Aerobic and anaerobic microbial degradation of crude (4-methylcyclohexyl)methanol in river sediments

Li Yuan ^{a,b,c}, Wei Zhi ^{a,d}, Yangsheng Liu ^{a*}, Elizabeth Smiley ^b, Daniel Gallagher ^b, Xi Chen ^c, Andrea Dietrich ^b, Husen Zhang ^{b*}

^a College of Environmental Sciences and Engineering, Peking University, Haidian District, Beijing 100871, China

^b Department of Civil and Environmental Engineering, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, United States

^c Department of Earth and Environmental Engineering, Columbia University, New York, New York 10027, United States

^d John and Willie Leone Department of Energy and Mineral Engineering, The Pennsylvania State University, University Park, Pennsylvania 16802, United States

* Corresponding author. Tel.: +86-1062751756. *E-mail address:* yshliu@pku.edu.cn, address: College of Environmental Sciences and Engineering, Peking University (Y. Liu)

* Corresponding author. Tel.: +1 5402310011. *E-mail address:* husen@vt.edu, address: Department of Civil and Environmental Engineering, Virginia Polytechnic Institute and State University (H. Zhang)

Abstract: Cyclohexane and some of its derivatives have been a major concern because of their significant adverse human health effects and widespread occurrence in the environment. The 2014 West Virginia chemical spill has raised public attention to (4-methylcyclohexyl)methanol (4-MCHM), one cyclohexane derivative, which is widely used in coal processing but largely ignored. In particular, the environmental fate of its primary components, *cis*- and *trans*-4-MCHM, remains largely unexplored. This study aimed to investigate the degradation kinetics and mineralization of *cis*- and *trans*-4-MCHM by sediment microorganisms under aerobic and anaerobic conditions. We found the removal of *cis*- and *trans*-4-MCHM was mainly attributed to biodegradation with little contribution from sorption. A nearly complete aerobic degradation of 4-MCHM occurred within 14 d, whereas the anaerobic degradation was reluctant with residual percentages of 62.6% of *cis*-4-MCHM and 85.0% of *trans*-4-MCHM after 16-d incubation. The *cis*-4-MCHM was degraded faster than the *trans* under both aerobic and anaerobic conditions, indicating an isomer-specific degradation could occur during the 4-MCHM degradation. Nitrate addition enhanced 4-MCHM mineralization by about 50% under both aerobic and anaerobic conditions. Both *cis*- and *trans*-4-MCHM fit well with the first-order kinetic model with respective degradation rates of 0.46-0.52 and 0.19-0.31 d⁻¹ under aerobic condition. Respective degradation rates of 0.041-0.095 and 0.013-0.052 d⁻¹ occurred under anaerobic condition. One bacterial strain capable of effectively degrading 4-MCHM isomers was isolated from river sediments and identified as *Bacillus pumilus* at the species level based on 16S rRNA gene sequence and 97% identity. Our findings will provide critical information for improving the prediction of the environmental fate of 4-MCHM and other cyclohexane derivatives with similar structure as well as enhancing the development of feasible treatment

technologies to mitigate these compounds.

Keywords: West Virginia chemical spill; Crude (4-methylcyclohexyl)methanol;
Degradation kinetics; Mineralization; 4-MCHM-degrading bacteria; 4-MCHM isomers.

1. Introduction

Chemical spills and accidents have been a well-known environmental problem for decades because of their widespread occurrence and multiple threats to human beings and the environment (Capel et al., 1988; Bryant and Abkowitz, 2007; Jiang et al., 2012; Lim and Huang, 2007). Thus, when a contamination event occurs, it is of significant importance to comprehensively assess the fate and transport of the spilled chemical in order to provide critical information for accurately evaluating its environmental risks and developing feasible cleanup strategies. Cyclohexane and its derivatives have been widely used in various manufacturing processes (US EPA, 1994) and some of them are found to exhibit significant adverse human health effects with large amounts of environmental releases; e.g. the release of cyclohexane in US totaled about 3.7 million pounds in 2014 according to the Toxics Release Inventory (US EPA, 2014). However, to date, limited data are available on the environmental fate of cyclohexane and its derivatives, making it difficult to predict environmental risks brought by these compounds.

(4-methylcyclohexyl)methanol (MCHM, $\text{CH}_3\text{C}_6\text{H}_{10}\text{CH}_2\text{OH}$), one cyclohexane derivative, has been widely used in coal processing but largely ignored before the West Virginia chemical spill (Savoia et al., 2015). On January 9, 2014, an estimated 10,000 gallons of crude 4-MCHM (a mixture that contains 68–89% *cis* and *trans*-4-MCHM, 4–10% water, and other cyclohexane compounds) (Eastman Chemical, 2015) leaked from a storage tank, down the river embankment, and into a small river, the Elk River in West Virginia. Only 1.6 km downstream of the leak was the intake to the drinking water treatment plant. Thus, 4-MCHM contaminated drinking water for 300,000 people and cost at least \$61 million losses for local businesses and residents (Manuel, 2014). A “do

not use” order was issued to advise affected residents not to use tap water for any purpose, other than flushing toilets, to reduce the health impacts of 4-MCHM (Rosen et al., 2014). Residents were immediately and readily aware of the 4-MCHM contamination as its air odor threshold is in the range of 0.06 ppb-v (Gallagher et al., 2015). Due to the initial lack of gas chromatographic methods for monitoring, 4-MCHM (measured as the combined *cis* and *trans* isomers) concentration was first measured in water about two days after the initial spill; soon after monitoring began, concentrations as high as 3.8 mg L⁻¹ were detected (WVDHSEM, 2014; Whelton et al., 2015). 4-MCHM concentrations were not measured in the soil or river sediment at the site of the spill. Monitoring in drinking water continued through April 2014; concentrations of several µg L⁻¹ were detected (Foreman et al., 2015; Whelton et al., 2015; WVDHSEM, 2014).

More than 1900 patients reported symptoms like rashes and nausea after exposure to 4-MCHM-contaminated tap water (Toxicology Data Network, 2014). Since very limited data were available about crude 4-MCHM, various studies regarding the physical, chemical, biological, and toxicological properties of crude 4-MCHM were conducted soon after the spill. A recent study revealed that 4-MCHM at 10 mg L⁻¹ can lead to DNA damage in human cells, indicating 4-MCHM can be related to genotoxicity and may cause chronic carcinogenesis (Lan et al., 2015). The National Toxicology Program (2015) reported that 4-MCHM was a developmental toxicant that decreased fetal weight and induced fetal malformations at an oral dose of 400 mg kg⁻¹ d⁻¹ using the rat model. Foreman et al. (2015) observed the presence of 4-MCHM in the Ohio River (Louisville, Kentucky) which is ~630 km downriver from the spill location, indicating that 4-MCHM could linger long and travel far to impact a large geographic area and

population.

Possible fates for 4-MCHM released into the environment include hydrolysis, photolysis, biodegradation, volatilization, and sorption on sediments ([Toxicology Data Network, 2014](#)). Among them, biodegradation is a desirable pathway because it can lead to complete mineralization instead of transferring pollutants from one phase to another ([Pandey et al., 2007](#)). One of the few previous studies on 4-MCHM biodegradation was conducted in activated sludge. During a 28-day period, about 50% of the crude 4-MCHM [dissolved organic carbon (DOC) concentration of 20 mg L⁻¹] was degraded after a 9-day lag phase; degradation was based on carbon dioxide (CO₂) evolution ([Beglinger, 1997](#)). So far as we know, no effort has been made to understand the biodegradation of 4-MCHM by microorganisms in river sediments.

Given that river sediments exhibit both aerobic (surface layer) and anaerobic (significant quantities) conditions ([Rhee et al., 1989](#)), biodegradation of organic contaminants can be aerobic and/or anaerobic depending on the chemical structure and microbial community. Anaerobic biodegradation is generally much slower than aerobic biodegradation due to lack of sufficient amounts of electron acceptors ([Shim et al., 2002](#); [Zhang et al., 2010](#)). Addition of oxygen ([Zhi et al., 2015](#)), nitrate ([Burland and Edwards, 1999](#)), sulfate ([Kohring et al., 1989](#)), or Fe(III) oxides ([Villatoro-Monzón et al., 2008](#)) to sites where anaerobic biodegradation predominates has proved to be feasible for enhanced biodegradation of many compounds. In particular, nitrate has an oxidative potential which is close to that of oxygen ([Chen and Strous, 2013](#); [Zhang et al., 2010](#)). Few studies have addressed the biodegradation of 4-MCHM in river sediments under aerobic and anaerobic conditions, or investigated the effect of nitrate. Moreover, biodegradation of individual geometric isomers, *cis*- and *trans*-4-MCHM, is also little

reported in the literature partly because of the previous lack of available analytical methods that were capable of detecting them individually. Once analytical methods for *cis*- and *trans*-4MCHM were established, investigations into their individual physicochemical properties demonstrated clear differences. The *cis*-isomer was more water soluble and less sorptive to organic matter than the *trans*-isomer. Specifically, for the *cis*-isomer, its room temperature properties were $K_{OW} = 225$, aqueous solubility = 1300 mg L^{-1} , and activated carbon capacity = 12.8 mg g^{-1} ; the *trans*-isomer properties were $K_{OW} = 291$, aqueous solubility = 1010 mg L^{-1} , and activated carbon capacity $26.5 = \text{mg g}^{-1}$ (Dietrich et al., 2015). The *cis*-isomer was also less volatile than the *trans*-isomer; the room temperature unitless Henry's Law Constant for *cis*-4-MCHM was 1.42×10^{-4} while for *trans*-4-MCHM was 3.08×10^{-4} (Sain et al., 2015).

As reported by Dietrich et al. (2015), the difference in the physicochemical properties of *cis*- and *trans*-4-MCHM is expected to impact their biodegradation behaviors. Thus, the individual biodegradation of these two geometric isomers needs to be studied for more accurately assessing the environmental fate of 4-MCHM. The aim of this study was to investigate the biodegradation kinetics of *cis*- and *trans*-4-MCHM in aerobic and anaerobic river sediments as well as determine the effect of nitrate on the biodegradation behaviors of *cis*- and *trans*-4-MCHM. To reveal whether sediment microorganisms were able to completely mineralize 4-MCHM into inorganic carbon, production of CO_2 and methane (CH_4) were also measured at the end of the biodegradation period. Isolation and identification of 4-MCHM-degrading strain(s) were conducted to investigate which bacterial isolates contributed to the loss of 4-MCHM in river sediments. To the best of our knowledge, this is the first report on the degradation of *cis*- and *trans*-4-MCHM in natural aquatic systems under aerobic and

anaerobic conditions and isolation of 4-MCHM-degrading bacteria.

2. Materials and Methods

2.1. Chemicals

Crude 4-MCHM was provided by Eastman Chemical (Kingston, TN, USA). Previous analyses in our laboratories demonstrated that this crude mixture was 86.5% 4-MCHM with a *cis:trans* ratio of 1:1.75 and contained small amounts of several other substituted cyclohexanes (Gallagher et al., 2015). The crude 4-MCHM was dissolved in autoclaved tap water to prepare crude 4-MCHM stock solution (2000 mg L⁻¹ 4-MCHM), which was then diluted to 80 mg L⁻¹ for the biodegradation experiments. This 4-MCHM concentration was chosen because the median-lethal concentration (LC50) of crude 4-MCHM was 30-111 mg L⁻¹ (95% confidence interval) in the acute (24-96 h) aquatic effects test with the Fathead minnow as a fish model (Light, 1998). Stock solution of pure 4-MCHM (1000 mg L⁻¹ 4-MCHM), prepared by dissolving pure 4-MCHM (~98% purity, *cis:trans* ratio of 2.08:1, TCI America) in methanol-water (90:10), was used to make calibration standards for gas chromatograph (GC) analysis. Luria–Bertani broth (LB, Lennox, Difco) that contained 10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, and 5 g L⁻¹ sodium chloride (NaCl) was adjusted to pH of 7.0 before autoclaving at 121 °C for 15 min. Sodium nitrate (NaNO₃, Fisher Chemical) was dissolved in autoclaved tap water to prepare nitrate stock solution (2000 mg L⁻¹ nitrate). Tap water that contained 2.5 mg L⁻¹ nitrate, 5.8 mg L⁻¹ sulfate, 6 mg L⁻¹ chemical oxygen demand (COD), 10.1 mg L⁻¹ sodium, 5.5 mg L⁻¹ magnesium, 11.7 mg L⁻¹ calcium, 1.5 mg L⁻¹ potassium, and other trace elements was autoclaved at 121 °C for 15 min and used in the biodegradation experiments to provide trace nutrients for sediment microorganisms. All solutions were

stored at 4 °C and warmed to room temperature before use.

2.2. Sediment sampling and characterization

River sediment that was not previously exposed to 4-MCHM was collected from nearshore surface sediments (0-5 cm) at duck pond in Virginia Polytechnic Institute and State University (Blacksburg, Virginia) for the biodegradation experiments. The sediment was sieved (2-mm-diameter wire mesh) to remove coarse rock fragments and homogenized thoroughly before use. The blank determinations of the sediment and sediment extract (sediment to nanopure water = 1:20 w/w) were performed. The sediment contained $20.7 \pm 1.3\%$ water and $1.2 \pm 0.2\%$ organic matter; the sediment extract contained $0.05 \pm 0.01 \text{ mg L}^{-1}$ soluble total iron without detection of soluble manganese, nitrate, nitrite, or sulfate (Table 1). River sediment was stored at 4°C and used with 24 h.

[Insert Table 1 here]

2.3. Biodegradation experiments

The degradation kinetics of *cis*- and *trans*-4-MCHM in sediment was assessed in 20-mL transparent vials that contained 0.5 g fresh sediments and 10 mL of 80 mg L^{-1} 4-MCHM (prepared by crude 4-MCHM stock solution). To test the effect of nitrate on 4-MCHM degradation, another set of microcosms was performed as described above but further amended with 200 mg L^{-1} nitrate (prepared by nitrate stock solution). The following controls were also set up: (1) blank controls (0.5 g fresh sediments without 4-MCHM addition) aimed for determining the indigenous production of inorganic carbon (e.g. CO_2 and CH_4), (2) nitrate-amended blank controls (0.5 g fresh sediments and 200

mg L⁻¹ nitrate) aimed for determining the indigenous production of inorganic carbon (e.g. CO₂ and CH₄) and consumption of nitrate, (3) sterile controls (0.5 g autoclaved sediments and 80 mg L⁻¹ 4-MCHM) aimed for determining the sorption effect of sediment on the loss of 4-MCHM, and (4) abiotic controls (80 mg L⁻¹ 4-MCHM without sediments) aimed for determining the abiotic loss of 4-MCHM during the experiment. All vials were sealed with Teflon-coated butyl rubber septa and aluminum crimp caps without opening for oxygen replenishment during the whole degradation period. The vials were incubated statically in an incubation shaker (25 °C), in which no special attention was paid for lighting schedule and condition.

For the aerobic degradation, all vials mentioned above were set up under atmospheric conditions (0.21 atm. O₂). In this system, the total O₂ amount was 96.3 μmol, which included 93.7 μmol of headspace O₂ (calculated based on equation 1) and 2.6 μmol of dissolved oxygen (DO, calculated based on equation 2). The total O₂ amount was higher than the theoretical O₂ demand (71.9 μmol) for 4-MCHM complete mineralization, indicating it was possible that 4-MCHM can be completely mineralized into CO₂ under this condition. While for the anaerobic degradation, all vials (except sterile and abiotic controls which were only performed under aerobic condition) were set up in an anaerobic glovebox (818-GB, Plas Labs) under N₂ conditions. All experiments were performed at least twice. During the degradation period, headspace concentrations of 4-MCHM isomers were periodically monitored as an indicator of the dissolved 4-MCHM isomers using a gas chromatograph coupled with a flame ionization detector (GC-FID, Thermo-Trace Ultra). To quantify the mineralization of 4-MCHM, headspace concentrations of CO₂ and CH₄ were measured using a gas chromatograph coupled with a thermal conductivity detector (GC-TCD, Shimadzu 14A). At the end of

the biodegradation experiments, solution pH was measured immediately by a pH meter after the vials were opened.

$$n_1 = \frac{P \times V_1}{R \times T} \quad (1)$$

$$n_2 = \frac{DO \times V_2}{M} \quad (2)$$

Where n_1 and n_2 were the amounts of headspace O_2 and DO, respectively; P was headspace O_2 pressure, V_1 and V_2 were the volumes of headspace O_2 and DO, respectively; R was the ideal gas constant ($8.314 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$), T was the temperature, DO was 8.2 mg L^{-1} in this work, and M was O_2 molecular weight.

2.4. Determination of nitrate consumption

To reveal whether 4-MCHM biodegradation was linked to nitrate reduction, nitrate concentration was measured using the sacrificial method. A series of identical vials that containing 200 mg L^{-1} nitrate with and without 4-MCHM (80 mg L^{-1}) addition were set up under aerobic and anaerobic conditions, respectively. Vials were opened at scheduled times, and the supernatants were immediately centrifuged ($10,000 \text{ g}$ for 1 min) and filtered (Millipore, $0.45 \text{ }\mu\text{m}$) before residual nitrate measurement using an ion chromatograph with a conductivity detector (Dionex DX-120). Nitrite concentration was also determined. All experiments were performed at least twice.

2.5. Isolation and identification of 4-MCHM-degrading bacteria

To identify which bacterial strains contributed to 4-MCHM degradation in river sediments, approximately 1.0 g fresh sediments were well mixed with $10 \text{ mL } 1 \times$ phosphate-buffered saline (PBS, pH 7.0), and the obtained sediment extracts were serially diluted in $1 \times$ PBS. Aliquots of 0.1 mL of each dilution were spread on the surface of LB agar plates by a heat-sterilized loop using the streak plate technique. After

incubation overnight at room temperature ($21 \pm 1^\circ\text{C}$), visually distinct colonies were selected and streaked for purity. Then, the colonies were incubated in 10 mL of LB medium and grown overnight at 25°C in an orbital shaker (120 rpm). The resulting bacterial cultures were used for testing their ability for 4-MCHM degradation, which were performed in 20-mL serum vials containing 10 mL of 4-MCHM (80 mg L^{-1}), bacterial cell suspension (2% v/v), and fresh LB medium (1% v/v). Out of these isolates, only one was found to significantly degrade *cis*- and *trans*-4-MCHM. To investigate whether this isolate was able to utilize *cis*- and *trans*-4-MCHM in the absence of additional carbon source, vials were set up under the same condition mentioned above but without addition of LB medium. To test whether this isolate was able to utilize nitrate for enhanced degradation of *cis*- and *trans*-4-MCHM, vials were set up with addition of 30, 60, 100, and 200 mg L^{-1} nitrate, respectively. All experiments were generally performed in triplicate, and at least in duplicate, under atmospheric condition; uninoculated (no cells) served as controls.

The genomic DNA of this 4-MCHM-degrading isolate was extracted by using the MoBio PowerSoil DNA isolation kit following the manufacture's instruction. The 16S rRNA genes were amplified using the bacterial primers 27F and 1492R. The sequencing of the PCR products were performed by the Virginia Bioinformatics Institute of Virginia Tech. The consensus sequences were then analyzed by the Basic Local Alignment Search Tool (BLAST) program ([Johnson et al., 2008](#)).

2.6. Other chemical analyses

The water content and organic matter content of the sediment were measured gravimetrically after drying at 105°C for 12 h and at 550°C for 25 min, respectively ([Wu and Gschwend, 1986](#)). Sediment mixed with nanopure water at sediment to water

ratio of 1:20 w/w was stirred for 1 h and the resulting sediment extract was filtered (Millipore, 0.45 μm) prior to the following analyses: ammonium and COD were measured as previously described (Zhi and Ji, 2014); soluble total iron and manganese were determined by an inductively coupled plasma mass spectroscopy (ICP-MS, Agilent 7500 Series) (Yuan et al., 2015); total carbon, organic carbon, and inorganic carbon were quantified using a total organic carbon analyzer (TOC, Sievers 5310C); nitrate, nitrite, and sulfate were monitored by the Dionex DX-120 ion chromatograph. The Shimadzu 14A GC-TCD for headspace CO_2 and CH_4 measurement was coupled with a Supelco Analytical Haysept D packed column (3.05 m $\times$ 6.35 mm ID). 100- μL gas samples were taken from the headspace of each vial using a 100- μL gastight syringe (Hamilton, Reno, Nevada) and immediately injected into GC. The column flow was 28 mL min^{-1} helium; temperatures of injector, detector, and column were maintained at 70, 110, and 40 $^{\circ}\text{C}$, respectively.

The Thermo-Trace Ultra GC-FID for headspace 4-MCHM measurement was coupled with an Agilent J&W Scientific DB-624 column (30 m $\times$ 0.53 mm ID $\times$ 3.0 μm film) and a headspace autosampler (HS 2000). The column flow was 5 mL min^{-1} nitrogen; headspace sample of 200 μL was injected in the splitless mode at 250 $^{\circ}\text{C}$. The oven temperature was started at 100 $^{\circ}\text{C}$ (held for 1 min), ramped to 140 $^{\circ}\text{C}$ at 20 $^{\circ}\text{C min}^{-1}$, and held for 3 min. The limits of detection (US EPA, 2000) for the headspace GC method were determined; the values were 0.126 mg L^{-1} *cis*-4-MCHM and 0.195 mg L^{-1} *trans*-4-MCHM. This method was previously applied to determine the concentrations of both *cis*- and *trans*-4-MCHM (Sain et al., 2015). The structures and chromatographic results of *cis*- and *trans*-4-MCHM were illustrated in Fig. 1.

[Insert Fig. 1 here]

2.7. Data analysis

The degradation data of 4-MCHM isomers were fitted with the first-order kinetic model:

$$\ln \frac{C_t}{C_0} = -kt \quad (3)$$

$$t_{1/2} = \frac{\ln 2}{k} \quad (4)$$

where C_t/C_0 was the residual percentage of *cis*- and *trans*-4-MCHM at time t (d), k (d^{-1}) was the first-order kinetic rate constant, and $t_{1/2}$ (d) was the half-life for *cis*- and *trans*-4-MCHM.

The geo-chemical modelling Visual MINTEQ (version 3.0) was used to calculate concentrations of inorganic carbon species including carbonic acid (H_2CO_3), bicarbonate ion (HCO_3^-), and carbonate ion (CO_3^{2-}) based on solution pH and headspace CO_2 concentration (measured by GC-TCD). The 4-MCHM mineralization (M) was defined as the following equation: $M = \text{NIC}/\text{TIC}$, where NIC was the net inorganic carbon ($\Sigma \text{CO}_2 + \text{H}_2\text{CO}_3 + \text{HCO}_3^- + \text{CO}_3^{2-}$) production which was calculated as inorganic carbon production in treatment vials (with 4-MCHM addition) subtracted that in blank controls (no 4-MCHM addition) and TIC was the theoretical inorganic carbon production which was calculated as $50 \mu\text{mol}$ if the added 10 mL of 80 mg L^{-1} 4-MCHM was completely converted into CO_2 based on reaction (5). The t-test (two-sample assuming unequal variances) was applied to compare the inorganic carbon production between treatment vials and blank controls in order to test whether 4-MCHM addition stressed or stimulated CO_2 emission from sediment organic matters. A difference was

considered significant when $p < 0.05$. The 4-MCHM mineralization was considered as 0 if $p > 0.05$. The stoichiometric relationships between net inorganic carbon production and nitrate reduction were calculated as (net inorganic carbon production in the presence of nitrate subtracted that in the absence of nitrate) divided by nitrate reduction.

3. Results and Discussion

3.1. Biodegradation kinetics of *cis*- and *trans*-4-MCHM under aerobic and anaerobic conditions

As shown in Fig. 2, the concentrations of *cis*- and *trans*-4-MCHM in abiotic (no sediment addition) and sterile (with autoclaved sediment) controls remained relatively constant, wherein the latter was less than 10% lower than the former, indicating sorption contributed little to 4-MCHM removal. This coincides well with *cis* and *trans*-4-MCHM physicochemical properties as both isomers have moderately high water solubilities that exceed 1000 mg L⁻¹ (Dietrich et al., 2015). In contrast, both isomers with fresh sediments were completely degraded by day 14 without a lag phase under aerobic condition (Fig. 2a), indicating sediment microorganisms were adapted readily to aerobically utilize 4-MCHM as carbon source. Both *cis*- and *trans*-4-MCHM fit well with the first-order kinetic model (Fig. 2 and Table 2). The aerobic degradation half-lives for *cis*- and *trans*-4-MCHM were calculated as 1.5 and 3.6 d, respectively; with nitrate addition, respective half-lives were 1.3 and 2.2 d (Table 2). In other words, under aerobic condition, the addition of nitrate had little effect on *cis*-4-MCHM degradation rate but enhanced *trans*-4-MCHM degradation rate, indicating these two isomers may have different biodegradation pathways. The faster degradation rate for *cis*-isomer is

consistent with *cis*-4-MCHM having a higher aqueous solubility and lower K_{OW} than the *trans*-isomer (Dietrich et al., 2015).

[Insert Fig. 2 here]

Under anaerobic condition, *cis*- and *trans*-4-MCHM failed to be completely degraded within 16-day incubation (Fig. 2). The respective residual percentages of *cis*- and *trans*-4-MCHM were 62.6% and 85.0% when nitrate was absent. Under this condition, the half-lives were calculated as 17.0 d for *cis*-4-MCHM and 55.0 d for *trans*-4-MCHM (Table 2). This indicates that *cis*- and *trans*-4-MCHM are probably long-term pollutants under anaerobic condition. Enhanced biodegradation was observed with nitrate addition; i.e. the respective residual percentages of *cis*- and *trans*-4-MCHM were reduced to 29.5% and 41.9% with half-lives of 7.3 and 13.2 d. This result was consistent with previous reports of the effectiveness of nitrate for enhanced anaerobic biodegradation (Dou et al., 2008; Silva and Corseuil, 2012).

[Insert Table 2 here]

As described in Table 2, the aerobic degradation rate constants (0.460 and 0.520 d^{-1}) of *cis*-4-MCHM were higher than those of *trans*-4-MCHM (0.193 and 0.314 d^{-1}) in the absence and presence of nitrate, respectively. Similar observations were made under the anaerobic condition. This indicates that *cis*-4-MCHM is degraded faster than *trans*-4-MCHM under both aerobic and anaerobic conditions, suggesting *cis*-4-MCHM is preferentially utilized compared with *trans*-4-MCHM by sediment microorganisms.

This *cis*-preferred degradation for 4-MCHM was in accordance with several studies investigating other organic compounds (Guckert et al., 1986; Bashir, 1982). For example, *cis*-monoenoic fatty acids were preferentially utilized during nutrient deprivation of *Vibrio cholerae* (Guckert et al., 1986). Bashir (1982) observed that larvae of the common green lacewing degraded *cis*-permethrin 1.7-fold faster than *trans*-permethrin. Despite that the mechanism is still unclear, we show that the degradation of 4-MCHM by river sediments is isomer-sensitive, which underlines the importance of understanding the individual environmental fate of 4-MCHM isomers.

3.2. Mineralization of 4-MCHM

The production of inorganic carbon in treatment vials (with 4-MCHM) and blank controls (without 4-MCHM) is shown in Table 3. Under aerobic condition, 4-MCHM mineralization was calculated as 60.5% with inorganic carbon production of 30.2 μmol ; with nitrate addition, the mineralization was enhanced to 107.4% with inorganic carbon production of 53.7 μmol . The additional 7.4% mineralization was probably due to the degradation of other minor cyclohexane components in crude 4-MCHM such as 4-(methoxymethyl)cyclohexanemethanol and methyl 4-methylcyclohexanecarboxylate in the crude 4-MCHM mixture (Eastman Chemical, 2015; Gallagher et al., 2015). These results suggest that sediment microorganisms are capable of completely mineralizing 4-MCHM into inorganic carbon under nitrate-amended aerobic condition. By contrast, under anaerobic condition, the 4-MCHM mineralization was 0 without significant production of inorganic carbon from treatment vials compared with their corresponding blank controls ($p = 0.40$). This also indicates that inorganic carbon production from sediment organic matter is not altered by 4-MCHM addition. With nitrate addition, 4-MCHM mineralization under anaerobic

condition was enhanced to 46.0% with inorganic carbon production of 23.0 μmol . The insufficient mineralization under anaerobic condition was probably attributed to the lack of electron acceptors, which was confirmed by the characteristics of the sediments with limited concentrations of nitrate, sulfate, iron, and manganese (Table 1). No CH_4 was detected under either aerobic or anaerobic conditions, indicating that CH_4 is probably not a degradation product of 4-MCHM in river sediments. Further studies are needed to identify 4-MCHM biodegradation intermediate metabolites because of the possible formation of toxic by-products.

[Insert Table 3 here]

Compared with Eastman's 4-MCHM degradation study in which 4-MCHM biodegradability was ~50% using an activated sludge inoculum after 28 days (Beglinger, 1997), a higher biodegradability of 4-MCHM was observed in this study. This can have several explanations. The first difference was inocula. As reported by previous studies (Yang et al., 2013; Yuan et al., 2015), the microbial communities in wastewater treatment sludge and river sediment were significantly different, and thus may have differential abilities to degrade 4-MCHM. Second, the present experiments were carried out at 25 °C, whereas the Eastman's study was at 19-22 °C. It is possible that the sediment microorganisms exhibited higher microbial activity at 25 °C for 4-MCHM mineralization than sludge microorganisms at 19-22 °C since microbial metabolism and populations are highly temperature-dependent (Liang et al., 2003).

3.3. Reduction of nitrate

Figure 3 presents data on the variation of nitrate and nitrite under aerobic and

anaerobic conditions. No apparent loss of nitrate was observed in blank controls (no 4-MCHM addition) during the whole incubation (Figs. 3a and 3c). This coincided well with the observations of Hu et al. (2007), who reported that no nitrate reduction occurred probably due to lack of the carbon sources or the biodegradation cultures. In contrast, with 4-MCHM addition, nitrate decreased by 63.5% and 100% under aerobic and anaerobic conditions, respectively, indicating sediment microorganisms were capable of using nitrate as an electron acceptor for 4-MCHM degradation. This was consistent with the results mentioned above (Table 2 and Fig. 2) that enhanced biodegradation and mineralization of 4-MCHM were observed in the presence of nitrate. The stoichiometric relationships between net inorganic carbon production and nitrate reduction were calculated as about 1:1 and 2:3 (mol/mol) under aerobic and anaerobic conditions, respectively. This indicated that more nitrate was needed under anaerobic conditions than under aerobic conditions to achieve equivalent mineralization, probably due to the contribution of oxygen to 4-MCHM mineralization under aerobic conditions. At the same time, nitrite was detected as the intermediate product that was below 5 mg L⁻¹, indicating nitrite was not likely accumulated during 4-MCHM degradation.

[Insert Fig. 3 here]

3.4. Isolation and identification of 4-MCHM-degrading bacteria

We tested 12 sediment isolates for their 4-MCHM degradation capability under aerobic conditions. One isolate, phylogenetically identified as *Bacillus pumilus* at the species level (97% identity) based on 16S rRNA gene sequence analysis, was able to degrade ~62% of *cis*-4-MCHM and ~88% of *trans*-4-MCHM within 18 d (Table 2 and

Figs. 4a and 4b). In particular, this isolate could utilize 4-MCHM as sole carbon source with a slightly lower degradation compared with that in the presence of LB medium. However, unlike the mixed culture under similar aerobic conditions, *trans*-4-MCHM exhibited a faster degradation than *cis*-4-MCHM (Table 2). Without LB addition, the calculated half-life for *cis*-4-MCHM was 11.3 d, but only 5.1 d for *trans*-4-MCHM. A similar pattern, but with slightly shorter half-lives, was found with LB addition (Table 2).

As illustrated in Figs. 4c and 4d, nitrate addition (30-200 mg L⁻¹) had insignificant effect ($p > 0.05$) on the degradation of *cis*- and *trans*-4-MCHM, indicating the isolated strain of *Bacillus pumilus* may be not able to utilize nitrate as an electron acceptor for 4-MCHM degradation under aerobic condition. In contrast, nitrate was consumed by the mixed sediment culture for enhanced degradation and mineralization of 4-MCHM. This difference could be attributed to multiple bacterial groups being involved in 4-MCHM degradation and mineralization; some groups were capable of utilizing nitrate as electron acceptor.

[Insert Fig. 4 here]

It has been found that *Bacillus* spp. are widely present in the environment (i.e. soil, natural waters, sewage sludge, pulp and paper sludge, textile wastewater drains, and tannery effluent) (Cadmus et al., 1982; Maier et al., 2004; Raj et al., 2007; Suntornsuk and Suntornsuk, 2003; Vargha et al., 2005) and capable of degrading various pollutants (i.e. textile dyes, crude oil, and benzo[a]pyrene (Antai, 1990; Dan et al., 2006; Dawkar et al., 2008, 2009)). The present study observed the ability of *Bacillus*

pumilus in 4-MCHM removal, which broadened the application of *Bacillus* spp. in bioremediation of environmental pollutants. However, the degradation of 4-MCHM by the *Bacillus pumilus* isolate is not fully understood. For example, it remains unknown about the degradation pathway and environmental factors affecting the degradation kinetics. Further work is needed to systematically evaluate the ability of this and other *Bacillus* spp. for 4-MCHM degradation, which can serve as the foundation to enhance development of bioremediation technologies to mitigate 4-MCHM.

4. Conclusions

In summary, biodegradation was considered as a key mechanism for eliminating 4-MCHM in river sediments with little contribution from sorption. In particular, both *cis*- and *trans*-4-MCHM were readily biodegraded by sediment microorganisms under aerobic conditions but very resistant to biodegradation under anaerobic conditions. Addition of nitrate enhanced the degradation kinetics and mineralization of 4-MCHM under both aerobic and anaerobic conditions. A newly isolated strain of *Bacillus pumilus* was observed to effectively degrade *cis*- and *trans*-4-MCHM, and this may offer an opportunity for in situ bioremediation for 4-MCHM when future event occurs.

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the isolation and identification of 4-MCHM-degrading bacteria. We also thank Amanda Sain for assistance with analytical methods and GC-headspace measurements.

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Figure Captions

Fig. 1 Molecular structures (a) of the two 4-MCHM isomers and their chromatographic results (b) determined by the Thermo-Trace Ultra GC-FID.

Fig. 2 First-order degradation kinetics model for *cis*- and *trans*-4-MCHM by sediment microorganisms. (a) *cis*-4-MCHM under aerobic condition, (b) *trans*-4-MCHM under aerobic condition, (c) *cis*-4-MCHM under anaerobic condition, and (d) *trans*-4-MCHM under anaerobic condition.

Fig. 3 Variations of nitrate and nitrite concentrations under aerobic and anaerobic conditions.

Fig. 4 Degradation of *cis*- and *trans*-4-MCHM by *Bacillus pumilus* under aerobic condition. (a) degradation of *cis*-4-MCHM in the presence and absence of LB medium, (b) degradation of *trans*-4-MCHM in the presence and absence of LB medium, (c) nitrate-amended degradation of *cis*-4-MCHM after 18-day incubation, and (d) nitrate-amended degradation of *trans*-4-MCHM after 18-day incubation.

Table Captions

Table 1 Characteristics of sediment extract (sediment to water = 1:20 w/w) used in this study.

Table 2 The degradation rate constants (k), half-lives ($t_{1/2}$), and residual percentages (RP) of *cis*- and *trans*-4-MCHM under aerobic and anaerobic conditions.

Table 3 Mineralization of 4-MCHM under aerobic and anaerobic conditions.

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Table 1 Characteristics of sediment extract (sediment to water = 1:20 w/w) used in this study

River sediment	Properties
pH	7.8 ± 0.1
Total carbon	$15.0 \pm 0.6 \text{ mg L}^{-1}$
Organic carbon	$3.0 \pm 0.2 \text{ mg L}^{-1}$
Inorganic carbon	$12.0 \pm 0.4 \text{ mg L}^{-1}$
COD	$43 \pm 2 \text{ mg L}^{-1}$
Total soluble iron	$0.05 \pm 0.01 \text{ mg L}^{-1}$
Total soluble manganese	N.D. ^a
Nitrate	N.D. ^a
Nitrite	N.D. ^a
Ammonium	N.D. ^a
Sulfate	N.D. ^a

^a The detection limits were: manganese (1 ppb), nitrate (0.05 mg L^{-1}), nitrite (0.05 mg L^{-1}), ammonium (0.4 mg L^{-1}), and sulfate (0.1 mg L^{-1}).

Table 2 The degradation rate constants (k), half-lives ($t_{1/2}$), and residual percentages (RP) of *cis*- and *trans*-4-MCHM under aerobic and anaerobic conditions.

Condition	Addition	<i>cis</i> -4-MCHM				<i>trans</i> -4-MCHM			
		RP (%)	k (d ⁻¹) ^a	R ²	$t_{1/2}$ (d)	RP (%)	k (d ⁻¹) ^a	R ²	$t_{1/2}$ (d)
Aerobic	no addition	0	0.460 ± 0.028	0.94	1.5	3.4 ± 3.6	0.193 ± 0.023	0.78	3.6
	+ nitrate	0	0.520 ± 0.044	0.91	1.3	0	0.314 ± 0.038	0.79	2.2
Anaerobic	no addition	62.6 ± 0.02	0.041 ± 0.005	0.75	17.0 ^b	85.0 ± 3.5	0.013 ± 0.002	0.72	55.0 ^b
	+ nitrate	29.5 ± 4.2	0.095 ± 0.005	0.92	7.3	41.9 ± 4.3	0.052 ± 0.002	0.97	13.2
<i>Bacillus pumilus</i>	no addition	40.4 ± 3.1	0.062 ± 0.004	0.92	11.3	14.2 ± 3.1	0.135 ± 0.009	0.93	5.1
	+ LB ^c	37.9 ± 1.9	0.071 ± 0.007	0.87	9.8	11.9 ± 2.1	0.151 ± 0.011	0.92	4.6

^a Slopes ± standard errors. All k 's statistically different from 0 ($p < 0.05$)

^b Calculated $t_{1/2}$ beyond the experiment duration of 16 days.

^c Luria–Bertani broth

Table 3 Mineralization of 4-MCHM under aerobic and anaerobic conditions.

Conditions	Nitrate addition	Treatment vials (with 4-MCHM, μmol)			Blank controls (without 4-MCHM, μmol)			t-test p	Net carbon Production (μmol)	4-MCHM mineralization (%)
		CO_2 (g)	H_2CO_3 (aq)	HCO_3^- (aq)	CO_2 (g)	H_2CO_3 (aq)	HCO_3^- (aq)			
Aerobic	no addition	7.4 ± 1.5	6.2 ± 1.5	35.5 ± 5.7	1.7 ± 0.5	1.4 ± 0.5	15.7 ± 3.8	0.013	30.2 ± 6.7	60.5 ± 13.5
	+nitrate	9.4 ± 0.4	7.8 ± 0.4	56.5 ± 3.6	1.5 ± 0.2	1.2 ± 0.2	17.3 ± 2.3	0.0002	53.7 ± 2.6	107.4 ± 5.3
Anaerobic	no addition	2.6 ± 0.2	2.1 ± 0.2	16.5 ± 1.9	0.6 ± 0.2	0.5 ± 0.2	16.0 ± 6.1	0.40	0 ^a	0 ^a
	+nitrate	1.3 ± 0.1	1.1 ± 0.1	46.0 ± 1.5	0.6 ± 0.1	0.5 ± 0.1	24.3 ± 4.6	0.015	23.0 ± 3.4	46.0 ± 6.8

Note: CO_2 (g) was measured by GC-TCD and other inorganic carbon species were calculated with Visual MINTEQ. CO_3^{2-} was not taken into account due to its negligible presence in the pH range (7.1-8.0) of this study. ^a The net inorganic carbon production and 4-MCHM mineralization were considered as 0 given $p > 0.05$ for treatment vials vs blank control under anaerobic condition without nitrate addition.

Graphical Abstract