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Effects of *Alpha*-Pinene and Trichloroethylene on Oxidation Potentials of Methanotrophic Bacteria

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Trichloroethylene (TCE), a widely used solvent notable for its degreasing properties, is a common environmental contaminant that poses significant risk to public health (ATSDR 1999). TCE has been shown to be effectively removed from soil and water by phytoremediation, often favored over other methods because of its effectiveness, low cost, and aesthetic benefits. More rapid TCE removal has been observed in the root zone of plants (rhizosphere) used in phytoremediation (Anderson and Walton 1995; Brigmon et al. 1999), and methanotrophs, methane-oxidizing bacteria that thrive on oxygen and methane and are capable of co-oxidizing TCE (Wilson and Wilson 1985; Little et al. 1988), have been implicated in this increased activity (Brigmon et al. 1999).

Loblolly pines (*Pinus taeda*), shown to support large rhizosphere populations of methanotrophs (Brigmon et al. 1999), have been considered for TCE remediation. These trees produce and release significant quantities of monoterpenes, the most predominant being (R)-α-pinene, composing over 65% of the total oleoresin composition in different plant tissues (Phillips et al. 1999). Since concentrations of (R)-α-pinene have been observed to be as high as 1.4 mg g^{-1} in fresh litter layers of pine forest soils (White 1994), the probability that soil microorganisms encounter these compounds in nature is high. Previous studies have shown that (R)-α-pinene has a concentration-dependent inhibitory effect on methane oxidation by methanotrophs (Amaral and Knowles 1997; Amaral et al. 1998), and, thus, may impact not only the growth of these bacteria in the rhizosphere but also their ability to co-oxidize TCE. While methanotrophs were shown to regain methane oxidation activity one to three days after exposure to (R)-α-pinene (Amaral et al. 1998), the implications of the long-term presence of this monoterpene on methanotrophic activity in the rhizosphere, in particular concentration effects of this chemical and its influence on TCE removal potentials, are not clear.

To this end, this study sought to first assess the ability of representative Type I, II, and X methanotrophs, grouped by their differences in carbon assimilation pathways, intracytoplasmic membrane structures, fatty acid carbon lengths, and phylogeny (Bowman et al. 1993), to oxidize (R)-α-pinene over a range of concentrations using oxygen uptake analysis. Secondly, this study sought to gain

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a better preliminary understanding of the variation in oxygen uptake responses to mixtures of (R)-α-pinene and TCE by representative methanotrophs, thus ultimately providing insight into the effect of (R)-α-pinene on TCE oxidation potentials of these bacteria and guidance for the phytoremediation practitioner to more accurately predict the extent of TCE rhizodegradation when using monoterpene-releasing plants. We report herein observations of the potential of methanotrophs to oxidize (R)-α-pinene over a broad range of concentrations and (R)-α-pinene/TCE mixture effects on methanotrophic oxygen uptake activity.

MATERIALS AND METHODS

Methanotroph strains used in this study included Type I *Methylomicrobium album* BG8 (American Type Culture Collection (ATCC) 33003) and Type II *Methylosinus trichosporium* OB3b (ATCC 35070), obtained from Dr. Jeremy Semrau (University of Michigan, Ann Arbor, MI, USA), and Type X *Methylococcus capsulatus* (Bath) (ATCC 33009), purchased from the ATCC (Manassas, VA, USA). Cultures were grown in nitrate mineral salts (NMS) medium (Whittenbury et al. 1970), with and without 10 μM $Cu(NO_3)_2$ to provide conditions for expression of the particulate and soluble forms of methane monooxygenase (pMMO and sMMO), respectively. With the exception of *M. capsulatus* (Bath), incubated at 45 °C with 50% methane (99.99% pure, Strate Welding, Jacksonville, FL, USA) in the headspace, all organisms were routinely subcultured in sealed erlenmeyer flasks containing 20% methane in the headspace and incubated at 30°C in a rotary shaker at 250 rpm, as previously described (Lindner et al. 2000). Purity of the cultures was verified by routine streaking on 2% (w/v) nutrient agar plates (Difco, Sparks, MD, USA). Expression of sMMO was qualitatively verified by a naphthalene assay modified from Brusseau et al. (1990) and described by Lindner et al. (2000).

Oxygen uptake analysis was performed in this study, as it has been shown to be a rapid, effective means of assessing oxidative potential of whole cells (Lindner et al. 2000, 2003). (R)-α-pinene was chosen to represent monoterpenes because it is a major component of loblolly pine oleoresin (Phillips 1999). (R)-α-pinene and TCE were obtained in the highest purity available from Aldrich Chemical Co. (Milwaukee, WI, USA). Standard solutions of 10 μmol/ml were prepared in 1,4-dioxane (Fisher Scientific, Pittsburgh, PA, USA), used as the carrier solvent because it easily solubilized the substrates, was not oxidized by any of the cultures studied, and caused no probe effects during oxygen uptake analysis (Lindner et al. 2000). Resting-cell suspensions were prepared from 500 ml cultures harvested at ¾-log phase by centrifugation in a J2-HS Beckman floor model centrifuge (Beckman-Coulter, Fullerton, CA, USA) at 2460 *x g*, 4°C, for 20 min. To ensure removal of all methane, the cells were washed with NMS medium, recentrifuged, and resuspended in the NMS medium to a wet cell concentration of 0.2 g/ml. The oxygen uptake system was composed of a 1.9 ml, well-stirred, enclosed reactor held at room temperature, as described by Lindner et al. (2000). After assessing the ability of methanotrophs to oxidize TCE and (R)-α-pinene alone, the study proceeded to investigate the effect of α-pinene on

TCE oxidation by adding both substrates simultaneously into the oxygen uptake system before addition of the resting cells.

Despite storage of the resuspended cells on ice throughout the oxygen uptake experiments, loss of cell activity over time was observed. To ensure comparability of measurements throughout the 2-3-day testing period, all rates of oxygen uptake were normalized to the rates observed with 4 ml of methane gas, measured just prior to a change to a new substrate concentration. Details of this normalization procedure are presented in Lindner et al. (2000). The electrode was calibrated at least daily with a saturated sodium sulfite solution, and "live" runs were performed at least in triplicate for each concentration tested. All runs were corrected for endogenous metabolism. Controls without cells and with 4 ml of acetylene gas, a known inhibitor of MMO (Prior and Dalton 1985), were routinely run to verify that depletion of oxygen, hence, oxidation activity, was a result of MMO activity. Initial rates of oxygen uptake were calculated by linear or polynomial fits to the data points using Microsoft Excel software (Microsoft Corp., Redmond, WA, USA).

RESULTS AND DISCUSSION

M. trichosporium OB3b and *M. capsulatus* (Bath), when cultured with no copper, expressed positive sMMO activity, as evidenced by a bright pink-to-purplish color in the assay. All of the strains tested negative for sMMO activity (no color change observed) when cultured with 10 μM $Cu(NO_3)_2$. sMMO and pMMO expression under culturing conditions without and with copper, respectively, was thus assumed, a reasonable conclusion given that enzyme expression in these methanotrophs under these conditions is well characterized. Active resting cells of all three representative methanotrophs consumed oxygen over a range of TCE and (R)-α-pinene concentrations, regardless of the type of MMO expressed (Fig. 1, A-E). No oxygen uptake was observed after addition of acetylene or without cells present, verifying MMO activity in all cases. As shown in Figure 1, regardless of the methanotroph or substrate tested, a maximum rate of oxygen uptake was observed, followed by a rapid decrease in rates, suggesting toxic effects of either the substrate itself or of oxidation products formed. This oxygen uptake behavior has been reported previously for methanotrophs with aromatic substrates (Lindner et al. 2000), and, while both substrates have been shown to have toxic effects on methanotrophic activity (Alvarez-Cohen and McCarty 1991; Henry and Grbic-Galic 1991; Oldenhuis et al. 1991; White 1994; Amaral and Knowles 1997; Amaral et al. 1998), there have been no previous reports on the effects of a range of substrate concentrations on relative activities.

As shown in Figure 1, methanotrophs expressing sMMO (plots A, C) oxidized TCE at higher maximum rates than those expressing pMMO (plots B, D, E), as previously reported (Little et al. 1988; DiSpirito et al. 1992; Lontoh and Semrau 1998). The maximum normalized rates of oxidation by *M. trichosporium* OB3b and *M. capsulatus* (Bath) expressing sMMO or pMMO were 0.11 ± 0.01 and 0.03 ± 0.00 and 0.05 ± 0.01 and 0.03 ± 0.01, respectively (Fig. 1, A-D), while the

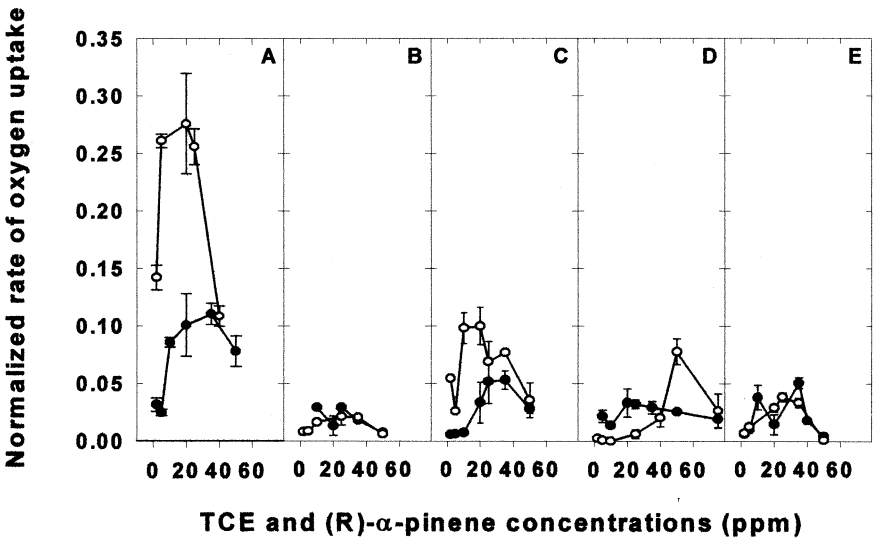

Figure 1. Normalized rate of oxygen uptake by the representative methanotrophs in the presence of varying concentrations of TCE (•) and (R)-α-pinene (○).
A, B: *M. trichosporium* OB3b cultured without and with copper, respectively. **C, D**: *M. capsulatus* [Bath] cultured without and with copper, respectively. **E**: *M. album* BG8 cultured with copper. Error bars represent the standard deviation for triplicate samples.

maximum rate expressed by *M. album* BG8, capable of pMMO expression only, was 0.05 ± 0.01 (Fig. 1, E). The TCE concentrations where the observed normalized oxygen uptake rate was the highest ranged from 20 to 35 ppm for the tested strains. *M. trichosporium* OB3b and *M. capsulatus* (Bath) expressing sMMO exhibited oxygen uptake maxima at higher TCE concentrations (35 ppm) than when expressing pMMO (20-25 ppm), and *M. album* BG8 expressing pMMO showed a maximum observed rate at 35 ppm TCE. These results do suggest differing sensitivity levels to TCE, depending on the methanotroph and type of MMO expression.

As observed with TCE, both sMMO-expressing methanotrophs were also capable of oxidizing (R)-α-pinene at higher rates than their pMMO-expressing counterparts (Fig. 1, A-D). The maximum normalized rate of oxygen uptake by *M. trichosporium* OB3b expressing sMMO was almost 10 times the rate observed with pMMO-expressing cells (0.28 ± 0.04 and 0.02 ± 0.01, respectively); however, both rate maxima occurred at 20 ppm (R)-α-pinene (Fig. 1, A, B). The maximum normalized oxygen uptake rate with *M. capsulatus* (Bath), expressing sMMO, was 0.10 ± 0.02 at 20 ppm (R)-α-pinene, compared to 0.08 ± 0.01 at 50 ppm (R)-α-pinene under pMMO expression (Fig. 1, C and D). The observed

maximum normalized rate of oxygen uptake by M. *album* BG8 was 0.04 ± 0.00, between the values observed for the other two strains under pMMO expression (Fig. 1E). Previous studies have reported higher TCE oxidation rates by pure methanotrophs under sMMO expression (Little et al. 1988; DiSpirito et al. 1992; Lontoh and Semrau 1998); however, this is the first report of such a trend with (R)-α-pinene. These results bring direct relevance to the environment, as sMMO expression in methanotrophs occurs only at very low copper concentrations (Lontoh and Semrau 1998). Measurement of bioavailable copper is essential, therefore, for effective prediction of methanotrophic activity potential.

The response of each methanotroph in the presence of 20 ppm TCE over a range of (R)-α-pinene concentrations is shown in Figure 2, A-E. This plot presents the change in normalized oxygen uptake rate with 20 ppm TCE alone caused by the presence of different concentrations of (R)-α-pinene and thus represents the influence of (R)-α-pinene on TCE oxidation and provides insight into mixture effects on methanotroph activity. The concentration of 20 ppm TCE was chosen because it was not observed to be toxic to any of the methanotrophs tested previously (Fig. 1).

The responses to (R)-α-pinene were highly dependent on the type of methanotroph and MMO expression, with *M. trichosporium* OB3b showing decreased rates relative to 20 ppm TCE alone regardless of (R)-α-pinene concentration (Fig. 2, A, B) and *M. capsulatus* (Bath) and *M. album* BG8 showing mostly increased rates (Fig. 2, C, D, E). With the exception of *M. capsulatus* (Bath) under pMMO expression, the highest observed rates in the presence of the mixture were lower than those observed with (R)-α-pinene alone. *M. trichosporium* OB3b expressing pMMO showed consistently small decreases in oxygen uptake activity in the presence of the mixture compared to 20 ppm TCE alone; however, the activity of this strain when expressing sMMO appeared to be inhibited to a greater extent in the presence of all tested concentrations (2.5 to 20 ppm) of (R)-α-pinene in the mixtures (Fig. 2, A and B). Regardless of MMO expression, *M. capsulatus* (Bath) yielded increased normalized oxygen uptake rates in the presence of the mixture above approximately 20 ppm (R)-α-pinene relative to its observed rate at 20 ppm TCE alone. The greatest rate increase shown by *M. capsulatus* (Bath) expressing sMMO in the presence of the mixture was observed at 40 ppm (R)-α-pinene. This maximum rate observed with the mixture was 1.8 times the rate with 20 ppm TCE alone, suggesting a lessoning of toxicity effects on the cells. The maximum increase with this strain under pMMO expression was observed at 30 ppm (R)-α-pinene and was approximately 3.5 times higher than with 20 ppm TCE alone and 1.5 times higher than observed at 50 ppm (R)-α-pinene alone. Increase in oxidation potential of *M. album* BG8 was also observed when (R)-α-pinene was in the presence of 20 ppm TCE (Fig. 2, E). At the highest concentration of (R)-α-pinene tested (30 ppm) with this strain, the increase in normalized oxygen uptake rate was 1.8 times the rate observed with TCE alone.

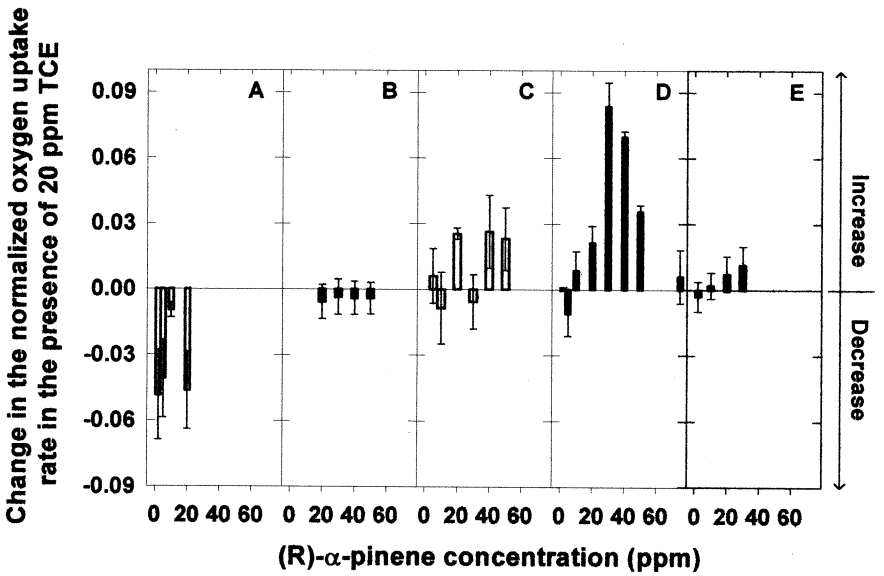

Figure 2. Change in the normalized oxygen uptake rate by representative methanotrophs observed in the presence of 20 ppm TCE at varying concentrations of (R)-α-pinene. **A, B**: *M. trichosporium* OB3b cultured without and with copper, respectively. **C, D**: *M. capsulatus* [Bath] cultured without and with copper, respectively. **E**: *M. album* BG8 cultured with copper. Error bars represent the standard deviation for triplicate samples.

In conclusion, all of the tested methanotrophs expressing either sMMO or pMMO were capable of oxidizing (R)-α-pinene over a range of environmentally relevant concentrations. However, toxicity effects of this monoterpene, similar to those shown with TCE, were observed. When both (R)-α-pinene and TCE were introduced to the representative methanotrophs, varying responses in the rates—decreases with the Type II methanotroph and increases with the Types I and X methanotrophs—were observed in comparison to those observed in the TCE-only experiments. Whether TCE and/or (R)-α-pinene were oxidized in the mixture is not known, given the indirect measurement method of oxygen uptake analysis; however, it is suggested here that the total oxidation potential of methanotrophs is affected, either antagonistically or synergistically, in the presence of TCE and (R)-α-pinene mixtures. These results emphasize the importance of not only assessing the concentration levels of both contaminants and monoterpenes and but also of measuring the oxidation potentials and diversity of rhizosphere methanotrophs at phytoremediation sites where plants that release large amounts of monoterpenes are being contemplated for use.

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