COMMUNICATION TO THE EDITOR

Aerobic Degradation of Mixtures of Chlorinated Aliphatics by Cloned Toluene-*o*-Xylene Monooxygenase and Toluene *o*-Monooxygenase in Resting Cells

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Abstract: Recombinant strains of *Escherichia coli* constitutively expressing toluene-*o*-xylene monooxygenase (ToMO) of *Pseudomonas stutzeri* OX1 and toluene *o*-monooxygenase (TOM) of *Burkholderia cepacia* G4 were investigated for their ability to oxidize trichloroethylene (TCE), 1,1-dichloroethylene (1,1-DCE), *cis*-1,2-dichloroethylene (*cis*-DCE), *trans*-1,2-dichloroethylene (*trans*-DCE), vinyl chloride (VC), and chloroform (CF), individually as well as in various mixtures. ToMO oxidized all of these individual compounds well, whereas TOM did not degrade VC significantly (16-fold less) and degraded *cis*-DCE and *trans*-DCE less well (3.7- and 2.4-fold, respectively). For mixtures of these chlorinated aliphatics, ToMO was again more robust than TOM. For example, in binary mixtures including TCE, ToMO degraded all three DCE isomers and CF, but the presence of TCE inhibited VC degradation; TOM degraded both TCE/1,1-DCE and TCE/*trans*-DCE, but not *cis*-DCE for TCE/*cis*-DCE, and the addition of CF or VC to TCE completely inhibited degradation of both compounds and TCE. The addition of CF or *trans*-DCE stimulated VC degradation in the presence of TCE for ToMO, and the addition of any of the three DCE isomers stimulated VC degradation for TOM. Significant degradation of all ternary mixtures of TCE and less chlorinated ethenes, as well as a mixture of TCE, three DCEs, and VC, was achieved with ToMO (but not TOM). In mixtures of these chlorinated compounds, degradation was found to occur simultaneously rather than sequentially, and the mineralization of many of these compounds could be confirmed through detection of chloride ions. *Biotechnol Bioeng* **70**: 693–698, 2000.

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INTRODUCTION

Both tetrachloroethylene (PCE) and trichloroethylene (TCE) are suspected carcinogens and are the most common groundwater pollutants at hazardous waste sites (McCarty, 1997). These effective solvents are degraded anaerobically (only one known aerobic microorganism can degrade PCE [Ryoo et al., 2000]) via reductive dehalogenation to the less chlorinated ethenes *trans*-1,2-dichloroethylene (*trans*-DCE), *cis*-1,2-dichloroethylene (*cis*-DCE), 1,1-dichloroethylene (1,1-DCE), vinyl chloride (VC), and ethene as well as to ethane (Sharma and McCarty, 1996); however, the dechlorination of PCE and TCE is generally incomplete when it does occur (primarily VC and *cis*-DCE have been formed), and the potent VC generated is a known human carcinogen (McCarty, 1997). Both VC and *cis*-DCE are U.S. Environmental Protection Agency priority pollutants (Bradley and Chapelle, 1998). CF is also a common groundwater contaminant and a potential carcinogen (McClay et al., 1996).

Because of this ecological risk posed by soil and water contaminated simultaneously by TCE, DCEs, and VC (as well as mixtures with chloroform [CF]), the ability of two monooxygenases to degrade mixtures of these compounds has been evaluated. ToMO from *Pseudomonas stutzeri* OX1 has a relaxed regiospecificity (hydroxylates toluene in the *ortho*, *meta*, and *para* positions and *o*-xylene in both the 3 and 4 positions) (Bertoni et al., 1996) as well as a broad substrate range as it oxidizes *o*-xylene, *m*-xylene, *p*-xylene, toluene, benzene, ethylbenzene, styrene, and naphthalene (Bertoni et al., 1996) and also TCE, 1,1-DCE, and CF (Chauhan et al., 1998). ToMO is a multicomponent enzyme encoded by the genes *touABCDEF* with greatest similarity to the aromatic monooxygenases of *Burkholderia pickettii* PKO1 and *Pseudomonas mendocina* KR1 (Bertoni et al., 1998). TOM of *Burkholderia cepacia* G4 is also encoded by

six genes (*tomA012345*) (Shields and Francesconi, 1996) and is a three-component enzyme consisting of a 211-kDa hydroxylase with a catalytic oxygen-bridged binuclear iron center, a 40-kDa reductase, and a 10.4-kDa protein involved in electron transfer between the hydroxylase and reductase (Newman and Wackett, 1995). TOM oxidizes TCE, all three DCEs, and VC (Shields and Francesconi, 1996; Shields et al., 1994), and TCE is degraded primarily to CO_2 and Cl^- in vivo (Luu et al., 1995; Nelson et al., 1986).

There are relatively few reports of degradation of mixtures of pollutants and most involve mixed cultures (not pure cultures expressing single degradative enzymes). The best-studied system is that of the five-component benzene, toluene, ethylbenzene, *o*-xylene, and *p*-xylene mixture, which Bielefeldt and Stensel (1999) showed, using mixed cultures from a manufactured gas site, could be modeled by competitive inhibition kinetics. For chlorinated aliphatics, binary mixtures of TCE and CF have been studied for toluene-oxidizing bacteria (McClay et al., 1996) and for a mixed methanotrophic culture (Alvarez-Cohen and McCarty, 1991); only the mixed methanotrophic culture successfully degraded both CF and TCE (many toluene and phenol oxidizers cannot oxidize CF at all [Chang and Alvarez-Cohen, 1995]). In addition, in a mixture of aromatic solvents and chlorinated aliphatics, two propane-grown pure *Rhodococcus* species have been shown to degrade CF, VC, 1,1-DCE, *cis*-DCE, and 1,1,2-trichloroethane (but nonsignificant amounts of PCE, TCE, 1,1,1-TCE, and *trans*-DCE) over the course of 2 weeks (Malachowsky et al., 1994).

Because there are few data on the simultaneous degradation of mixtures of chlorinated compounds by any monooxygenase, the degradation of mixtures TCE, 1,1-DCE, *cis*-DCE, *trans*-DCE, VC, and CF by ToMO and TOM was investigated using *E. coli* so that a single degradative enzyme could be studied. This is the first report of successful, simultaneous degradation of mixtures of chlorinated aliphatics by a pure culture expressing a single degradative enzyme and the first report of degradation of VC, *cis*-DCE, and *trans*-DCE by ToMO as well as degradation of CF by TOM.

MATERIALS AND METHODS

Organisms and Growth Conditions

Escherichia coli JM109 was used as the host with either pBZ1260 (Bertoni et al., 1996) containing the *P. stutzeri* OX1 6-kb *touABCDEF* (a pGEM3Z derivative; Promega, Inc., Madison, WI) or pMS64 (Shields et al., 1995) containing the *B. cepacia* G4 11.2-kb *tomA012345* (a pGEM4Z derivative; Promega). In pBZ1260, ToMO is expressed under the control of the constitutive *lac* promoter and, in pMS64, TOM is expressed under the control of the IS*50* promoter of Tn*5* (Shields and Francesconi, 1996). JM109/pBZ1260 and JM109/pMS64 were grown at 37°C overnight in Luria–Bertani (LB) medium (Sambrook et al., 1989) containing 150 and 100 μg/mL ampicillin (Sigma Chemical Co., St. Louis, MO), respectively, and cells were harvested by centrifugation at 13,800*g* for 5 min at 25°C (JA-17 rotor in a J2 series centrifuge; Beckman Instruments, Palo Alto, CA), resuspended in 0.1 *M* potassium phosphate buffer (PPB; pH 7), and diluted to an optical density of 1.0 at 600 nm (unless specified otherwise).

Chemicals

TCE and CF were purchased from Fisher Scientific Co. (Pittsburgh, PA); 1,1-DCE, *cis*-1,2-DCE, and *trans*-1,2-DCE from Aldrich Chemical Co., Inc. (Milwaukee, WI); and VC from Supelco Inc. (Bellefonte, PA). All materials were of the highest purity available and used without further purification.

Extent of Degradation

Ten milliliters of the PPB cell suspension (OD 1) was added to 60-mL glass vials that were then covered with a Teflon-coated septum and aluminum crimp seal. The six chlorinated compounds were injected directly into the cell suspension from 10 m*M* dimethylformamide (Fisher Scientific Co.) stock solutions (except for VC at 32 m*M* in methanol) using a liquid-tight syringe (Hamilton Co., Reno, NV) to yield (assuming all of the volatile compound to be in the liquid phase) TCE at 10 μ*M*, CF at 10 μ*M*, VC at 32 μ*M*, and DCEs at 50 μ*M*. The inverted vials were shaken at room temperature at 300 rpm on a Vibrax VXR shaker (IKA-Works, Inc., Cincinnati, OH). The concentrations of the chlorinated compounds in the headspace were determined by gas chromatography (GC) after 24 h (or more frequently for the simultaneous degradation experiments) by injecting a 50-μL headspace sample with a 100-μL Hamilton gas-tight syringe into a gas chromatograph (5890 Series II; Hewlett-Packard Co., Palo Alto, CA) equipped with a flame-ionization detector and fitted with a 0.1% AT-1000 on 80/100 Graphpac packed column (Alltech Associates, Inc., Deerfield, IL). The samples were analyzed with the column and injector at 200°C and the detector at 250°C. Abiotic (no cells plus chlorinated compound) vials as well as JM109 plus chlorinated compound vials were used as negative controls, and each set of aliphatic compounds was degraded two to seven times to confirm the results.

Extents of Mineralization

Overnight LB cultures were washed with PPB three times to remove chloride and then resuspended in PPB at 1.0 to 3.7 OD_{600}. Ten milliliters of cells was placed into 60-mL glass vials, sealed as noted earlier, and the chlorinated aliphatics were injected at 50 to 100 μ*M*. After centrifuging the cells,

the supernatant chloride ion concentrations generated from the mineralization of the chlorinated aliphatics were measured spectrophotometrically using the procedure of Bergmann and Sanik (1957), which involved adding 200 μL of 0.25 *M* $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ in 9 *M* HNO_3 and 200 μL of $Hg(SCN)_2$ in 95% ethanol to 0.6 mL of the supernatant. After 5 min, the absorbance of the $Fe(SCN)^{2+}$ product at 460 nm was measured with a DU640 spectrophotometer (Beckman Instruments). The colored $Fe(SCN)^{2+}$ product and $HgCl_2$ were both formed as free chloride ions displaced the thiocynate ion of $Hg(SCN)_2$. Duplicate vials were used for each chlorinated aliphatic/cell concentration, and chloride ion concentrations were calculated relative to the average of duplicate JM109 negative controls, which contained the chlorinated aliphatic at the same concentration. The minimum detectable chloride concentration with this method was 8 μ*M*.

RESULTS

In *E. coli* JM109, the degradative genes for both monooxygenases were expressed downstream of constitutive promoters in these high-copynumber constructs (the *lac* promoter for ToMO was constitutive due to lack of *lacI* repressor on plasmid pBZ1260 and thus the high copynumber titrated the F′-derived repressors); therefore, there was no need to add inducers (e.g., 58% of 50 μ*M* *cis*-DCE was degraded overnight by JM109/pBZ1260 with 1 m*M* isopropyl-β-D-thiogalactopyranoside vs. 54% when no inducer was added). Expression levels of the two enzymes appeared to be comparable because similar amounts of TCE were degraded by both strains (Table I), and both enzymes converted similar amounts of naphthalene to naphthol (1340 ± 75 nmol/mg protein for ToMO vs. 5400 ± 250 nmol/mg protein for TOM) (Ryoo et al., 2000).

ToMO and TOM were initially tested for the ability to oxidize TCE, 1,1-DCE, *cis*-DCE, *trans*-DCE, CF, or VC (nonmixtures) by looking for the overnight disappearance of the starting compounds (Table I) relative to the chlorinated aliphatic that was lost in the plasmid-free negative controls for the same period of time (usually 5% to 30% depending on the compound). Some variability probably arose in these extent-of-degradation experiments because the different JM109 cultures were not all the same age (stationary-phase cultures with slightly different ages were diluted to the same OD). All six chlorinated compounds were degraded to a

Table I. Degradation of TCE (10 μ*M*), three DCEs (50 μ*M* each), chloroform (CF, 10 μ*M*), and vinylchloride (VC, 32 μ*M*) by resting cells expressing ToMO and TOM (relative to negative controls). Actual initial liquid concentrations are 3.3 μ*M* for TCE, 20 μ*M* for *cis*-DCE, 21 μ*M* for *trans*-DCE, 6.3 μ*M* for 1,1-DCE, 6.3 μ*M* for CF, and 5.4 μ*M* for VC (Bradley and Chapelle, 1998; Mercer and Cohen, 1990).

Compound(s)	Percent removal after 24 h with resting cells[a]		Number of replicates	
	ToMO	TOM	ToMO	TOM
TCE	82 ± 16	62 ± 15	6	5
1,1-DCE	33 ± 6	20 ± 2	3	4
cis-1,2-DCE	70 ± 22	19 ± 2	6	4
trans-1,2-DCE	50 ± 25	21 ± 9	5	4
CF	41 ± 13	54 ± 22	7	4
VC	32 ± 21	2 ± 2	3	4
TCE and 1,1-DCE	50 ± 24, 46 ± 23	44 ± 6,60 ± 20	6	2
TCE and *cis*-1,2-DCE	47 ± 16, 63 ± 30	26 ± 23,12 ± 11	3	2
TCE and *trans*-1,2-DCE	55 ± 13, 66 ± 42	52 ± 19,60 ± 9	2	2
TCE and CF	57 ± 27, 41 ± 29	0.25 ± 0.5, 0	4	4
TCE and VC	71 ± 1, 11 ± 2	5 ± 8,2 ± 3	2	4
1,1-DCE and *cis*-1,2-DCE	35 ± 9, 33 ± 4	27 ± 9, 33 ± 21	2	4
1,1-DCE and *trans*-1,2-DCE	68 ± 2, 64 ± 1	32 ± 13,40 ± 12	2	2
1,1-DCE and CF[b]	15 ± 5	5 ± 1	3	2
1,1-DCE and VC	17 ± 8, 18 ± 12	86 ± 3,89 ± 0	4	2
cis- and *trans*-1,2-DCE[b]	49 ± 23	23 ± 0	2	2
cis-1,2-DCE and CF[b]	93 ± 2	33 ± 4	3	2
cis-1,2-DCE and VC	77 ± 2, 44 ± 5	29 ± 11,41 ± 18	4	2
trans-1,2-DCE and CF[b]	2.5 ± 3	Not determined	2	ND
trans-1,2-DCE and VC	67 ± 13, 43 ± 30	42 ± 6,65 ± 10	4	2
CF and VC	67 ± 8, 39 ± 28	17 ± 4,3 ± 1	4	2
TCE, 1,1-DCE, and CF	75 ± 35, 9 ± 13[c]	17 ± 11, 0[c]	2	2
TCE, 1,1-DCE, and VC	37 ± 27, 32 ± 20, 20 ± 20	1 ± 1,4 ± 4,11 ± 4	5	2
TCE, *cis*-1,2-DCE, and VC	16 ± 28, 51 ± 29, 8 ± 14	20 ± 3,43 ± 1,12 ± 10	3	2
TCE, *trans*-1,2-DCE, and VC	64 ± 15, 68 ± 15, 56 ± 31	24 ± 4,2 ± 13, 0	6	2
TCE, CF, and VC	55 ± 24, 50 ± 24, 40 ± 26	17 ± 16,36 ± 7, 0	6	2
TCE, three DCEs, and VC	32 ± 14, 33 ± 11,[d] 37 ± 8,[e] 32 ± 16	5 ± 5,4 ± 0[d],3 ± 4[e],1 ± 1	4	2

[a]Compared to negative controls after the same time period (24 h). [b]Combined peak. [c]Combined peak for 1,1-DCE and CF. [d]1,1-DCE. [e]Combined peak for *cis*- and *trans*-1,2-DCE.

significant extent overnight for ToMO, whereas there was less degradation of *cis*-, *trans*-, and 1,1-DCE and no significant degradation of VC for TOM (Table I). *P. stutzeri* OX1 has previously been reported to degrade TCE, 1,1-DCE, and CF at 10 μ*M* (Chauhan et al., 1998), whereas *B. cepacia* G4 TOM has previously been reported to degrade TCE, all three DCEs, and VC at 10 μ*M* (Shields and Francesconi, 1996; Shields et al., 1994). Hence, these nonmixture results extend the range of ToMO to include *cis*-DCE, *trans*-DCE, and VC, and extend the range of TOM to include CF, but call into question its ability to degrade VC well (in nonmixtures).

To corroborate the degradation of CF by both enzymes, the extent of mineralization (conversion to chloride ions) was investigated (Table II). For TOM, chloroform was clearly degraded, because 39% of 50 μ*M* and 43% of 100 μ*M* was mineralized (13 and 35 μ*M* chloride detected). ToMO also clearly mineralized CF, because 71% to 100% of the initial CF was mineralized (30 and 130 μ*M* chloride detected).

Because *cis*- and *trans*-DCE were previously reported to not be degraded by ToMO, their degradation was also confirmed through chloride ion analysis. The results in Table II indicate that 100% of *cis*-DCE was mineralized, whereas 89% of *trans*-DCE was mineralized. TCE was also mineralized by both ToMO (100%) and TOM (100%), and TOM mineralized 43% to 100% of *cis*-DCE.

Strains expressing the two enzymes were also characterized for their ability to degrade overnight mixtures of two, three, and five chlorinated aliphatics (Table I). For the binary mixtures containing TCE, ToMO degraded all three DCE isomers and CF, but the presence of TCE inhibited VC degradation. Binary combinations of the DCE isomers and CF and VC were degraded well except for 1,1-DCE/CF, 1,1-DCE/VC, and *trans*-DCE/CF. For ternary mixtures including TCE and VC, ToMO acquired the ability to oxidize VC upon addition of 1,1-DCE, *trans*-DCE, or CF. In general, all ternary combinations of TCE and the less chlorinated ethenes (and TCE/CF/VC), as well as the mixture of five chlorinated ethenes (TCE/3 DCEs/VC), were very effectively degraded by ToMO.

As compared with ToMO, degradation of mixtures of chlorinated aliphatics by TOM was not as robust (Table I). For the binary mixtures including TCE and DCE, TOM degraded TCE/1,1-DCE and TCE/*trans*-DCE well, but not TCE/*cis*-DCE; hence, the addition of TCE enhanced degradation of 1,1- and *trans*-DCE. Although both CF and TCE were degraded independently, combining the two resulted in the inhibition of degradation of both substrates. Addition of VC also completely inhibited TCE degradation. The binary combinations of DCE were degraded well, but the presence of CF prevented the degradation of CF and 1,1-DCE. Interestingly, in binary mixtures, the addition of any of the three DCE isomers stimulated VC degradation, and this pattern was repeated in ternary mixtures with TCE because the presence of 1,1-DCE or *cis*-DCE stimulated VC degradation. However, for ternary mixtures of TCE and less-chlorinated ethenes, only TCE/*cis*-DCE/VC was transformed (although VC was only slightly degraded).

ToMO also degraded TCE, CF, VC, and *trans*-1,2-DCE simultaneously when these compounds were present in mixtures of TCE/CF, TCE/CF/VC, or TCE/*trans*-DCE/VC (Table III). For example, ToMO in *E. coli* JM109/pBZ1260 degraded 43% of the TCE, while degrading 29% of the chloroform. Chloride ion analysis indicated that 100% of the two degraded compounds was mineralized in 24 h. Similar simultaneous degradation occurred with ToMO and mixtures of TCE/CF/VC and TCE/*trans*-DCE/VC as well with TOM and *trans*-DCE/VC.

DISCUSSION

It has been reported that TOM cannot degrade CF (McClay et al., 1996); however, we showed both significant overnight degradation (54%; see Table I) as well as mineralization (43% and 39% at 100 and 50 μ*M*, respectively). Perhaps the difference seen was due to the different strains and concentrations, as we used the *B. cepacia tom* genes expressed in *E. coli* at 6.3 μ*M* (actual concentration taking into account volatilization for degradation; see Table I), whereas, previously, wild-type toluene-induced *B. cepacia* was used at 16.2 μ*M* (actual). Note that, in this study, CF (but not VC) was also degraded by TOM in mixtures of

Table II. Mineralization of chlorinated aliphatics by ToMO and TOM (average of duplicate vials shown).

Enzyme	Compound (μ*M*)	OD	Degradation (GC) (μ*M*)	Cl^- measured (μ*M*)	Theoretical Cl^- (μ*M*)	Mineralization (%)
ToMO	*cis*-1,2-DCE (100)	3.7	79 ± 3	198 ± 1	158	100
		1.0	41 ± 1	122 ± 8	82	100
	trans-1,2-DCE (100)	3.2	58 ± 4	103 ± 3	116	89
	CF (100)	3.2	34 ± 4	130 ± 9	102	100
	CF (50)	1.0	13.8 ± 0.4	30 ± 3	42	71
	TCE (75)	1.0	26 ± 3	93 ± 8	78	100
TOM	*cis*-1,2-DCE (100)	3.7	23 ± 0	20 ± 0	46	43
		1.0	20 ± 6	87 ± 11	40	100
	CF (100)	3.2	27 ± 1	35 ± 0	81	43
	CF (50)	1.0	11.3 ± 0.4	13 ± 1	33	39
	TCE (75)	1.0	18.6 ± 0.2	63 ± 2	54	100

Table III. Simultaneous degradation of mixtures of chlorinated aliphatics by ToMO and TOM (average of duplicate vials from one of two separate sets of experiments shown).

		Percent decrease of compounds in mixtures (compared to *E. coli* JM109 without enzyme)						
Enzyme	Contact time (h)	Compounds (concentrations, μ*M*)				Measured Cl^- (μ*M*)	Theoretical Cl^- (μ*M*)	Mineralization (%)
ToMo		TCE (50)	CF (100)					
	2	0	2					
	5	5	6					
	9	26	18					
	16	43	31					
	24	43	29			152	152	100
ToMO		TCE (50)	CF (100)		VC (32)			
	6	17	10		0			
	12	10	32		28			
	19.5	43	55		48			
	30	38	51		42	277	224	100
ToMO		TCE (10)		*trans*-DCE (50)	VC (32)			
	1	2		3	10			
	3	15		18	23			
	7	24		41	44			
	14	35		46	47			
	30	51		63	61			
TOM				*trans*-DCE (100)	VC (32)			
	6			13	11			
	12			9	8			
	19.5			11	12			
	30			28	40	98	70	100

CF/VC and CF/TCE/VC (Table I). As reported previously with toluene-*p*-monooxygenase (McClay et al., 1996), TCE inhibited CF oxidation for TOM, but ToMO degraded significant amounts of both TCE and CF.

Many of the stimulation and inhibition results of the experiments involving mixture degradation can be explained by the fact that these monooxygenases have two active sites (two binuclear iron centers) per hydroxylase subunit (Bertoni et al., 1998; Newman and Wackett, 1995). Hence, for TOM, VC degradation is not stimulated until perhaps *cis*-, *trans*-, or 1,1-DCE binds to one site and facilitates the binding and oxidation of VC at the other active site (Tables I and III). This stimulation was again seen with TOM oxidation of VC in ternary mixtures with TCE and *cis*- or 1,1-DCE (Table I). Similarly, for ToMO, TCE inhibits VC degradation in binary mixtures, but in ternary mixtures the binding of *trans*-DCE in the presence of TCE stimulates VC oxidation, possibly by binding at one active site (and excluding TCE during VC oxidation); this stimulation was also seen in the mixtures of TCE, three DCEs, and VC (Tables I and III). The presence (binding) of CF at the expense of TCE also appears to facilitate VC oxidation by ToMO in ternary mixtures of TCE/CF/VC (Tables I and III).

The proposed importance of the role of the two active sites of each enzyme is supported by the experiments in which it was checked to see whether the chlorinated compounds were degraded simultaneously or sequentially. For both TCE/*trans*-DCE/VC and TCE/CF/VC with ToMO, all three compounds are degraded simultaneously rather than sequentially. Hence, it is reasonable to suggest that *trans*-DCE facilitates VC degradation for TCE/*trans*-DCE/VC and that CF facilitates VC degradation for TCE/CF/VC, because all these compounds are degraded simultaneously.

For ToMO, quintuple and ternary mixtures of TCE/DCE/VC are more readily degraded than binary mixtures of TCE/VC; for TOM, binary mixtures of DCEs/VC as well as TCE/DCEs are more readily degraded than VC or DCE alone, but ternary and quintuple mixtures are not readily degraded. Hence, ToMO is capable of degrading mixtures of chlorinated aliphatics, which arise at sites throughout anaerobic degradation of PCE and TCE.

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