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Biodegradation Kinetics of 1,4-Dioxane in Chlorinated Solvent Mixtures

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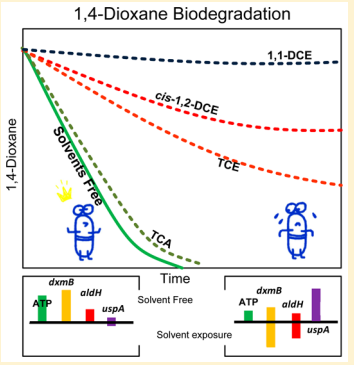
Supporting Information

ABSTRACT: This study investigated the impacts of individual chlorinated solvents and their mixtures on aerobic 1,4-dioxane biodegradation by *Pseudonocardia dioxanivorans* CB1190. The established association of these co-occurring compounds suggests important considerations for their respective biodegradation processes. Our kinetics and mechanistic studies demonstrated that individual solvents inhibited biodegradation of 1,4-dioxane in the following order: 1,1-dichloroethene (1,1-DCE) > *cis*-1,2-diochloroethene (cDCE) > trichloroethene (TCE) > 1,1,1-trichloroethane (TCA). The presence of 5 mg L^{-1} 1,1-DCE completely inhibited 1,4-dioxane biodegradation. Subsequently, we determined that 1,1-DCE was the strongest inhibitor of 1,4-dioxane biodegradation by bacterial pure cultures exposed to chlorinated solvent mixtures as well as in environmental samples collected from a site contaminated with chlorinated solvents and 1,4-dioxane. Inhibition of 1,4-dioxane biodegradation rates by chlorinated solvents was attributed to delayed ATP production and down-regulation of both 1,4-dioxane monooxygenase (*dxmB*) and aldehyde dehydrogenase (*aldH*) genes. Moreover, increasing concentrations of 1,1-DCE and *cis*-1,2-DCE to 50 mg L^{-1} respectively increased 5.0-fold and 3.5-fold the expression of the *uspA* gene encoding a universal stress protein. In situ natural attenuation or enhanced biodegradation of 1,4-dioxane is being considered for contaminated groundwater and industrial wastewater, so these results will have implications for selecting 1,4-dioxane bioremediation strategies at sites where chlorinated solvents are present as co-contaminants.



1. INTRODUCTION

Successful in situ bioremediation of contaminated aquifers is heavily influenced by site-specific conditions, such as microbial communities, dissolved oxygen, groundwater chemistry, and co-occurring contaminants.[1−3] These issues are increasingly important for emerging water contaminants, such as 1,4-dioxane, a probable human carcinogen.[4] While many laboratory-based studies focus on individual contaminants to determine the feasibility of a biodegradation approach, the complexity of contaminant mixtures and biogeochemical conditions might result in significantly different outcomes.[3,5−7] Groundwater contamination with 1,4-dioxane is a widespread problem, and affected aquifers are typically impacted with mixtures of multiple chlorinated solvents.[8−10] Although 1,4-dioxane was primarily used as a stabilizer of 1,1,1-trichloroethane (TCA), many facilities alternated between trichloroethylene (TCE) and TCA in response to a perceived lower toxicity for TCA or availability and pricing.[8] A recent study determined that co-occurrence of 1,4-dioxane and TCE accounted for 64% of 1,4-dioxane detected in monitoring wells,[11] further suggesting that mixed contamination is a potential challenge for 1,4-dioxane remediation. Importantly, the incomplete biological and abiotic degradation of these cocontaminating compounds can result in chlorinated solvent mixtures of TCE, 1,1-dichloroethene (1,1-DCE), *cis*-1,2-dichloroethene (cDCE), and vinyl chloride at contaminated sites.[12]

Chlorinated organic solvents are known to inhibit bacterial growth rates,[13−15] reduce membrane permeability,[15] and damage nucleic acids.[15] In studies using pure and mixed methanotrophic cultures, TCE exposure slowed both the metabolic and the cometabolic biotransformation rates of methane and dichloroethenes, respectively.[16,17] Chlorinated solvents have been reported to inactivate specific enzymes,[18] such as cytochrome P450 related monooxygenases.[19] For example, TCE was reported to decrease toluene-2-monooxygenase activities in *Burkholderia cepacia* G4[20] and inactivated the oxygenase in a methanotrophic culture.[16] These findings indicate chlorinated solvents may significantly impact the monooxygenase-driven degradation of 1,4-dioxane by aerobic bacteria. Several studies with Gram positive bacteria have shown that a large number of stress response proteins are produced under conditions that compromise the cell membrane,[21] such as solvent exposure. The *uspA* gene encodes a universal stress protein, which is induced during stationary-phase growth, starvation, and exposure to a wide range of stimuli including heat, oxidants, metals, and antibiotics.[22]

Pseudonocardia dioxanivorans CB1190 (hereafter "CB1190") is a model 1,4-dioxane-degrading bacterium that has previously

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been shown to grow on a wide range of carbon sources and rapidly degrade 1,4-dioxane at rates ranging from 1.1 to 19.8 mg min^{-1} mg^{-1} protein[23,24] (Table S1). It was determined, using multiple lines of evidence, that a monooxygenase enzyme (DXMO) was responsible for catalyzing the initial biodegradation of 1,4-dioxane, while aldehyde dehydrogenase (ALDH) degraded intermediates in the biodegradation pathway.[25,26] Whole-genome sequencing of CB1190[27] revealed genes encoding 1,4-dioxane monooxygenase (*dxmADBC*) and aldehyde dehydrogenase (*aldH*), some of which have been used as biomarkers to predict the biotransformation potential in samples collected from contaminated sites.[28−31]

The objectives of this study were to provide a quantitative analysis of 1,4-dioxane biodegradation kinetics in mixtures with relevant chlorinated solvents (TCE and TCA) and their degradation products (1,1-DCE and cDCE). We also determined the relationship between solvent exposure, cellular energy, and expression of genes coding for stress response and key reactions in the biochemical pathway to better understand mechanisms influencing 1,4-dioxane biodegradation kinetics in mixed-contaminant environments.

2. MATERIALS AND METHODS

Chemicals. 1,4-Dioxane (99.8%, ACS grade), 1,1-DCE (≥99.9%), cDCE (97%), and TCE (≥99.5%, ACS grade) were purchased from Sigma-Aldrich. 1,1,1-TCA (>95%) was obtained from Ultra Scientific (North Kingstown, RI). Saturated chlorinated solvent stock solutions were prepared as previously described.[16]

Bacterial Strain and Culture Conditions. CB1190 was harvested with a 1% (v/v) transfer from an actively growing pure culture and incubated at 30 °C with 150 rpm agitation to maintain aerobic conditions in the ammonium mineral salts (AMS) medium[23] amended with 1,4-dioxane. Cultures (late-exponential- or early-stationary-phase) were used to inoculate the experimental bottles when 1,4-dioxane in the stock fell below the detection limit (<1.0 mg L^{-1}).

Biodegradation of 1,4-Dioxane in the Presence of Individual Chlorinated Solvents. For examination of the effects of each chlorinated solvent on 1,4-dioxane biodegradation, each 120 mL serum bottle contained 30 mL of active CB1190 culture with 0.5 to 100 mg L^{-1} 1,4-dioxane and 1,1-DCE, cDCE, TCE, or TCA at final concentrations of 0.5, 5, or 50 mg L^{-1}. 1,4-Dioxane activity (eq 1) and biodegradation rates (eq 2) were calculated as follows:

$$\text{observed activity } (\text{mg L}^{-1}\text{hr}^{-1}) = \frac{C_{\text{Initial}} - C_{\text{Final}}}{\Delta t} \quad (1)$$

$$\text{biodegradation rate } (\text{mg}_{\text{dioxane}}\text{hr}^{-1}\text{mg}^{-1}_{\text{protein}}) = \frac{C_{\text{Initial}} - C_{\text{Final}}}{X_{\text{protein}} \times \Delta t} \quad (2)$$

where C_{Initial} and C_{Final} were the initial and final concentrations of 1,4-dioxane (mg L^{-1}), respectively. X was the biomass measured as total protein (mg L^{-1}), and Δt was time (h). In solvent-free controls, 1,4-dioxane biodegradation followed first-order kinetics in the first 8 h; thus, we use $\Delta t = 8$ for these calculations.

The maximum specific rate of 1,4-dioxane degradation (V_{max}), 1,4-dioxane half saturation coefficient (K_s), and inhibition constants (K_I) were determined by fitting the equation $v_0 = \frac{V_{max} \times C_{Initial}}{K_s + C_{Initial}}$ to match the observed nonvolatile 1,4-dioxane concentrations using the Prism version 6.0 (Graphpad Software, Inc., San Diego, CA) as previously described.[5]

Microcosm Study. A total of 8 L of groundwater was collected from a monitoring well, and 2.26 kg of soil was collected by direct-push sampling near the monitoring well. The well was located in the source zone of a contaminated groundwater plume containing >700 μg L^{-1} 1,4-dioxane and chlorinated solvents ranging from 0−1000 μg L^{-1}. Microcosms were prepared in sterile 250 mL Boston round bottles equipped with Mininert valves (Restek Corporation, Bellefonte, PA) and designed according to the following conditions: (1) "Untreated Control" (natural attenuation; (+) solvents; (2) "Heat Sterilized Control"; (3) natural attenuation after flushing to remove solvents ("Natural attenuation; (−) Solvents"); (4) bioaugmention with CB1190 ("Bioaugmented CB1190; (+) Solvents"); and (5) bioaugmention with CB1190 without solvents ("Bioaugmented CB1190; (−) Solvents"). For conditions 1−3, 50 mL of groundwater was transferred into each bottle. Bioaugmented conditions (4 and 5) received 25 mL of groundwater from each location, 20 mL of ammonium mineral salts (AMS) medium, and 5 mL of CB1190 cultured in AMS medium to a final cell concentration of approximately 1.0×10^4 cells mL^{-1}. The addition of AMS was performed to provide the necessary nutrients for CB1190, as described in a previous microcosm study.[32] All microcosms received 10 g of soil, and all (−) solvent conditions were flushed with N_2 gas to remove volatile solvents from solution by cycling gas for 15 min, followed by 15 min of rest for a total of five cycles.

Heat-sterilized controls were prepared by autoclaving microcosm bottles for 30 min at 121 °C and 15 psi. All microcosms were prepared in triplicates with the exception of a single sterile control for each location. Microcosms were incubated at 30 °C with 150 rpm agitation. At each time point, aliquots of 200 μL were collected for 1,4-dioxane quantification, and 300 μL samples of the soil and groundwater slurry from each microcosm were collected and stored at −80 °C for the extraction of total nucleic acids.

Dissolved Oxygen, Oxidation−Reduction Potential, and pH Measurements. Dissolved oxygen (DO), oxidation−reduction potential (ORP), and pH values were measured using an Orion 5-Star Plus multiparameter meter (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions in samples collected at the beginning and end of microcosm incubations (Table S2).

Biodegradation of 1,4-Dioxane in Solvent Mixtures. To rank the relative importance of inhibitory solvents on 1,4-dioxane biodegradation in environmental mixtures, the impact of solvent mixtures in CB1190 culture was evaluated using similar concentrations of chlorinated solvents, as determined in the microcosm study described above (Table S3). The following chlorinated solvent mixtures were studied for their combined effects on the biodegradation of 1 mg L^{-1} 1,4-dioxane. Mixture 1 contained 5000 μg L^{-1} 1,1-DCE, 500 μg L^{-1} cDCE, 700 μg L^{-1} TCE and 120 μg L^{-1} TCA, while mixture 2 had 500 μg L^{-1} cDCE, 700 μg L^{-1} TCE, and 120 μg L^{-1} TCA, and mixture 3 contained only 700 μg L^{-1} TCE and 120 μg L^{-1} TCA. This approach was used to determine chlorinated solvent(s) with the greatest impact on 1,4-dioxane metabolism. The aqueous concentrations of chlorinated volatile organic compounds was calculated based on Henry's Law constants.[33] At every time point (0, 2, 4, 6, 8, 10, 12, 14, 16, and 24 h), 200 μL aqueous samples were collected for 1,4-dioxane analysis by using a 1 mL syringe equipped with a sterile needle (23 gauge, 0.64 mm o.d., 1.5 in.

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length) and stored at −20 °C; 500 μL samples were collected and stored at −80 °C for total nucleic acids extraction. Chlorinated solvent concentrations were quantified from 100 μL headspace samples collected from each bottle at 0, 1, 7, and 10 days using a gas chromatograph equipped with a flame ionization detector (GC−FID) or an electron capture detector (GC−ECD).

Analytical Methods. 1,4-Dioxane (1−2000 μg L^{-1}) was monitored using an Agilent 6890 chromatograph equipped with a 5973 mass spectrometer (GC−MS) and a Supelco SPB-1 Sulfur column (30 m × 0.32 mm id ×4 μm). The collected aqueous sample was prepared for GC−MS analysis using a frozen microextraction procedure as previously described,[34] and 5 μL of processed sample was injected into the GC−MS equipped with a pulsed split-less injection with an inlet temperature of 150 °C, 77 kPa, a pulsed pressure of 170 kPa for 2 min, and a purge flow of 15.0 mL min^{-1}. The oven temperature was initially held constant at 35 °C for 5 min, then increased to 100 °C at a rate of 20 °C min^{-1}, and further increased to 275 °C at a rate of 50 °C min^{-1}. The mass-selective detector (MSD) was operated to use an electron multiplier (EM) offset of 400 and EM voltage of 2000. The MS quadrupole was programmed for 150 °C with a source for 230 °C. 1,4-Dioxane (1−100 mg L^{-1}) was monitored using an Agilent 6890 GC−FID as previously described.[7]

TCE, TCA, 1,1-DCE, and cDCE were quantified by a GC−FID equipped with a Restek Stabilwax-DB capillary column (30 m × 0.53 mm × 1 μm) for experiments investigating the influence of individual chlorinated solvents. The injector and detector temperatures were set at 220 °C with a 7.4 mL min^{-1} flow rate and 250 °C, respectively. The oven was initially set at 100 °C for 1 min then increased at 25 °C min^{-1} to 150 °C and maintained for 30 s.

In experiments with solvent mixtures, a Varian 3500 capillary gas chromatograph equipped with an ECD was used to quantify 100−5000 μg L^{-1} TCE, TCA, and 1,1-DCE with an Agilent DB-5 capillary column (30 m × 0.25 mm × 0.25 μm). Headspace samples of 50 μL were injected into the injector set at 260 °C, whereas the detector was set at 310 °C. The oven temperature was 35 °C and held for 30 min with 9.6 mL min^{-1} of column flow rate. Concentrations of cDCE from 500 to 5000 μg L^{-1} were measured by GC−FID with a constant oven temperature at 130 °C for 7 min. The flow rate was 1.7 mL min^{-1}, and other parameters were the same as those described for individual solvents.

ATP Assay for Energy Generation. ATP production was measured in 100 μL of cell culture using the BacTiler Glo assay (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, 50 μL of sample was transferred to a 96 well plate and mixed with 50 μL of BacTiter Glo reagent. Samples were incubated in the dark for 5 min at room temperature and luminescence measurements were recorded using the VICTOR 3 V plate reader (PerkinElmer, Waltham, MA).

Total Nucleic Acids Extraction and cDNA Synthesis. Total nucleic acids were extracted from samples using a modified phenol-chloroform extraction method as described previously.[28] Briefly, 500 μL of cell cultures was centrifuged at 13000*g* for 3 min, and the supernatant was discarded. The cells were lysed by adding 250 μL of lysis buffer (50 mM sodium acetate, 10 mM EDTA [pH 5.1]), 100 μL 10% sodium dodecyl sulfate, 1.0 mL pH 8.0 buffer-equilibrated phenol, and 1 g of 100 μm-diameter zirconia−silica beads (Biospec Products, Bartlesville, OK), followed by heating at 65 °C for 2 min, bead beating for 2 min with a Mini-Beadbeater 16 (Biospec Products, Bartlesville, OK), incubating for 8 min at 65 °C, and bead beating again for 2 min. The lysate was collected by centrifugation at 13000*g* for 5 min, followed by phenol−chloroform−isoamyl alcohol purification and chloroform−isoamyl alcohol purification. Precipitation of total nucleic acids was performed by addition of 0.1 volume of 3 M sodium acetate and 1 volume of isopropanol followed by incubation at −20 °C overnight. Nucleic acid pellets were collected by centrifugation at 4 °C for 30 min at 20000*g*. The precipitate was washed with 70% ethanol and resuspended in 100 μL of DNase- and RNase-free water. The concentrations of DNA and RNA were determined by a Nanodrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE). For gene expression analyses, RNA was isolated from total nucleic acid extracts using a RapidOUT DNase Kit (Thermo Scientific, Waltham, MA). The cDNA was synthesized from purified total RNA using a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA). All samples were stored at −80 °C until further amplification and analyses.

Quantitative Polymerase Chain Reaction. To study the influence of chlorinated solvents on gene expression in pure cultures, 1,4-dioxane biomarker targets (*dxmB* and *aldH*) were selected because of the significance of monooxygenase and dehydrogenase enzymes in the 1,4-dioxane biodegradation pathways as described previously.[28] Universal stress gene (*usp*A) was also selected to investigate the response of CB1190 cells exposed to chlorinated solvents.[35] Gene expression was quantified using the $2^{-\Delta\Delta CT}$ method, as described by Livak and Schmittgen.[36] Gene expression data were first normalized to the housekeeping gene, RNA polymerase σ subunit D (*rpoD*), followed by normalization to the values obtained at time 0. All reactions were run on a StepOnePlus thermocycler (Life Technologies, Carlsbad, CA) using a total volume of 20 μL containing 1× Luminaris Color HiGreen−HiROX qPCR Master Mix (Thermo Scientific, Waltham, MA), 0.3 mM primers, and 2 μL of DNA (1−10 ng/μL) template. Primer sequences are listed in Table S4, and additional details following MIQE reporting requirements can be found in Table S5. The cycling parameters to amplify the gene fragment included sample holds at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. All reactions were accompanied by a melt-curve analysis to confirm the specificity of quantitative polymerase chain reaction (qPCR) products. Melt-curve analyses within 78.1−80.5 °C (*rpod*), 81.5−83.6 °C (*dxmB, aldH,* and 16S rRNA), and 86.9−88.7 °C (*uspA*) were considered specific to target genes.

3. RESULTS

Kinetics of 1,4-Dioxane Biodegradation by CB1190 Exposed to Chlorinated Solvents. TCE, TCA, 1,1-DCE, and cDCE were individually examined for their potential inhibitory effects on 1,4-dioxane biodegradation (Figure 1). In solvent-free controls, CB1190 (32.1 ± 4.2 mg protein) biodegraded 1 mg L^{-1} 1,4-dioxane to below detection limits in 8 h at a rate (mean ± SD) of 100.3 ± 1.0 μg L^{-1} h^{-1}, which was similar to rates observed in the presence of 0.5 mg L^{-1} TCE and 0.5 mg L^{-1} cDCE, 95.4 ± 1.4 μg L^{-1} h^{-1} ($p = 0.26$) and 93.9 ± 8.2 μg L^{-1} h^{-1} ($p = 0.23$), respectively. Comparatively, TCA at concentrations of 0.5 mg L^{-1} ($p = 0.37$), 5 mg L^{-1} ($p = 0.34$), and 50 mg L^{-1} ($p = 0.41$) had little effect on 1,4-dioxane biodegradation by CB1190 (37.8 mg of protein). At 50 mg L^{-1} TCE, 1,4-dioxane biodegradation rate decreased to 35.3 ± 8.5 μg L^{-1} h^{-1}. cDCE had a stronger inhibitory effect at 5 mg L^{-1} and completely inhibited 1,4-dioxane biodegradation at 50 mg L^{-1}. In 1,1-DCE exposed cultures, 1,4-dioxane was degraded in the presence of

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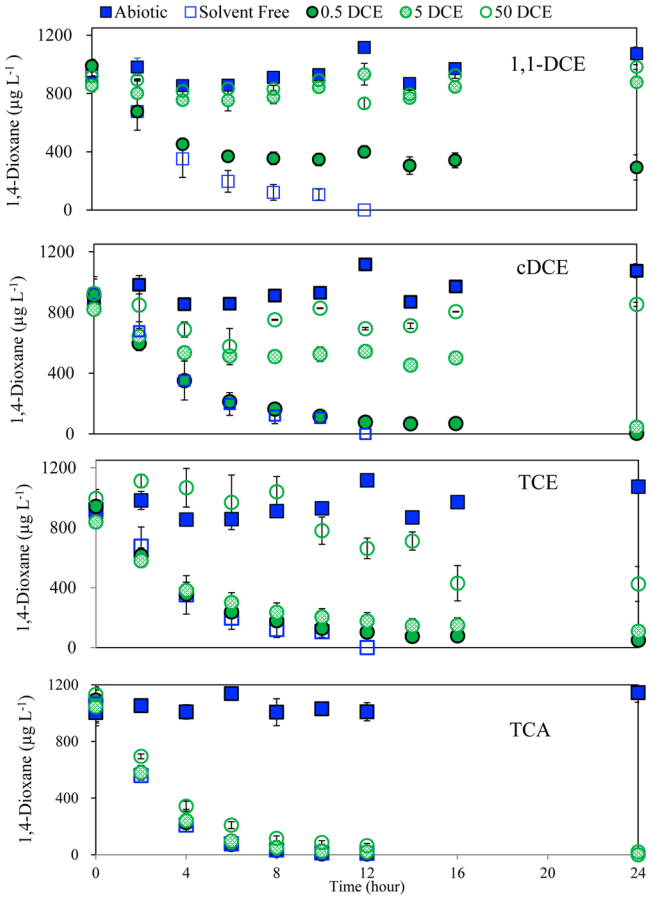

Figure 1. Inhibition of 1,4-dioxane biodegradation by chlorinated solvents. *P. dioxanivorans* CB1190 was exposed to final concentrations of 1 mg L^{-1} 1,4-dioxane and 0.5, 5, or 50 mg L^{-1} (A) TCA; (B) TCE; (C) cDCE; and (D) 1,1-DCE individually. Error bars represent standard deviation of triplicates.

low 1,1-DCE concentrations at the rate of 79.4 ± 3.7 μg L^{-1} h^{-1} in the first 12 h, but 1,4-dioxane degradation ceased in the following 12 h. Thus, 1,1-DCE was the strongest inhibitor of 1,4-dioxane biodegradation among tested chlorinated solvents. The inhibition constants (K_I, mg L^{-1}) for 1,1-DCE, cDCE, and TCE were determined as 1.51 ± 0.26, 26.63 ± 6.63, and 8.60 ± 1.74 by the Michaelis–Menten model, respectively (Table S6). The most appropriate model was selected on the basis of fit (R^2; coefficient of determination) and the Akaike information criterion (AIC). AIC uses estimates of goodness-of-fit and model variability to provide a quantitative ranking for different models in their abilities to describe each data set. This approach has been used in several studies to identify best-fit models.[37,38] According to R^2 values and Akaike's information criterion,[39] the uncompetitive model was the best fit for 1,1-DCE and TCE, and the noncompetitive model was the most suitable for cDCE. As observed earlier, the presence of TCA had little impact on 1,4-dioxane biodegradation, so the K_I could not be determined.

Hindering of Energy Production in CB1190 by Chlorinated Solvents. The ability of CB1190 to grow using 1,4-dioxane as the sole energy and carbon source was previously reported.[24] ATP content was measured in actively growing CB1190 cultures exposed to cDCE and 1,1-DCE amended with 100 mg L^{-1} 1,4-dioxane and 0.5 mg L^{-1} 1,1-DCE or 5 mg L^{-1} cDCE. ATP generation correlated with 1,4-dioxane oxidation (R^2 = 0.91; p < 0.01) and the total ATP yields were similar in the solvent-free control and 1,1-DCE-, and cDCE-exposed cultures (4.7–5.9 mol-ATP mol-1,4-dioxane^{-1}) (Figure 2). However, maximum ATP yield was delayed by 4 days in both solvent-exposed conditions compared to the unexposed controls (Figure 2).

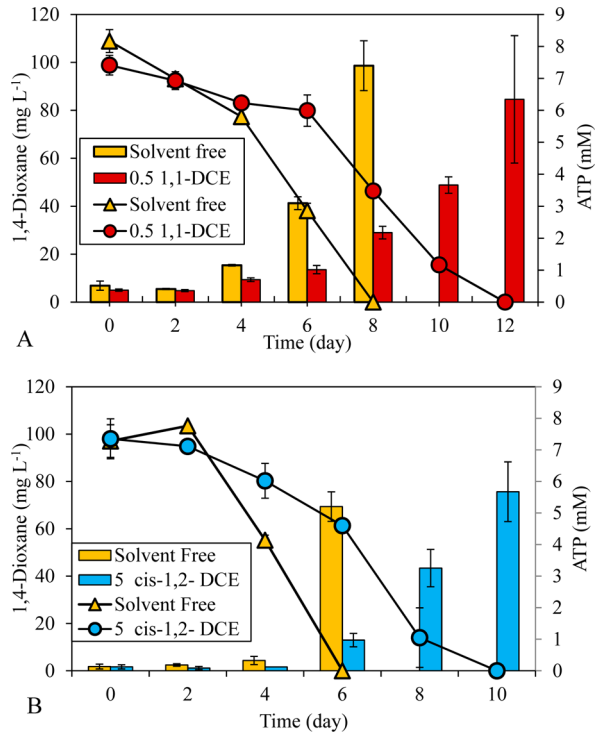

Figure 2. Effect of chlorinated solvents on energy production in CB1190. Actively growing *P. dioxanivorans* CB1190 culture (1% transfer of active seed culture) amended with 100 mg L^{-1} 1,4-dioxane (lines) was exposed to 0.5 mg L^{-1} 1,1-DCE (A) and 5 mg L^{-1} cDCE (B). The ATP production (bars) was delayed in the presence of both chlorinated solvents. 1,4-Dioxane (1–100 mg L^{-1}) was monitored using an Agilent 6890 GC–FID as previous study described.[7] Error bars represent ranges of duplicates.

Solvent Inhibition of 1,4-Dioxane Biodegradation by CB1190 in Environmental Mixtures. In aerobic microcosms prepared using groundwater and soil from a site contaminated with 1,4-dioxane and chlorinated solvents, no 1,4-dioxane removal occurred in 10 weeks in untreated bottles (Figure 3A). This suggested that the indigenous microorganisms had limited potential to biodegrade 1,4-dioxane. Even when volatile chlorinated solvents were removed by flushing, 1,4-dioxane biodegradation was not observed. However, bioaugmentation with CB1190 successfully removed 1,4-dioxane within 7 days in flushed bottles. The presence of chlorinated solvents slowed 1,4-dioxane biodegradation in CB1190 amended microcosms to a rate of 6.65 μg L^{-1} day^{-1} over 56 days, which confirmed the inhibitory effects of chlorinated solvents.

1,1-DCE as the Major Inhibitor of 1,4-Dioxane Biodegradation among Individual Chlorinated Solvents and in Chlorinated Solvent Mixtures. Synthetic mixtures of chlorinated solvents were designed to investigate the inhibitory effects caused by mixtures of chlorinated solvents in the environment and to help interpret the results from the microcosms. The concentrations of solvents (TCE, TCA, 1,1-DCE, and cDCE) and 1,4-dioxane were selected to be similar to concentrations found in groundwater at the site used for the

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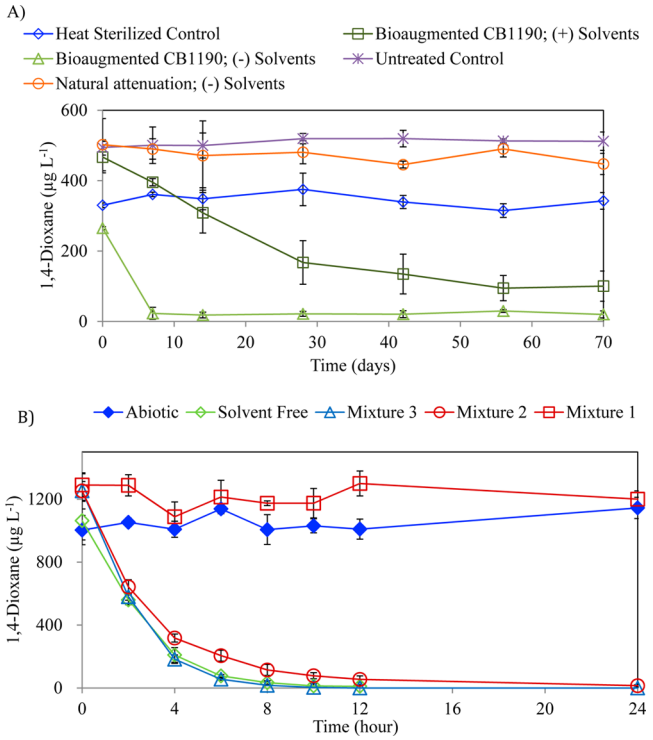

Figure 3. 1,4-Dioxane biodegradation in the presence of chlorinated solvent mixtures. (A). Microcosm study: "Untreated Control" (natural attenuation; (+) solvents); "Heat-Sterilized Control"; natural attenuation after flushing to remove solvents ("Natural attenuation; (−) Solvents"); bioaugmention with CB1190 ("Bioaugmented CB1190; (+) Solvents"); bioaugmention with CB1190 without solvents ("Bioaugmented CB1190; (−) Solvents"). (B). Pure culture study: Mixture 1 contained 5000 μg L^{-1} 1,1-DCE, 700 μg L^{-1} TCE, 450 μg L^{-1} cis-1,2-DCE, and 120 μg L^{-1} TCA; Mixture 2 contained 700 μg L^{-1} TCE, 450 μg L^{-1} cis-1,2-DCE, and 120 μg L^{-1} TCA; Mixture 3 contained 450 μg L^{-1} cis-1,2-DCE and 120 μg L^{-1} TCA. Error bars represent standard deviation of triplicates.

microcosm study. 1,4-Dioxane biodegradation was completely inhibited in Mixture 1, containing approximately 5 mg L^{-1} 1,1-DCE, but was successful in Mixtures 2 and 3 (Figure 3B). These results agreed with our results from individual chlorinated solvents demonstrating that 5 mg L^{-1} 1,1-DCE was the strongest inhibitor of 1,4-dioxane by CB1190 (Figure 1).

Down-regulation of Dioxane Monooxygenase (*dxmB*) and Aldehyde Dehydrogenase (*aldH*) genes in the Presence of Chlorinated Solvents. Gene abundance (Figures S1 and S2) and expression were quantified in studies of individual chlorinated solvents as well as solvent mixtures. CB1190 exposed to 5 mg L^{-1} TCE or cDCE showed up-regulation of *dxmB* compared to the initial levels in the first 2 h, during which 1,4-dioxane was being degraded at a high rate (Figure 4). When the cells were exposed to 50 mg L^{-1} TCE, insignificant 1,4-dioxane degradation occurred in the first 12 h, but *dxmB* remained induced by 1,4-dioxane (Figures 1 and S3), while *aldH* expression was low due to lack of formation of 1,4-dioxane degradation products containing aldehyde functional groups.[28] In contrast to TCE and cDCE, lower concentrations of TCA did not inhibit 1,4-dioxane degradation by CB1190, which resulted in *dxmB* and *aldH* fluctuating with no significant difference in expression comparing to solvent-free control (Figures S3 and 4). Similar expression levels occurred in the presence of 50 mg L^{-1} TCA, at which 1,4-dioxane degradation rate was only marginally lower than that of the solvent-free control (Figure S3). However, at all concentrations of 1,1-DCE tested, *dxmB* and *aldH* were repressed about 6-fold at 6 h, and no further changes were measured in the following 6 h (Figure S3). In chlorinated solvent Mixture 1, the presence of 5 mg L^{-1} 1,1-DCE resulted in decreased expression of both *dxmB* and *aldH* (Figure S4), similar to that observed when 1,1-DCE was added individually (Figure 4).This result suggests that 1,1-DCE repressed the transcription of *dxmB* and *aldH* genes.

Dose-Dependent Universal Stress Protein Gene (*uspA*) Induction by 1,1-DCE and cDCE. The regulation of *uspA* gene was reported to be an important response of bacteria during growth arrest,[22] and it was induced by stress to protect the cells from nutrient depletion or accumulation of toxic chemicals.[40] The expression of *uspA* in CB1190 was examined in the presence of individual chlorinated solvents and mixtures (Figures 5 and S4). Exposure to TCE or TCA did not contribute to the *uspA* induction, which means *uspA* was not sensitive to TCE and TCA. Similarly, when low concentrations of cDCE were present in the culture, little influence on *uspA* expression was observed. However, *uspA* was significantly overexpressed approximately 5.0-fold and 3.5-fold when 1,1-DCE or cDCE concentrations were increased up to 50 mg L^{-1}, respectively. Interestingly, *uspA* transcription increased when approximately 50% 1,4-dioxane remained in Mixture 1. In Mixtures 2 and 3, *uspA* induction in CB1190 was limited without 1,1-DCE, even though 0.5 mg L^{-1} cDCE and low concentrations of TCE and TCA were present (Figure S4).

4. DISCUSSION

The inhibitory effects of individual chlorinated solvents on 1,4-dioxane biodegradation have been previously identified, e.g., the presence of TCE incurred incomplete 1,4-dioxane cometabolism in JOB5 and *Rhodococcus jostii* RHA1 cultures,[6] and the presence of 1,1-DCE reduced monooxygenase activity by 80% for *Rhodococcus erythropolis*.[41] Comparatively, this study determined that solvents inhibited biodegradation of 1,4-dioxane in the following order: 1,1-DCE > cDCE > TCE > TCA (Figure 1). However, Mahendra and co-workers examined the effects of pre-exposing CB1190 to individual chlorinated solvents prior to 1,4-dioxane biodegradation and reported stronger inhibition for TCA according to K_I values of 1.2 ± 1.0 μM and 3.3 ± 2.9 μM for TCA and 1,1-DCE, respectively.[5] Our study differs from that of Mahendra et al.[5] by focusing on 1,4-dioxane biodegradation inhibition in the presence of each chlorinated solvent as well as in mixtures and environmental samples (Table S6). The present approach adds improved representation of mixed contamination in groundwater, and the results provide a better understanding of chlorinated solvent influences on bioaugmentation strategies in co-contaminated environments. These studies differed significantly in exposure conditions and activity related to low substrate concentrations, indicating that cell density, growth phase,[14] and the activity of cells[42] vary the tolerance of bacteria against chlorinated solvents. Our study reports an inhibition constant, K_I, which was calculated according to initial biodegradation rates. However, this approach proved unreliable in determining overall solvent impacts, such as universal stress, energy depletion, transcriptional regulation, product toxicity, or any combination of these impacts. For example, K_I values from our study suggested a stronger inhibitory effect of TCE over cDCE on 1,4-dioxane biodegradation (Table S6). However, continuous 1,4-dioxane biodegradation still occurred in the presence of TCE,

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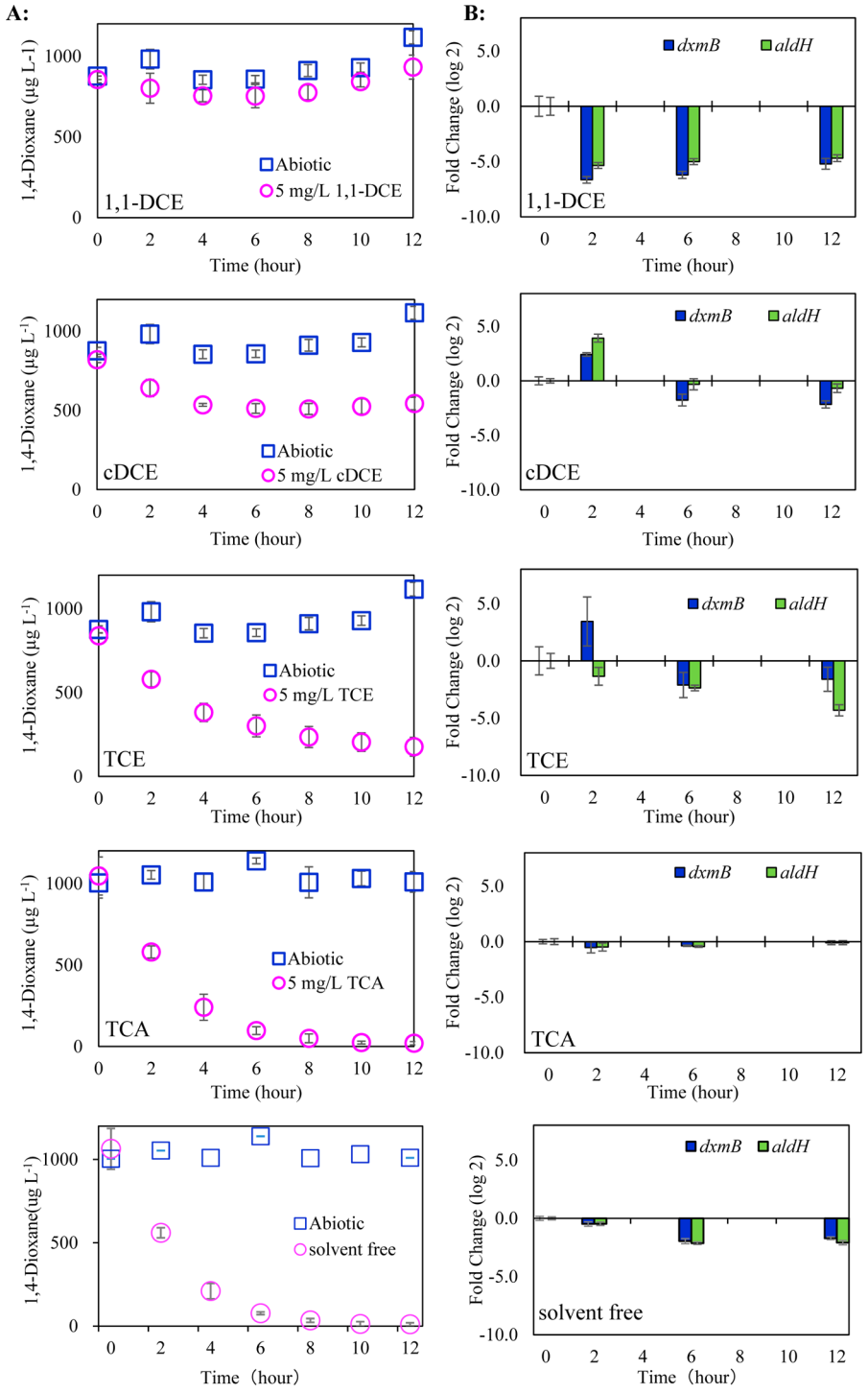

Figure 4. 1,4-Dioxane degradation and expression of relevant genes in CB1190 exposed to individual chlorinated solvents. (A) Kinetics of 1000 μg L^{-1} 1,4-dioxane biodegradation in the presence of 5 mg L^{-1} individual solvent (1,1-DCE, cis-1,2-DCE, TCE, and TCA). This figure was extracted from Figure 1 for comparison with the gene regulation figure. (B) Fold change in transcript copy numbers of dioxane monooxygeanse (*dxmB*) and aldehyde monooxygenase (*aldH*) at 0, 2, 6, and 12 h. CB1190 culture with 1000 μg L^{-1} 1,4-dioxane was exposed to 5 mg L^{-1} of solvents (1,1-DCE, cis-1,2-DCE, TCE, or TCA). The circles represent 1,4-dioxane concentrations.

while the degradation ceased after 6 h in the presence of cDCE (Figure 1).

Despite a higher degree of halogenation, TCE and TCA imparted less inhibition toward 1,4-dioxane biodegradation than dichloro-substituted ethenes. Similar results were reported in a mammalian toxicity study[43] as well as a bacterial cometabolism study[18] and were attributed to the chemical structures of the solvents that affect electron density distribution, polarity, and hydrophobicity of the molecules.[41] For example, more hydrophilic and polar solvents like 1,1-DCE and cDCE with lower log K_{ow} values (Table S2) may abundantly bind to cell membranes.[9,44] They can change the cell membrane composition, resulting in leakage of intracellular metabolites or forming channels for solvents to attack intracellular components.[14,45] The

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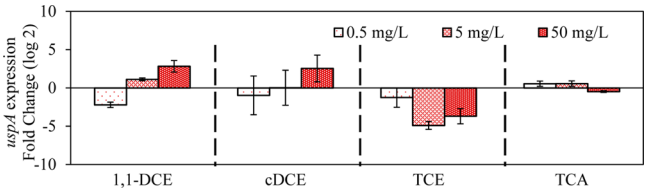

Figure 5. Dose-dependent *uspA* gene expression at 6 h in the presence of 0.5, 5, or 50 mg L^{-1} individual chlorinated solvents. All cDNA copy numbers were first normalized to *rpoD* housekeeping gene and then to solvent-free control. Error bars represent standard deviation of triplicates.

unsaturated carbon−carbon bond, isomerization, and number of chlorine atoms further determined the chemical interaction between each chlorinated solvent and macromolecules, such as monooxygenases.[46] In addition, carbon−carbon single bonds (1.54 Å) have longer interatomic distance than carbon−carbon double bonds (1.39 Å), which corresponded to the reduced reactivity of the molecule.[47,48] Thus, TCA has fewer cellular interactions than TCE, cDCE, and 1,1-DCE, as observed in the present study. Furthermore, three chlorine atoms attached to the carbon−carbon double bond in ethenes balanced resonance better than fewer chlorines attached to the unsaturated double bonds. Even in the abiotic oxidation of chlorinated solvents by potassium permanganate, the ease of attack was determined to be in the following order 1,1-DCE > cDCE > TCE > PCE, suggesting that 1,1-DCE has high reactivity.[49]

Previous studies of 1,4-dioxane biodegradation pathways have revealed that monooxygenases catalyze the initial steps of 1,4-dioxane biodegradation in both metabolic and cometabolic processes.[25,26] Because these multicomponent enzymes are membrane-bound in many bacteria,[28,50] their activity is susceptible to changes in structural conformation, covalent binding by inhibitors, and collapse in membrane potential.[51] A prior study of chlorinated solvent cometabolism by nitrifying bacteria demonstrated that 1,1-DCE, TCE, and 1,2-dichloroethane were able to inactivate enzymes.[51] It was also reported that 1,1-DCE had stronger inhibition effects than TCE and cDCE on nitrifying bacteria,[51] as was observed in our study. Furthermore, reactive epoxide formation was suggested as one of the possible causes to pose stronger inhibitory effects.[41] However, no significant loss of chlorinated solvents in 24 h was observed in our study, which indicated inhibition was not associated with product toxicity (Figure S5). Additionally, propidium monoazide−qPCR (PMA−qPCR) determined that exposure to cDCE, TCE, or TCA did not impact the abundance of cells with intact membranes compared to the solvent-free controls. Cells exposed to 1,1-DCE had a higher fraction of qPCR amplifiable cell numbers and were significantly different ($p < 0.01$) than the solvent-free control (Figure S6). This finding suggests that 1,1-DCE may bind or even damage nucleic acids, preventing PMA treatment. Previous studies determined that TCE and 1,1-DCE were able to inflict DNA damage in mammalian cells[52] and impacted gene transcription.[53] Those results are consistent with the observations in this study that *dxmB* and *aldH* were markedly suppressed when CB1190 was exposed to 5 or 50 mg L^{-1} solvents, especially 1,1-DCE. Because cell membranes are also associated with energy generation and consumption, this study determined that the ATP production rate was related to 1,4-dioxane degradation rate and that the total ATP yield was not affected by solvents. Taken together, these findings suggest that cell membrane damage does not explain inhibition at a 5 mg L^{-1} solvent concentration.

Exposure to chlorinated solvents influenced the expression of 1,4-dioxane biomarker genes (Figure 4). Expression of *dxmB* and *aldH* was down-regulated in the presence of 1,1-DCE, suggesting potential interactions between 1,1-DCE and the regulatory network of the *dxm* genes. Specifically, 1,1-DCE may bind to the *dxmB* promoter or activator regions in the nucleic acid sequence, reducing the affinity of RNA polymerase.[53] Studies on the toluene-3-monooxygenase of *Burkholderia pickettii* determined that the expression of this operon is linked to the expression of the activator TbuT.[54] Decreased concentrations of *dxmB* transcripts might result from interactions of 1,1-DCE with the CB1190 two-component signaling system[55] or repression following passive or active transport of 1,1-DCE through the cell membrane.[56,57] Our gene expression results suggest that further research into the regulation of this enzyme will help elucidate key mechanisms of solvent inhibition for bacterial multicomponent monooxygenases.

Chlorinated-solvent-induced stress on Gram positive bacteria has been linked to general stress response proteins.[44] We targeted the *uspA* gene encoding a universal stress protein to provide insights into the cellular response to chlorinated solvents. This protein is an important bacterial response against global cellular stress, and induction of *uspA* occurs in response to a wide range of environmental stimuli, including a transition into stationary-phase growth.[22,58] Our study determined that TCE exposure, TCA exposure, or both caused little stress on CB1190 cells, resulting in the down-regulation of *uspA* in a dose-dependent manner (Figure 5) and biodegradation of 1,4-dioxane was able to proceed (Figure 1). In contrast, concentrations of 50 mg L^{-1} cDCE and 1,1-DCE concentrations of >5 mg L^{-1} induced *uspA,* indicating a cellular stress response related to inhibition of 1,4-dioxane biodegradation (Figures 1 and 5). In other studies, cell membrane permeabilization,[15] transport activity,[15,59] and protein production[44] were also influenced by organic solvents. The regulation of *uspA* is an important new insight into the mechanism of inhibition among various organic solvents and valuable in evaluating the potential of bioremediation of various contaminants in the field. In pure cultures exposed to synthetic solvent mixtures, the total stress from all solvents resulted in the significant down-regulation of *dxmB* and *aldH* and up-regulation of *usp*A in Mixture 1 (Figure S4). Furthermore, our results determined that upregulation of *uspA* may prove useful in co-contaminated environments for predicting the potential of 1,4-dioxane biodegradation.

In conclusion, individual chlorinated solvents and their mixtures imposed time- and dose-dependent inhibition of 1,4-dioxane biodegradation via energy depletion and the down-regulation of genes coding for biodegradative enzymes. This study showed that chlorinated-solvent inhibition of 1,4-dioxane metabolism was not significantly related to the destruction of the cell membrane or formation of toxic by-products. Bioaugmentation successfully removed the 1,4-dioxane in the contaminated environmental samples, and low concentrations of TCA, TCE, and cDCE did not have significant effects on the metabolic transformation of 1,4-dioxane by CB1190, implying advantages for implementing metabolic biodegradation in certain co-contaminated aquifers. Therefore, aerobic bioremediation should be considered as a viable candidate strategy for 1,4-dioxane and chlorinated-solvent-contaminated groundwater.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b02797.

Details of quantification of cells with intact membranes and PMA-qPCR. Tables showing growth and 1,4-dioxane biodegradation kinetics parameters for *P. dioxanivorans* CB1190; geochemical parameters measured in the groundwater used to prepare microcosms; quantification of chlorinated solvents in synthetic mixtures at 0 and 24 hours; sequences of primers used for amplification of genes by qPCR; minimum information for publication of real-time PCR experiments; and kinetic parameters for 1,4-dioxane degradation in the presence of 1,1-DCE, cDCE, TCE, and TCA. Figures showing CB1190 population in the presence of 1 mg L^{-1} 1,4-dioxane and individual chlorinated solvents, CB1190 population in the presence of 1 mg L^{-1} 1,4-dioxane and chlorinated solvent mixtures, dose-dependent gene regulation in CB1190 pure culture exposed to chlorinated solvents, gene expression in *P. dioxanivorans* CB1190 exposed to chlorinated solvent mixtures, quantification of chlorinated solvents in CB1190 pure culture study at 0 and 24 hours, and quantification of cells with intact cell membranes. (PDF)

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Notes

The authors declare no competing financial interest.

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