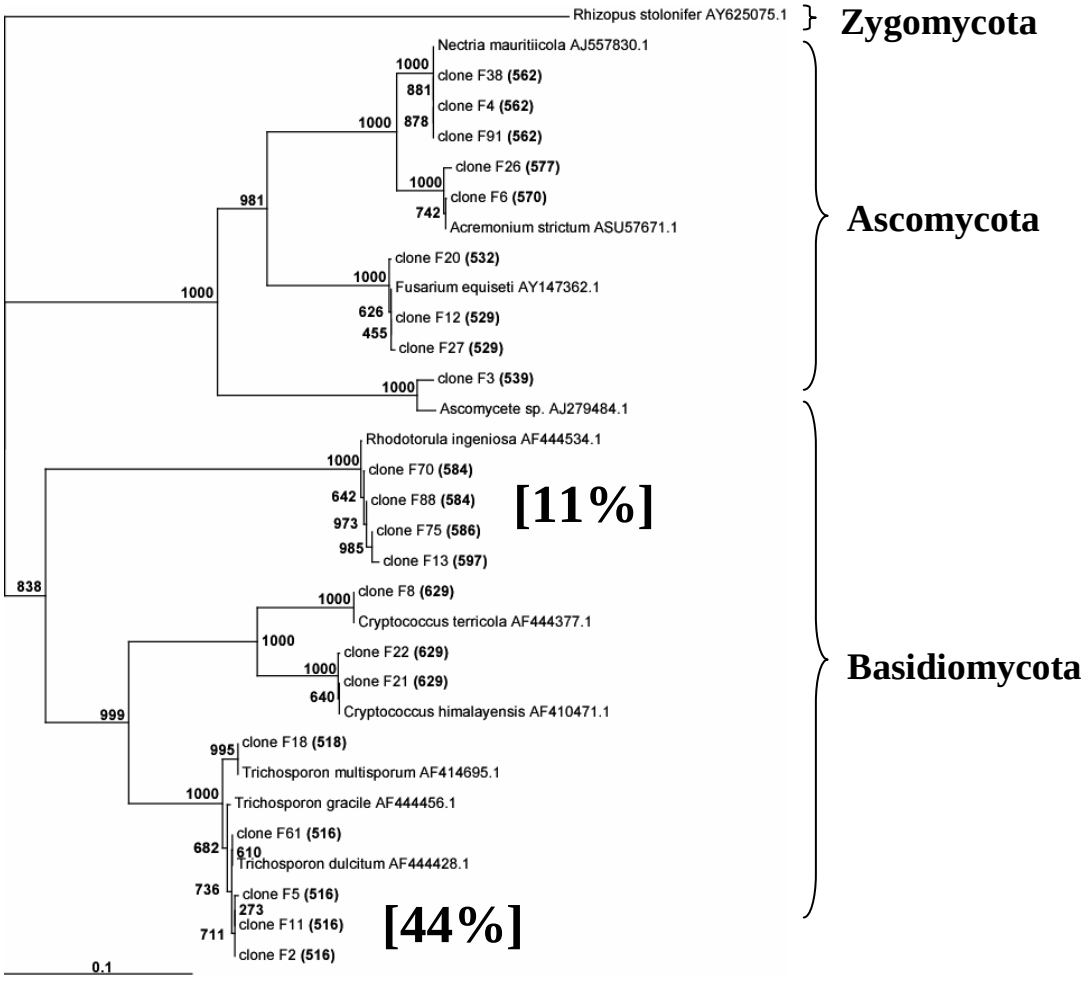

Figure 3.2 Phylogenetic analysis of cloned fungal genes (partial 18S rRNA, complete ITS1, 5.8S rRNA, and ITS2, and partial 28S rRNA) from the sediment-derived ^{13}C-DNA fraction of the 13/13 treatment. Potential clones were screened by RFLP and 21 were sequenced. Phylogenetic relationships were completed using the computational tools of DNASTAR (DNASTAR, Inc) and ClustalX version1.83. Numbers at nodes are bootstrap values based on a neighbor-joining bootstrap analysis with 1,000 replicates. Numbers in parentheses ( ) indicate the amplicon size for each clone. Numbers in brackets [ ] indicate the percentage of clones analyzed with identical RFLPs (eg. 44% of the clones had identical RFLPs and are most closely identified with the genus *Trichosporon.*

Trichosporon as playing a role in phenol catabolism. In the future, we expect that the combination of our modified field-based SIP methodologies with classical, culture-based methods in microbiology may provide clear ways to improve our understanding of trophic interactions in soil microbial communities.

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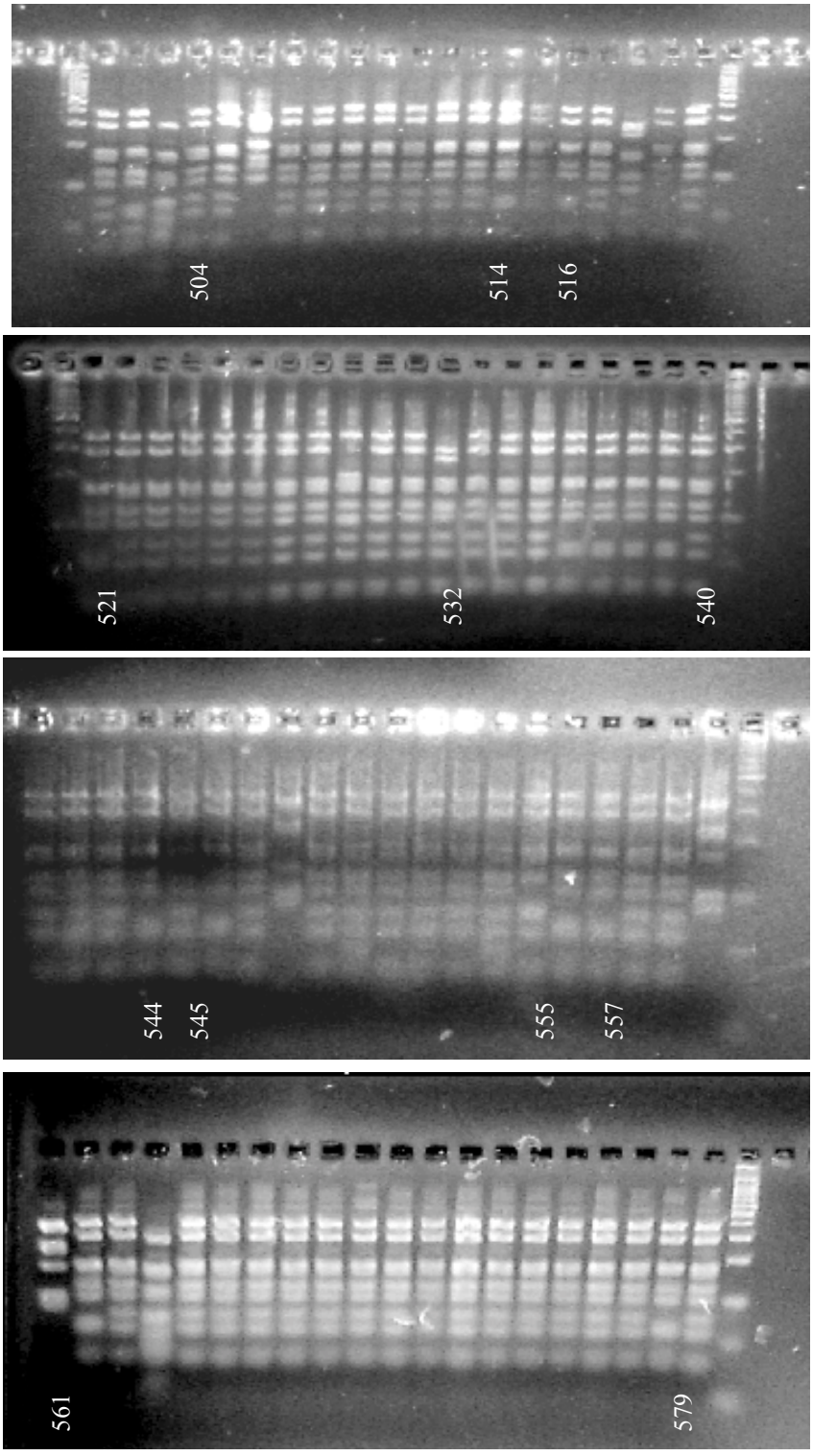
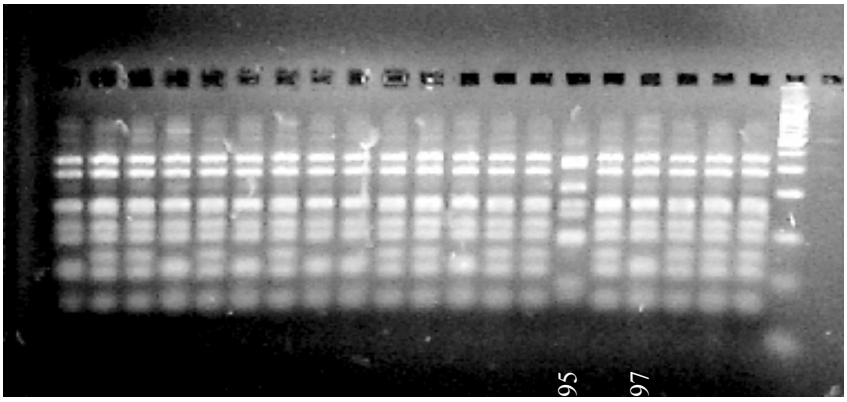
APPENDIX B

Bacterial and Fungal RFLP Patterns

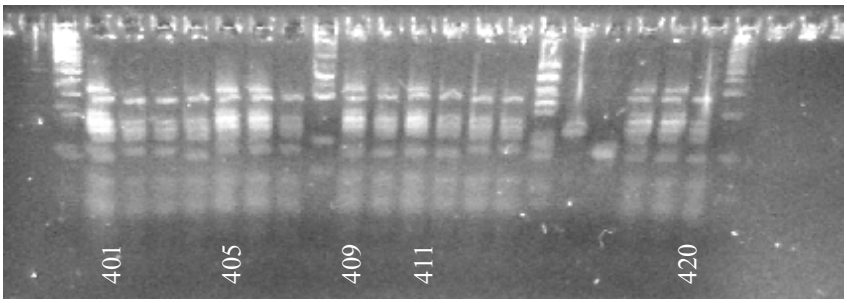
As described in Chapter 2, cloning from the ^{13}C-DNA derived from three distinct ^{13}C-phenol soil treatments proceeded when the corresponding band from ^{12}C-phenol treated soil failed to yield a PCR amplicon. Bacterial clones containing the expected 16S rDNA insert (~1500 bp) and fungal clones containing the expected 18S-ITS1-5.8S-ITS2-28S insert (~500-700 bp) were digested with *Hae*III and *Hha*I and restriction fragment length polymorphism (RFLP) patterns were analyzed on 3% MetaPhore agarose gels (BioWhittaker; Molecular Applications, Rockland, Maine) with a 100-bp ladder (Promega) as a marker. Metaphore agarose gel images of RFLPs are shown on the following four pages. Lanes are labeled with the clone numbers that were used for the phylogenetic analysis of bacterial and fungal populations in this study.

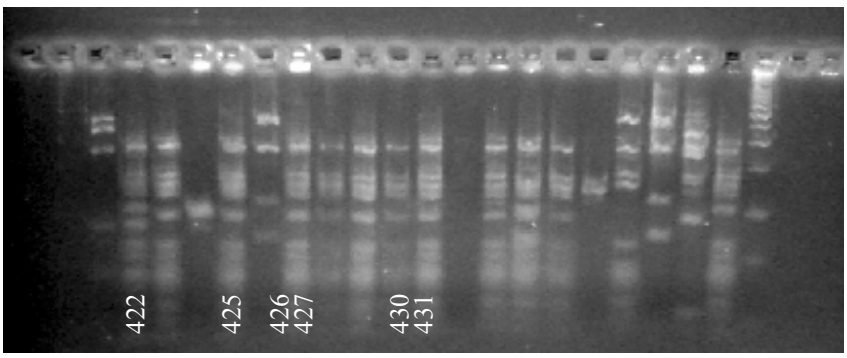
Bacterial RFLPs: 13/13 Treatment

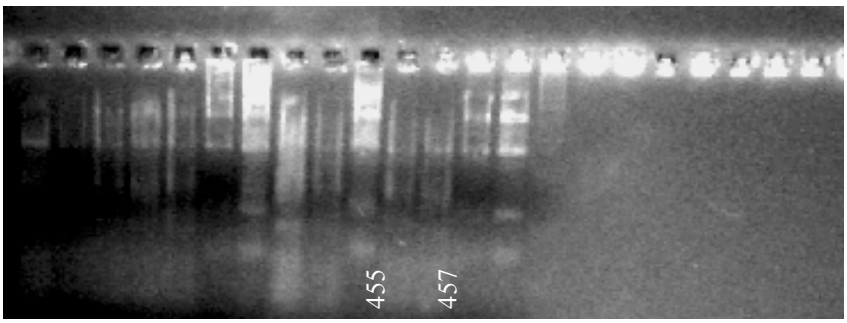


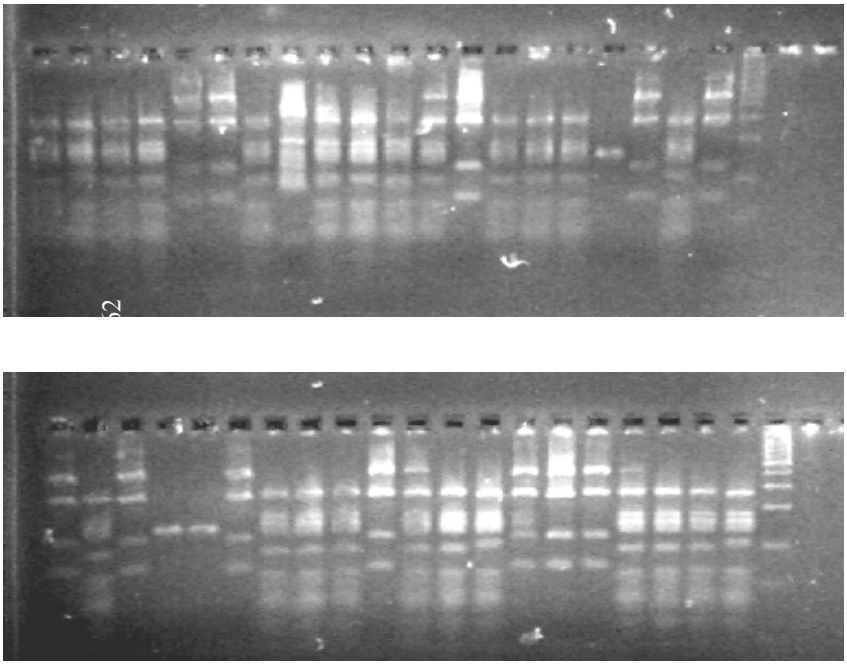


Bacterial RFLPs: 12/13 Treatment

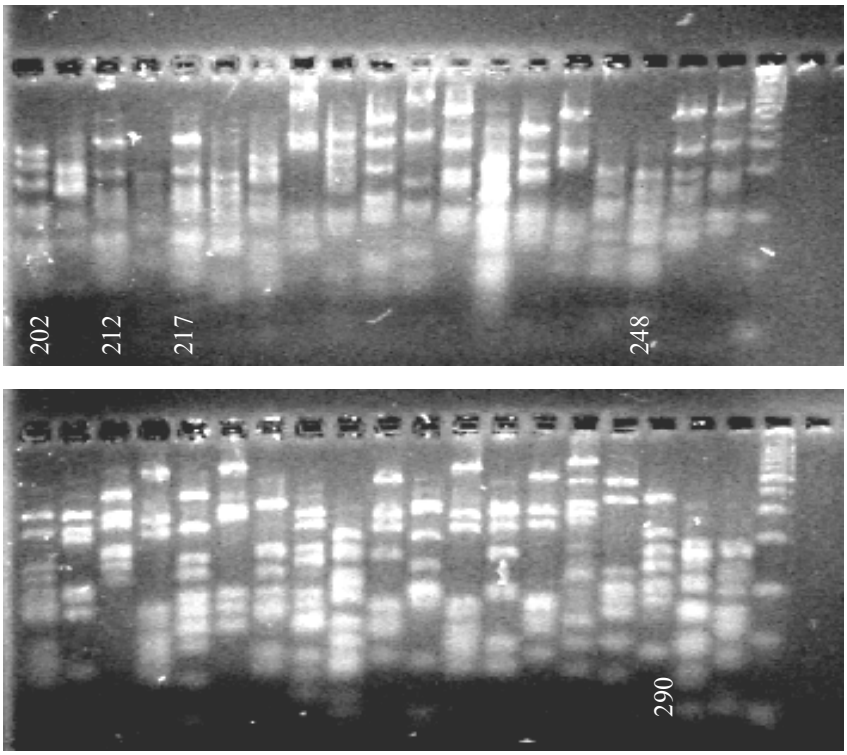








Bacterial RFLPs: N/13 Treatment



Fungal RFLPs: 13/13 Treatment