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Diversity and correlation of specific aromatic hydrocarbon biodegradation capabilities

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Abstract

This work investigated the biodegradation capabilities of indigenous microorganisms exposed to different combinations of aromatic hydrocarbons. Considerable diversity was found in the catabolic specificity of 55 strains. Toluene was the most commonly degraded compound, followed by *p*-xylene, *m*-xylene and ethylbenzene. Strains capable of degrading *o*-xylene and benzene, which were the least-frequently-degraded compounds, exhibited broader biodegradation capabilities. Kappa statistics showed a significant correlation between the abilities to degrade toluene and ethylbenzene, *p*-xylene and *m*-xylene, and *p*-xylene and *o*-xylene. The ability to degrade naphthalene was correlated to the ability to degrade other alkylbenzenes, but not benzene. In addition, the inability to degrade benzene was correlated to the inability to degrade *o*-xylene. Factorial analysis of variance showed that biodegradation capabilities were generally broader when aromatic hydrocarbons were fed as mixtures than when fed separately. Beneficial substrate interactions included enhanced degradation of benzene, *p*-xylene, and naphthalene when toluene was present, and enhanced degradation of naphthalene by ethylbenzene. Such heuristic relationships may be useful to predict biodegradation patterns when bacteria are exposed to different aromatic hydrocarbon mixtures.

Introduction

Aromatic hydrocarbons such as benzene, toluene, ethylbenzene, xylenes, and naphthalene (BTEXN) are common groundwater contaminants associated with petroleum product releases. Microbial degradation of these priority pollutants is widely regarded as a promising approach to clean up contaminated aquifers (NRC, 1994). Therefore, considerable effort has been devoted to elucidate the genetic and biochemical phenomena associated with BTEXN degradation (for reviews, see Smith, 1990; Zylstra, 1994). Most of these studies, however, have been conducted with a few model strains, and the vast majority of strains that can degrade BTEXN have not yet been well characterized (and perhaps, not even isolated). Therefore, the diversity of BTEXN biodegradation capabilities and limitations remains relatively unexplored.

Microbial catabolic diversity is reflected by the fact that five different pathways have been identified for the aerobic catabolism of toluene (Figure 1). These pathways were first identified in five different pseudomonads that have been widely studied (Gibson et al., 1990; Kukor and Olsen, 1991; Shields et al., 1989; Whited and Gibson, 1991; Williams and Murray, 1974). Many microorganisms, including these archetypes, express enzymes of relaxed specificity that enable them to degrade some of the other BTEXN compounds. However, differences in the effect that some substrates have on enzyme expression may contribute to variations in BTEXN degradation patterns when microbes are exposed to different BTEXN mixtures. For example, toluene, which is generally a good enzyme inducer, was reported to enhance the aerobic degradation of benzene by different pure and mixed cultures (Arvin et al. 1989; Alvarez and Vogel, 1991; Chang et al., 1993). Yet, toluene was also reported to

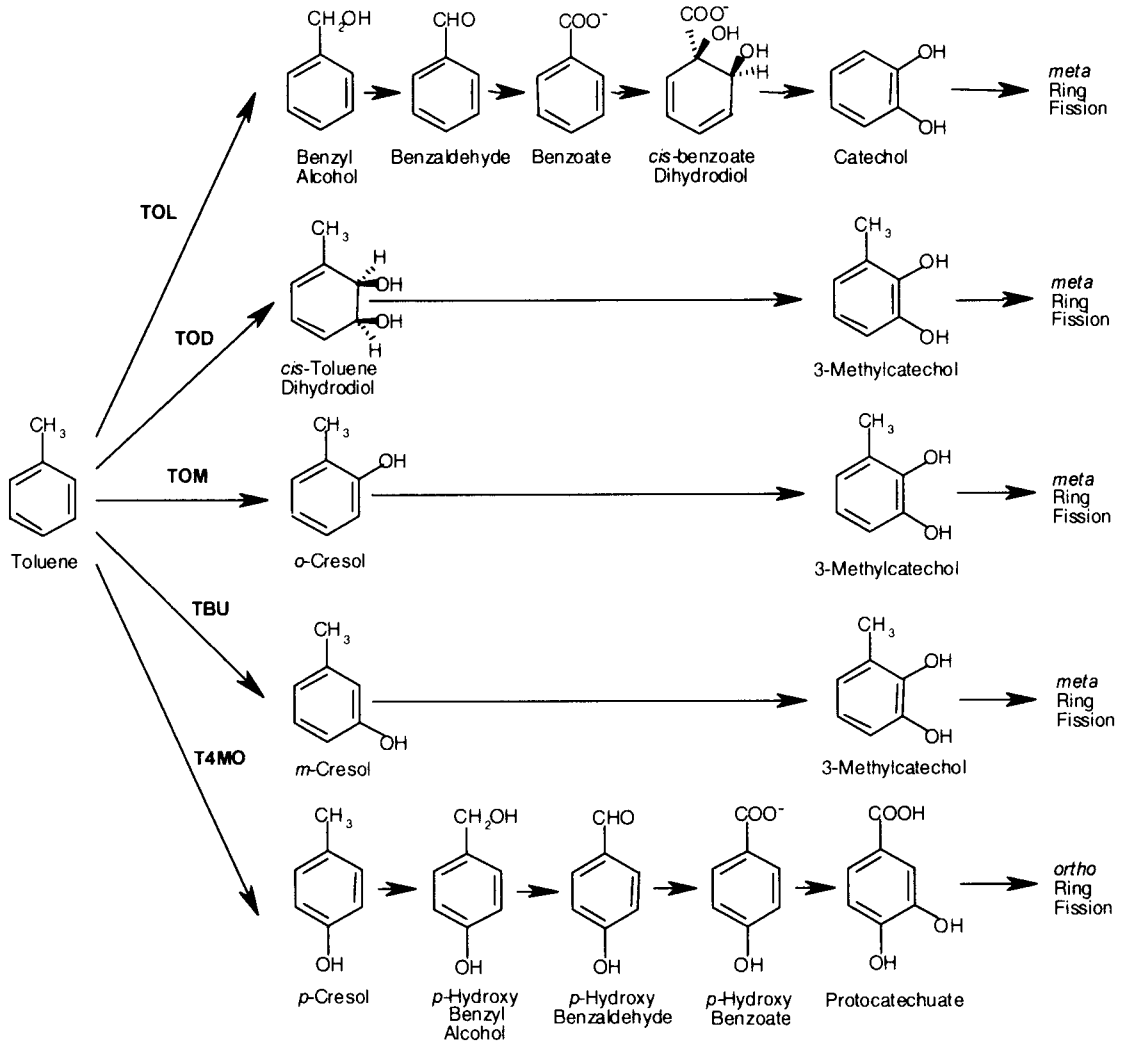

Figure 1. Diversity of aerobic toluene degradation pathways. The TOL pathway was discovered in a plasmid harbored by*Pseudomonas putida* mt-2, which initiates catabolism by oxidizing toluene at the methyl group (Williams and Murray, 1974). The TOD pathway was identified in *P. putida* F1, which uses toluene dioxygenase to add two oxygen atoms to the ring (Gibson et al., 1968). The TOM pathway was found in *Burkholderia cepacia* G4 (formerly known as *P. cepacia* G4), which uses toluene *ortho*-monooxygenase in the initial attack to form *o*-cresol (Shields et al., 1989). The TBU pathway occurs in *B. pickettii* PK01 (formerly known as *P. pickettii* PK01) which uses toluene *meta*-monooxygenase to form *m*-cresol (Kukor and Olsen, 1996). The T4MO pathway was identified in *P. mendocina* KR1, which uses toluene *para*-monooxygenase to form *p*-cresol (Whited and Gibson, 1991). The expression of these pathways is not exclusive to the five archetypes mentioned above.

competitively inhibit benzene degradation in similar experiments with different strains (Oh et al., 1994).

The substrate range of BTEXN degraders is also subject to considerable variability, even for different strains of a given species (Ridgway et al., 1990). Therefore, taxonomic identification of a given strain generally cannot be used to predict its ability to degrade different BTEXN compounds (Lang, 1996). These observations suggest that discerning general trends in biodegradation capabilities requires statistical analyses of BTEXN degradation patterns from numerous strains.

Several researchers have recognized the advantage of statistical analyses to identify stimulatory or inhibitory substrate interactions, and to obtain valuable insight on biodegradation capabilities and limitations (e.g., Arvin et al., 1989; Dyreborg et al., 1996a, 1996b; Ridgway et al., 1990). None of these studies, however, have correlated the specific biodegradation capabilities of multiple strains, or reported

how substrate interactions affect the range of BTEXN compounds that can be degraded. In this work, we surveyed the aerobic biodegradation capabilities of 55 different strains to address the following questions:

- Are there any correlations between the ability and inability of different strains to degrade specific BTEXN compounds?
- Which BTEXN compounds are more commonly (or less frequently) degraded?
- Do microorganisms that can degrade the more recalcitrant compounds have a broader catabolic range?
- How does the presence or absence of a given BTEXN compound affect the ability of bacteria to degrade other specific compounds?

Kappa statistics and analysis of variance were used to address these questions. Emphasis was placed on identifying heuristic relationships about probable degradation capabilities when indigenous microorganisms are exposed to different BTEXN combinations.

Materials and methods

General approach

Biodegradation assays and statistical analyses were conducted to investigate the substrate specificity and catabolic range of numerous strains that degrade aromatic hydrocarbons. Kappa statistics were used to analyze the catabolic specificity of 55 strains that degraded some BTEXN compounds when fed separately. The focus of this exercise was to identify any correlations on their ability or inability to degrade specific compounds. Factorial analysis of variance (ANOVA) was used to determine if the presence of any given BTEXN compound had a statistically significant effect on the ability of a strain to degrade other BTEXN compounds. Such substrate interactions were analyzed using data from the 16 strains listed in Table 1. For each strain, biodegradation capabilities were determined when the seven BTEXN compounds were fed separately, concurrently, and in several combinations in which one of the compounds was missing at a time. Thus, each strain was tested (in duplicate) under 15 different BTEXN combinations for a total of 480 degradation assays.

Strain sources and cultivation

The data analyzed by Kappa statistics included that reported by Ridgway et al. (1990), who isolated 39 strains from a shallow aquifer contaminated with gasoline (Table 2). All 39 strains were isolated on gasoline vapors, and were subsequently exposed to single-hydrocarbon vapors for up to two weeks at 23 °C in sealed chambers. The plates were examined for bacterial growth at different intervals, and growth was taken as a positive indication of biodegradation ability.

These data were augmented with data from our collection of 16 BTEXN degraders (Table 1). Three of these isolates were purchased from American Type Culture Collection (ATCC) based on their listed ability to degrade benzene (*Comamonas testosteroni* 27911 and *Rhodococcus zopfii* 51349) and naphthalene (*Pseudomonas putida* 17484). Three strains (*Pseudomonas corrugata* NG1, *P. corrugata* NG2, and *P. fluorescens* NG3) were isolated from a naphthalene-enriched culture originally seeded with uncontaminated topsoil. Serial dilutions of this enrichment were plated on agar plates amended with 200 mg/L naphthalene (not all of which was dissolved). The three different colony types that grew after 5 days were identified on the basis of their substrate utilization patterns using a Biolog® bacterial identification system. A similar approach was used to isolate and identify *P. putida* SI-1B, except that toluene was used instead of naphthalene in the soil enrichment and plating steps. Researchers from different laboratories kindly provided the remaining strains. David Gibson provided *P. putida* F1, *P. putida* mt-2, and *P. mendocina* KR1; Jerome Kukor provided *B. pickettii* PKO1 and *P*. CFS-215; Malcom Shields provided *B. cepacia* G4; and Loring Nies provided *Clavibacter michiganense* Bp11, *P. aeruginosa* N4, and*P. putida* Bp18.

All 16 strains were stored at −40 °C in Microbank cryovials (Pro-Lab Diagnostics, Ontario, Canada), and were grown on Bacto® Tryptic Soy Agar plates prior to conducting biodegradation assays.

Mineral medium

Basal mineral medium was used in biodegradation assays to provide essential inorganic nutrients for microbial growth and to buffer aqueous solutions at pH 7. The medium contained (in mg/L of deionized water): KH_2PO_4 (2104.7), K_2HPO_4 (1905.2), NH_4Cl (100.0), NaCl (10.0), $CaCl_2$ (5.0), $MgCl_2$ (10.0), $CuCl_2{\cdot}2H_2O$ (0.0392), $ZnCl_2$ (0.1363), $NiCl_2$ (0.0130), $FeCl_2{\cdot}4H_2O$ (0.7016), $AlCl_3$ (0.1106), $MnCl_2{\cdot}4H_2O$ (0.0281), $CoCl_2{\cdot}6H_2O$

Table 1. Catabolic profiles of 16 strains fed BTEXN compounds separately. Benzene (B), toluene (T), ethylbenzene (E), *p*-xylene (*p*-X), *m*-xylene (*m*-X), *o*-xylene (*o*-X), and naphthalene (N) were fed at 1 mg/L each, and cells were incubated at 20 °C on a horizontal shaker for 6 weeks. Instances when degradation occurred are indicated by "1", whereas "0" denotes that no degradation occurred

Organism	Strain	B	T	E	*p*-X	*m*-X	*o*-X	N
Reference strains (Figure 1):								
Burkholderia cepacia	G4	1	1	1	1	1	1	1
Burkholderia pickettii	PK01	1	1	1	1	0	1	0
Pseudomonas mendocina	KR1	1	1	1	1	1	0	0
Pseudomonas putida	F1	1	1	1	1	1	1	0
Pseudomonas putida	mt-2	0	1	1	1	1	0	1
Other tested isolates:								
Comamonas testosteroni	27911	1	1	1	0	0	0	0
Clavibacter michiganense	Bp11	0	0	0	1	0	0	0
Pseudomonas aeruginosa	N4	0	1	1	1	1	1	1
Pseudomonas corrugata	NG1	0	1	1	1	1	1	1
Pseudomonas corrugata	NG2	0	1	1	1	1	1	1
Pseudomonas flourescens Type F	NG3	0	1	1	1	1	1	1
Pseudomonas putida Biotype B	17484	1	1	1	1	1	1	1
Pseudomonas putida Type A1	Bp18	1	1	1	1	1	1	1
Pseudomonas putida Type A1	SI-1B	0	1	0	1	1	0	0
Pseudomonas sp.	CFS-215	1	1	1	1	1	1	0
Rhodococcus zopfii	51349	0	1	1	0	0	0	0

(0.0382), $Na_2MoO_4{\cdot}2H_2O$ (0.0254), H_3BO_4 (0.0382), and $NaSO_4$ (0.1420).

Biodegradation assays

The biodegradation capabilities of the 16 strains listed in Table 1 were studied using 20-mL GC vials (Kimble and Owens, IL) as batch reactors. Each vial contained 9.5 mL of mineral medium and approximately 1 mg/L of each BTEXN compound. This concentration is representative of groundwater contaminated by petroleum product releases. The 10-mL air headspace provided sufficient oxygen to meet the biochemical oxygen demand exerted by the added BTEXN. For inoculation, individual strains were grown on Bacto® Tryptic Soy Agar and transferred to the vials using a sterile loop. All vials were sealed with Teflon-lined rubber septa and aluminum crimp caps (The West Company, Lionville, PA). The vials were incubated on a horizontal shaker at room temperature (25 °C) for 4-6 weeks. These vials were then analyzed for residual BTEXN concentrations using a gas chromatograph equipped with a headspace autosampler as described in the analytical methods section.

Biodegradation was determined by the removal of a compound in viable incubations but not in controls. Two sets of controls were prepared. One set contained sterile mineral medium, 1 mg/L of each BTEXN compound, and no bacteria to monitor volatilization losses. The other set was seeded with bacteria and poisoned with 300 mg/L $HgCL_2$ to monitor potential BTEXN losses due to sorption onto biomass. Losses in both types of controls were minimal (<5%). At the end of each experiment, selected incubations were plated on Bacto® Trypticase Soy Agar to verify that the cultures in these vials were indeed pure.

Statistical analyses

Kappa statistics were used to identify any correlations between the ability and inability of multiple strains to degrade specific BTEXN compounds. The Kappa values of such dichotomous data indicate whether matches in biodegradation capabilities are higher than predicted by chance. Specifically, Kappa values range from −1 to 1, and a value of 1 corresponds to a perfect match in the presence or absence of any two specific biodegradation capabilities. Thus, broad substrate specificity is conducive to high Kappa values. A Kappa

Table 2. Catabolic profiles of 39 different strains isolated and tested by Ridgway et al. (1990). Strains were isolated on gasoline vapors, and then exposed to separate-hydrocarbon vapors in sealed chambers at 23 °C for up to two weeks. Based on their reported ability to grow on 16 separate hydrocarbons, these strains constitute 39 different phenotypes. Instances when cell growth (and thus, BTEXN degradation) occurred are indicated by "1", whereas "0" denotes that no degradation occurred

Organism	Strain	B	T	E	*p*-X	*o*-X	N
Pseudomonas aeruginosa	1	0	1	0	1	0	1
Pseudomonas aeruginosa	2	0	1	0	1	0	1
Pseudomonas aeruginosa	3	0	1	1	1	0	1
Pseudomonas aeruginosa	4	0	1	1	0	0	0
Pseudomonas aeruginosa	5	0	1	1	1	0	1
Pseudomonas aeruginosa	6	0	1	1	1	0	0
Pseudomonas aeruginosa	7	0	1	1	1	0	1
Pseudomonas aeruginosa	8	0	1	1	1	0	0
Pseudomonas aeruginosa	9	0	1	1	1	0	1
Pseudomonas aeruginosa	10	0	1	1	0	0	0
Pseudomonas aeruginosa	11	0	1	1	1	0	1
Pseudomonas aeruginosa	12	0	1	1	1	1	0
Pseudomonas aeruginosa	13	0	1	1	1	0	1
Nocardia spp.	14	1	0	0	0	1	0
Pseudomonas putida	15	1	1	1	0	0	0
Alc. denitrificans	16	1	1	1	0	0	0
Pseudomonas stutzeri	17	1	1	0	1	0	0
Pseudomonas malthophilia	18	1	1	1	1	1	1
Pseudomonas stutzeri	21	0	1	0	1	0	0
Pseudomonas malthophilia	22	0	1	0	1	0	0
Pseudomonas putida	24	1	1	1	1	1	1
Micrococcus	27	0	1	1	1	0	1
Pseudomonas alcaligines	28	1	1	1	0	0	0
Pseudomonas stutzeri	30	1	1	1	1	1	1
Pseudomonas putida	31	1	1	1	1	0	1
Pseudomonas putida	33	1	1	1	1	1	1
Pseudomonas stutzeri	34	1	1	1	1	1	1
Pseudomonas stutzeri	35	0	1	0	1	0	0
Pseudomonas putida	36	1	1	1	0	0	1
Pseudomonas alcaligines	38	0	1	0	0	0	0
Micrococcus	40	1	1	0	1	1	1
Micrococcus	41	1	1	1	1	1	1
Micrococcus	43	1	1	1	1	1	1
Micrococcus	44	0	0	0	0	0	1
Micrococcus	46	0	0	0	1	0	0
Micrococcus	47	0	0	0	1	0	0
Pseudomonas aeruginosa	49	1	1	0	1	1	1
Pseudomonas aeruginosa	50	1	0	0	1	1	0
Pseudomonas aeruginosa	51	0	1	1	1	1	0

value of 0 means that the level of agreement is the same as expected by chance. Negative Kappa values are rare, and represent agreement poorer than expected by chance (Tsuang et al. 1995). SPSS software was used to determine Kappa values and their statistical significance.

Factorial analysis of variance was performed to determine if the presence of any given BTEXN compound had a statistically significant effect (stimulatory or inhibitory) on the degradation of other BTEXN compounds. This analysis considered seven independent factors (i.e., the presence of B, T, E, *p*-X, *m*-X, *o*-X, or N) and eight dependent variables (i.e., whether degradation of B, T, E, *p*-X, *m*-X, *o*-X, or N occurred, and the catabolic range – indicated by the % of BTEXN compounds degraded). The 16 strains tested had different abilities to degrade BTEXN compounds, and were included in the model as a random factor to control for the variation in biodegradation ability. This statistical analysis was also performed with SPSS software (Norusis, 1993).

Chemicals

Toluene, ethylbenzene, naphthalene, mercuric chloride and all mineral medium chemicals were purchased from Fisher Scientific (Fair Lawn, NJ). All three xylene isomers were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI) while benzene was purchased from Mallinckrodt Chemical Works (St. Louis, MO). Bacto® Tryptic Soy Agar was purchased from Difco Laboratories (Detroit, MI).

Analytical methods

BTEXN compounds were analyzed by gas chromatography, using a Hewlett-Packard 5890 Series II gas chromatograph (GC) equipped with HP 19395A headspace autosampler and a flame ionization detector. ChemStation™ software was used for data acquisition and signal integration. The carrier gas (N_2) was supplied at 3 mL/min. Separation was accomplished with a 30-m DB-Wax column (J&W Scientific). The detection limit was approximately 5 ppb for each BTEXN compound.

Results and discussion

In this study, biodegradation refers to the removal of the BTEXN compounds as result of microbial action.

No attempts were made to analyze the metabolites or end products of oxidation, and in some cases it is unlikely that complete mineralization to CO_2 occurred. For example, reference strain F1 cannot grow on any xylene isomer, and strain mt-2 cannot grow on naphthalene. Therefore, the removal of these compounds by these strains (Table 2) probably resulted in a transformation to "dead-end" metabolites. It should be kept in mind, however, that products of BTEXN oxidative transformations (e.g., catechols and aromatic acids) are not priority pollutants. Furthermore, they are often polymerized and humified in soil systems (Tsao et al., 1998), if not mineralized by other members of the microbial consortium (i.e., cometabolic comensalism). Thus, from a practical point of view, BTEXN "biodegradation" as defined above reduces the risk to public health associated with environmental contamination, and helps achieve regulatory compliance.

Considerable diversity was found in the catabolic specificity of indigenous bacteria that degrade aromatic hydrocarbons. For example, 27 different profiles of catabolic capacity were expressed by 55 strains that were fed BTEXN compounds separately (Tables 1 and 2). Most of these strains exhibited relatively broad biodegradation capabilities. The average number of BTEXN compounds degraded by a given strain was 4.0, and ten strains (eight of which were pseudomonads) were capable of degrading all of the tested BTEXN compounds.

Kappa statistics were calculated for the dichotomous data shown in Tables 1 and 2 to quantify the level of agreement between the biodegradation capabilities and limitations of the 55 strains. The abilities to degrade the following pairs of compounds were significantly correlated, based on their high Kappa and low p-values (Table 3): benzene and *o*-xylene, toluene and ethylbenzene, ethylbenzene and naphthalene, *p*-xylene and *m*-xylene, *p*-xylene and *o*-xylene, *p*-xylene and naphthalene, *m*-xylene and naphthalene, and *o*-xylene and naphthalene. The positive (ability to degrade) and negative (inability to degrade) matches between *o*-xylene and naphthalene degradation capabilities were evenly distributed. However, the correlation between benzene and *o*-xylene biodegradability was primarily based on negative agreement. This infers that most microorganisms that cannot degrade benzene are also incapable of degrading *o*-xylene. In contrast, the correlations between the abilities to degrade toluene and ethylbenzene, *m*-xylene and naphthalene, and *p*-xylene and *m*-xylene were mostly due to positive agreement. This infers, for example, that a strain that degrades toluene is highly likely to be able to degrade ethylbenzene, and vice-versa, as is the case for strains that use the TOD or T4MO pathways (Gibson et al., 1990; Yen et al., 1991). This analysis also suggests that the abilities to degrade naphthalene and most alkylbenzenes (but not benzene) are correlated (Table 3).

A frequency analysis of the data shown in Tables 1 and 2 was conducted to assess the relative biodegradability of the tested compounds. Toluene was the most frequently degraded compound; it was degraded by 49 of the 55 strains tested (i.e., a success rate of 89%). The next most frequently degraded compounds were *p*-xylene (80%), *m*-xylene (75%), ethylbenzene (70%), naphthalene (52%), benzene (45%), and *o*-xylene (41%) (Figure 2(a)). Coincidentally, the least frequently degraded compounds, benzene and *o*-xylene, cannot be degraded by the enzymes coded in the TOL plasmid. This suggests that the TOL plasmid may have played a major role in BTEXN degradation by the tested strains. This untested hypothesis is supported by the fact that the TOL plasmid codes for the ability to degrade toluene, *p*-xylene, and *m*-xylene (Worsey et al., 1978), and this was the three-compound combination that was most frequently degraded (75%).

Figures 2(b) through 2(h) show the percentage of strains that could degrade a specific compound, given that they degraded the first compound in the series (black bars). To further explore relationships in biodegradation capabilities, these figures show also the percentage of strains that degraded a given compound when they could not degrade the first compound in the series (white bars). The fact that black bars are generally larger than white bars shows that strains capable of degrading any given BTEXN compound were more likely to degrade other aromatic hydrocarbons compared to strains that could not. This probably reflects the relaxed (cross) specificity of some catabolic enzymes, although the possibility that some strains used different enzymes to degrade different compounds cannot be ruled out. Incidentally, larger black bars correspond to higher percentages of positive matches in two degradation capabilities, while smaller white bars indicate higher percentages of negative matches. Therefore, the gap between respective black and white bars reflects how well correlated are the presence and absence of degradation abilities under consideration. For example, the relatively wide gap shown in Figure 2(b) for *o*-xylene is consistent with the correla-

Table 3. Kappa statistics for comparisons between the ability and inability of 55 strains to degrade different BTEXN compounds. Kappa values reflect the agreement between the evaluations of two raters, such as the ability to degrade toluene by a strain that can degrade benzene (positive agreement), and the inability to degrade toluene if that strain cannot degrade benzene (negative agreement). A Kappa value of 0 means that the agreement is the same as expected by chance, and a value of 1 represents perfect agreement (Tsuang et al., 1995). Bold entries represent comparisons where the agreement between a pair of biodegradation capabilities is statistically significant (i.e., p-value <0.05)

Degradative abilities compared	Kappa	p-value	Significant correlation?
Benzene and toluene	0.049	0.528	NO
Benzene and ethylbenzene	0.159	0.175	NO
Benzene and *p*-xylene	0.069	0.498	NO
Benzene and *m*-xylene	0	1	NO
Benzene and *o*-xylene	**0.483**	**<0.001**	**YES**
Benzene and naphthalene	0.059	0.657	NO
Toluene and ethylbenzene	**0.460**	**<0.001**	**YES**
Toluene and *p*-xylene	0.110	0.387	NO
Toluene and *m*-xylene	**0.333**	0.074	NO
Toluene and *o*-xylene	0.033	0.655	NO
Toluene and naphthalene	0.164	0.061	NO
Ethylbenzene and *p*-xylene	0.019	0.882	NO
Ethylbenzene and *m*-xylene	0.200	0.383	NO
Ethylbenzene and *o*-xylene	0.183	0.105	NO
Ethylbenzene and naphthalene	**0.256**	**0.041**	**YES**
***p*-Xylene and *m*-xylene**	**0.600**	**0.009**	**YES**
***p*-Xylene and *o*-xylene**	**0.238**	**0.014**	**YES**
p-Xylene and Naphthalene	0.286	0.010	YES
m-Xylene and *o*-xylene	0.429	0.074	NO
***m*-Xylene and naphthalene**	**0.500**	**0.021**	**YES**
***o*-Xylene and naphthalene**	**0.279**	**0.034**	**YES**

tion found between benzene and *o*-xylene degradation capabilities (Table 3).

Interestingly, the relatively few (23) strains that degraded *o*-xylene were also capable of degrading most of the other BTEXN compounds (Figure 2(g)). For example, these strains degraded an average of 6.2 out of the 7 BTEXN compounds tested in Table 1, and could grow on an average of 5 out of the 6 BTEXN compounds tested in Table 2. In contrast, strains that could not degrade *o*-xylene degraded an average of 3.1 compounds in Table 1 and grew on an average of 2.9 compounds in Table 2. A relatively broad catabolic range was also observed for the 25 strains capable of degrading benzene (Figure 2(b)) and for the 29 strains that degraded naphthalene (Figure 2(h)), which were the next "less-frequently-degraded" aromatic hydrocarbons. Apparently, strains capable of degrading the more recalcitrant compounds were more competent regarding the breadth of their biodegradation capabilities.

It should be noted that all of the above inferences are derived using data from 55 culturable strains, most of which were pseudomonads. The biodegradation capabilities of non-culturable and/or anaerobic strains that might participate in BTEXN bioremediation remain relatively unexplored.

Additional biodegradation assays were conducted with the 16 strains listed in Table 1 to determine how the presence of a given BTEXN compound affected the degradation of other compounds. For each strain, biodegradation capabilities were determined when the seven BTEXN compounds were fed separately, concurrently, and in several combinations in which one compound was missing at a time (data not shown). Several strains failed to degrade some BTEXN compounds when fed separately, but not when fed con-

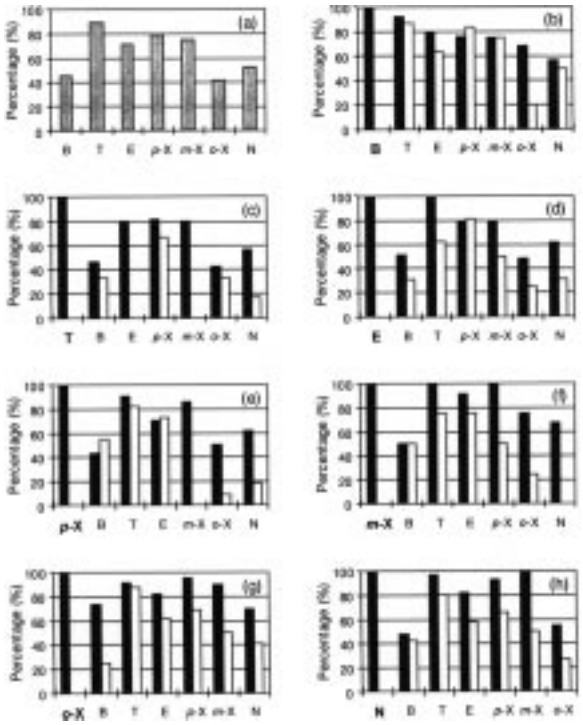

Figure 2. Frequency analysis of biodegradation capability of indigenous strains fed different aromatic hydrocarbons separately. Crosshatched bars [panel (a)] represent the percentage of 55 strains that was capable of degrading benzene (B), toluene (T), ethylbenzene (E), *p*-xylene (*p*-X), *o*-xylene (*o*-X), or naphthalene (N). The black bars [panels (b) to (h)] depict the percentage of strains that could degrade both the first compound in the series (in bold) and another compound indicated in the abscissa. The white bars [panels (b) to (h)] depict the percentage of strains that could not degrade the first compound in the series but could degrade another compound in the abscissa. Data includes that reported by Ridgway et al. (1990) for 39 isolates that were not tested for their ability to degrade *m*-xylene (Table 2).

currently. Therefore, beneficial substrate interactions enhanced the catabolic capacity of several strains. For example, *Rhodococcus zopfii* 51349 could not degrade benzene, *p*-xylene, or *o*-xylene when fed separately (Table 1). Yet, this strain degraded all of these compounds when the seven BTEXN compounds were fed concurrently. This strain also degraded benzene in all combinations containing toluene, but not when toluene was absent. A similar trend was observed with *P. corrugata* NG2, which did not degrade benzene when fed alone (Table 1) but degraded it in all other combinations. Furthermore, *B. pickettii* PKO1, *P. putida* F1, and *P.* sp. CFS-215 could not degrade naphthalene when fed alone (Table 1). Yet, all of these strains degraded naphthalene in all of the BTEXN mixtures tested. These observations reflect that some compounds can play a key role in inducing BTEXN degradation capabilities.

An analysis of variance of the collective experimental results corroborate that beneficial substrate interactions can enhance the catabolic capacity of many strains, possibly by stimulating the induction of enzymes with relaxed substrate specificity and/or co-oxidation. Specifically, toluene generally enhanced the ability to degrade benzene, *p*-xylene, naphthalene and the total number of BTEXN compounds degraded (Table 4). This corroborates previous reports that toluene can enhance the degradation of other BTEXN compounds under aerobic (Arvin et al., 1989; Alvarez and Vogel, 1991; Chang et al., 1993) and denitrifying conditions (Alvarez and Vogel, 1995; Evans et al., 1992; Hutchins, 1991). Other significant substrate interactions were the enhanced degradation of naphthalene by the presence of ethylbenzene, and the enhanced degradation of *p*-xylene when any of the other BTEXN compounds were present (Table 4). No negative substrate interactions were identified by this statistical analysis at the BTEXN concentrations tested (ca. 1 mg/L each).

Conclusions

Kappa statistics can be used to develop heuristic relationships about the probability of encountering strains that can (or cannot) degrade one or more related contaminants. This approach revealed significant correlations between the abilities to degrade toluene and ethylbenzene, *p*-xylene and *m*-xylene, and *p*-xylene and *o*-xylene. The ability to degrade naphthalene was correlated to the ability to degrade other alkylbenzenes, but not benzene. In addition, the inability to degrade benzene was correlated to the inability to degrade *o*-xylene.

Factorial ANOVA was useful to identify general trends regarding how the presence or absence of a compound is likely to affect biodegradation capabilities. This analysis showed that a broader (aerobic) degradation capability is generally expressed when a strain is exposed to contaminant mixtures than when the same compounds are fed separately. This enhancement was attributed to beneficial substrate interactions, such as enhanced enzyme induction and co-oxidation when toluene is present.

Table 4. Summary of positive substrate interactions identified as statistically significant in ANOVA output. Biodegradation assays were conducted with 16 strains fed different BTEXN combinations. This analysis considered 8 independent variables (strain, presence of B, or T, or E, or *p*-X, or *m*-X, or *o*-X or N) and 8 dependent variables (ability to degrade B, or T, or E, or *p*-X, or *m*-X, or *o*-X, or N, and substrate range – indicated as the percentage of BTEXN compounds degraded)

Independent variable (presence of X)	Dependent variable (enhanced degradation of Y)	*F*-Statistic	*p*-Value
Toluene	Benzene	7.08	0.009
Benzene	*p*-Xylene	7.50	0.007
Toluene	*p*-Xylene	7.50	0.007
Ethylbenzene	*p*-Xylene	7.50	0.007
m-Xylene	*p*-Xylene	7.50	0.007
o-Xylene	*p*-Xylene	7.50	0.007
Naphthalene	*p*-Xylene	7.50	0.007
Toluene	Naphthalene	5.54	0.021
Ethylbenzene	Naphthalene	5.54	0.021
Toluene	**% Total BTEXN**	**6.34**	**0.013**

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