Enhanced long-term attenuation of 1,4-dioxane in bioaugmented flow-through aquifer columns

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Enhanced long-term attenuation of 1,4-dioxane in bioaugmented flow-through aquifer columns

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Abstract Long term natural attenuation of 1,4-dioxane (dioxane) and its enhanced biodegradation after bioaugmentation with *Pseudonocardia dioxanivorans* CB1190 were assessed using flow-through aquifer columns. Natural attenuation of dioxane was not observed even after 2 years of acclimation. However, dioxane removal was observed in the bioaugmented columns (34% when the influent was 200 µg/L and 92% for 5 mg/L). The *thmA* gene that encodes the tetrahydrofuran monooxygenase that initiates dioxane degradation by CB1190 was only detected at the inoculation port and persisted for months after inoculation, implying the resiliency of bioaugmentation and its potential to offer long-term enhanced biodegradation capabilities. However, due to extensive clumping and limited mobility of CB1190, the augmented catabolic potential may be restricted to the immediate vicinity of the inoculation port. Accordingly, bioaugmentation with CB1190 seems more appropriate for the establishment of biobarriers. Bioaugmentation efficiency was associated with the availability of oxygen. Aeration of the column influent to increase dissolved oxygen significantly improved dioxane removal ($p < 0.05$), suggesting that (for sites with oxygen-limiting conditions) bioaugmentation can benefit from engineered approaches for delivering additional oxygen.

Keywords Bioaugmentation · Dioxane · Natural attenuation · *Pseudonocardia dioxanivorans* CB1190

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Introduction

1,4-Dioxane (dioxane) is a widespread groundwater contaminant (Zenker et al. 2003) classified as a probable human carcinogen (Class B2) (U.S.EPA 2014). Dioxane was frequently used as a stabilizer for chlorinated solvents and consequently it has been found as a co-contaminant in many chlorinated solvent-contaminated sites (Adamson et al. 2014). Dioxane removal from water has been demonstrated using advanced chemical oxidation processes with ozone (Khan et al. 2019; Kishimoto et al. 2007), hydrogen peroxide (Ikehata et al. 2016), Fenton's (Mohr et al. 2016) and/or photocatalytic titanium dioxide (Yamazaki et al. 2007). Other potential treatment technologies include sonication (Son et al. 2006), electrochemical (Blotevogel et al. 2019) and

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ultraviolet irradiation (Ikehata et al. 2016). However, implementing these technologies for full-scale groundwater remediation requires an ex situ aproach. This is generally costly to implement as it requires long periods of pumping, which is energetically demanding. In situ remediation approaches are thus often more cost-effective when applicable.

Phytoremediation (and associated rhizoremediation) have shown promise for dioxane removal (Aitchison et al. 2000) but this technique is limited by the contaminated soil depth and area accessible to plant roots. Monitored natural attenuation, which primarily relies on biodegradation, may be the most cost-effective approach for managing dioxane plumes. However, natural attenuation of dioxane is not always observed. Recently it was reported that only 22 out of 193 sites (from California GeoTracker) and 131 out of 441 wells (from Air Force data set) (Adamson et al. 2015) exhibited statistically significant attenuation rates. In the absence of natural attenuation, biostimulation or bioaugmentation approaches may be the next most cost-effective strategies, particularly for large dilute plumes.Biodegradation potential by native soil bacteria has been demonstrated (He et al. 2018; Sei et al. 2013) and it has been reported that several bacterial isolates or consortia can degrade dioxane metabolically (He et al. 2017; Huang et al. 2014; Kim et al. 2009; Parales et al. 1994; Sei et al. 2013) or cometabolically (Deng et al. 2018; Inoue et al. 2018; Mahendra and Alvarez-Cohen 2006). Therefore, the implementation of bioaugmentation strategies in contaminated sites where biodegradation activity potential by indigenous bacteria is absent could help accelerate dioxane removal (Grostern et al. 2012; He et al. 2017; Mahendra and Alvarez-Cohen 2006; Masuda et al. 2012).

Recently, dioxane biodegradation was demonstrated at field scale after the introduction of *Rhodococcus ruber* ENV425, which is a bacterium capable of breaking down dioxane cometabolically in the presence of a primary substrate (e.g., propane) (Lippincott et al. 2015). Nonetheless, supplementation with auxiliary co-substrates increases complexity and costs. Also, adequate distribution of the primary substrate can be challenging, and continuous injection of auxiliary substrates can lead to unintended consequences such as catabolic plasmid loss (Li et al. 2017). Therefore, bioaugmentation using bacteria capable of metabolically degrading dioxane may be preferable.

Successful bioaugmentation using metabolic strains has been reported in short-term microcosms (He et al. 2018; Zhang et al. 2016) and small flow-through column studies (e.g., 10-cm long, flow rate of 0.01 mL/min) (Zhao et al. 2018). These small-column experiments addressed the effects of bioaugmentation on high dioxane concentrations (3–10 mg/L), but did not consider low (ppb) levels typically found in diluted plumes, and no data were obtained on bacteria distribution from the injection point. Furthermore, neither microcosms nor small columns realistically simulate the temporal changes and natural gradients in substrates and oxidation–reduction potential that occur at contaminated sites, as well as provide information on bacteria transport and distribution after injection. Thus, larger scale and longer duration studies are needed to assess the merits and limitations of bioaugmentation to bioremediate dioxane plumes. Accordingly, in this work we used large flow-through aquifer columns (120-cm length, 5-cm i.d.) filled with uncontaminated soil. These columns were monitored for over 2 years to assess long-term natural attenuation of dioxane and its subsequently enhanced biodegradation after bioaugmentation with the archetype metabolizer *Pseudonocardia dioxanivorans* CB1190. Experiments were conducted using either low (200 µg/L) and high (5 mg/L) dioxane concentrations to assess the perseverance of the added strain (and the relative abundance of the pertinent catabolic gene, *thmA*), as well as its effectiveness in the removal of dioxane from both dilute plumes and source zones.

Materials and methods

Aquifer columns

Three flow through aquifer columns (120-cm length, 5-cm diameter, 19.6 cm^2 cross sectional area) equipped with eight sampling ports (at 2.5, 7.6, 14, 20, 40, 60, 80, and 100 cm from the inlet) were used to investigate the natural attenuation of dioxane and its enhanced biodegradation through aerobic bioaugmentation. Columns were filled with sandy aquifer material. The physical–chemical characteristics of the soil was (in mg/L): $N\text{-}NO_3^-$ (8), $P\text{-}PO_4^{2-}$ (35), K (115), Ca^{2+} (3,886), Mg^{2+} (161), S (23), Na (11), pH of 7.9, conductivity of 144 µmho/cm, and an organic matter of 0.83% (Soil, Water and Forage Testing Laboratory,

Department of Soil and Crops Sciences, College Station, TX). The columns were kept in the dark at room temperature (22 °C) and fed continuously with a carbonate-buffered synthetic groundwater for over 1 year in an up-flow mode. The mineral composition of synthetic groundwater (in mg/L) was as follows: NO_3^- (5.0); $FeSO_4$ (3.0); $CaCO_3$ (1,000); NH_4^+ (5.5); Mg^{2+} (1.5); PO_4^{3-} (0.06); and Ni(II), Cu(II), Zn(II), Co(II), and Mo(IV) (0.002 each) (modified from (von Gunten and Zobrist 1993). The groundwater was initially fed at flow rate of 7.2 mL/h using a peristaltic pump (Masterflex model 7519–15). Over the course of the experiment, the flow rate was decreased to 2 mL/h (using a syringe pump, Harvard Apparatus model 22) to reduce column's seepage velocity and increase residence time for improved biodegradation. The hydraulic characteristics of the columns were estimated by fitting bromide tracer data to the one-dimensional advection–dispersion equation as described previously (Da Silva and Alvarez 2002). The column's hydraulic parameters (with values in parentheses) were as follows: groundwater flow (Q = 7.2 mL/h), dispersion (D = 0.4–2.5 cm^2/h), effective porosity (η_e = 0.4–0.50) and seepage velocity (V = 1.5 cm/h). The obtained η_e were within typical values reported for sandy aquifers (Domenico and Schwartz 1998). Approximately 5 days were required to exchange one pore volume.

One flow-through aquifer column (column 1, Table 1) was not inoculated to serve as negative control and discern bioaugmentation from natural attenuation. Two other columns were inoculated with 120-mL of stock bacteria inoculum ($\sim 10^9$ cells/mL) directly into the port vial located at 40 cm from the column's inlet. This location was selected to assess the potential spatial distribution of the introduced bacteria through quantification of *thmA* catabolic genes along the column's length months after inoculation. Following 90 days after the first inoculation (column 3, Table 1), dioxane biodegradation activity was lost due to formation of anaerobic zones. This column was then re-inoculated (120 mL, $\sim 10^9$ cells/mL) this time near the column's influent (port located at 2.5 cm from the column's inlet) where the concentration of dissolved oxygen was higher. Dioxane influent concentrations were 200 µg/L and 5 mg/L. These two dioxane concentrations were selected to represent the typical dioxane levels in contaminated plumes and source zones, respectively (Adamson et al. 2015). Table 1 summarizes the experimental set up and operational changes made over time.

Bacteria inoculum

The well-characterized *Pseudonocardia dioxanivorans* CB1190 (Parales et al. 1994), capable of using dioxane as sole carbon and energy source, was utilized in this work as the inoculum. CB1190 was obtained directly from the American Type Culture Collection (ATCC 55486). Cells were grown in 2 L batch reactors containing Ammonium Mineral Salts (AMS) medium (Parales et al. 1994) amended with 800 mg/L dioxane. Batch reactors were kept at room temperature (23 °C) under continuous aeration (using 0.22 µm inline sterile filters to avoid contamination) using aquarium pumps. After 18 days, the grown cells were harvested

Table 1 Summary of the columns experimental set up and operational conditions

Experiment	Influent dioxane (mg/L)	Inoculation port	Flowrate used (pore velocity)	Re-inoculation	Addition of oxygen
Column 1- Natural attenuation	0.2	Not inoculated	7.2 mL/h (1.5 cm/h)	–	No
Column 2- Bioaugmentation	0.2	40 cm	7.2 mL/h (1.5 cm/h)	–	No
Column 3- Bioaugmentation	5	40 cm	7.2 mL/h (1.5 cm/h) Then 20 days after inoculation, the flow rate was decreased to 2 mL/h (0.36 cm/h)	After 90 days from inoculation, this column was re-inoculated at 2.5 cm from the inlet	Yes, 20 days after re-inoculation

by centrifugation (Heraeus MegaFuge8, Thermo Scientific), washed three times in phosphate buffer, and sonicated in a water bath (Ultrasonic Cleaner Bransonic 5510R-MT) for 40 s to disaggregate cell clumps. Bacteria concentration was estimated after sonication by optical density at 600 nm (OD_{600} of 1 = 8×10^8 cells/mL) using a Ultrospec^{TM} 2100 pro UV/Visible Spectrophotometer (GE Healthcare, Little Chalfont, UK). After sonication, cells were serially diluted and plated on Reasoner's 2A (R2A) plates to assess bacteria count (colony forming units, CFU) and viability. The optical density (OD600) of the culture after water bath sonication was 0.048 nm (equivalent to 3.8×10^7 cells/mL of *Escherischia coli*). Comparatively, the obtained number of colonies forming units (CFU) was $\sim 7 \times 10^8$ cells/mL indicating that bacteria remained viable after sonication.

Prior to inoculation of the columns, microcosms studies were performed to assess the metabolic activity of the harvested bacteria. 120 mL glass batch reactors were filled with 50 mL groundwater collected from a dioxane-contaminated site and capped with Teflon septa. Two reactors were prepared using groundwater from two independent sampling wells. Groundwater contained dioxane (1.5–4.5 mg/L) as well as chlorinated solvents present as co-contaminants (in mg/L): trichloroethene (2–3), 1,1-dichloroethene (3–5), and vinyl chloride (0.1). Microcosms were inoculated with approximately 1.6×10^8 cells/mL (final concentration). Two other microcosms, not inoculated (negative controls), served to distinguish the effects of bioaugmentation compared to natural attenuation alone. Microcosms were kept at room temperature (22 °C) and sampled to assess changes in dioxane concentrations over time. Dioxane was only degraded in the bioaugmented microcosms within 3–7 days, indicating the metabolic activity of CB1190.

Influent aeration

Air was injected inline directly into the influent of the bioaugmented column 3 (using a Harvard Apparatus 22) at flow rate of 5 mL/h in attempt to overcome the rapid depletion of dissolved oxygen inside the columns and to enhance dioxane removal by the inoculated bacteria. Higher air flow rate could increase oxygen and enhance biological activity, but due to limitations in column's dimensions, any further increase in air flow could result in the formation of air pockets and changes in hydraulic characteristics. A 60 mL plastic syringe filled with air and connected to a Tygon® tube was used to pump air directly into the column inlet. The syringe was refiled with air daily until the end of the experiments.

Soil DNA extraction and quantification of bacteria 16S rRNA and *thmA* catabolic gene

Aquifer sediments from the bioaugmented column (Table 1) were collected at selected locations (at 2.5, 40 and 80 cm from column inlet) 90 days after the first inoculation for bacterial genes analyses. About 2 g of soil was taken from the port vials located at 2.5, 40, and 80 cm from the columns' inlet. Soil samples were transferred into a lysing matrix tube for DNA extraction by using a QIAGEN DNEASY POWERSOIL kit according to the manufacturer's protocols. A bead-beating device (MINI Beadbeater) was utilized for soil lysis. A 50 µL soil DNA sample was collected in a 1.5 mL Eppendorf vial and stored in a freezer (IsoTemp® basic) at − 75 °C for further analysis.

The concentration of total bacteria and dioxane-degraders were quantified using real-time quantitative PCR (qPCR) analysis. Total bacteria were quantified based on 16S rRNA (Suzuki et al. 2000), and dioxane-degraders based on a previously developed primers/probe set targeting genes encoding the large hydroxylase subunit (*thmA*) of a putative tetrahydrofuran/dioxane monooxygenase (an enzyme proposed to initiate dioxane catabolism) (Li et al. 2013). Another primers/probe set previously designed to target *prmA* gene encoding the large hydroxylase subunit (*prmA*) of a propane monooxygenase enzyme that may be also involved in dioxane degradation by indigenous soil bacteria belonging to *Mycobacterium* spp. were also evaluated as biomarker (Deng et al. 2017; He et al. 2017). qPCR mixture contained 10 ng of genomic DNA, 300 ηM of forward and reverse primers, 150 nM of fluorogenic probe, 10 µL of TaqMan universal master mix II (Applied Biosystems, Foster City, CA, USA) and DNA-free water, to a total volume of 20 µL. The primers and probe used (Integrated DNA Technologies, Inc.) to target total bacteria 16S rRNA were: 5′-CGGTGAATACGTTCYCGG-3′ (forward primer), 5′-GGWTACCTTGTTACGACTT-3′ (reverse primer), and FAM-5′-CTTGTACACACCGCCCGTC3′-BHQ-1 (probe).

The primers and probe to target *thmA* was: 5′-CTGTATGGGCATGCTTGT-3′ (forward primer), 5′-CCAGCGATACAGGTTCAT C-3′ (reverse primer), and 5′-6-FAM-ACGCCTATT/ZEN/ACATC-CAGCAGCTCGA-IABkFQ-3′ (probe). The primers and probe to target prmA were: 5′-ACTGC-GATGCTGGTTGAC-3′ (forward primer), 5′-TCAGGTACGCCTCCTGATT-3′ (reverse primer), and 5′-6-FAM/TTCCTCGCG/ZEN/CAGATGATC-GACG-IABkFQ-3′ (probe). qPCR was performed in a CFX 96 real-time system (Bio-Rad, Hercules, CA, USA) with the following temperature program: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Serial dilutions (10^{-4}–10^{1} ng DNA/μL) of the extracted genomic DNA of CB1190 and *Mycobacterium dioxanotrophicus* PH-06 were utilized to prepare the calibration curves for *thmA* (1 copy/genome) and *prmA* genes (1 copy/genome), respectively. CB1190 DNA was also used to prepare calibration curves for 16S rDNA (3 copies/genome) gene. Assuming a genome size of 7.44 Mb (Sales et al. 2011) and 9.124×10^{14} bp/μg [i.e., (6.022×10^{17} Da/μg of DNA/660 Da/bp)], the gene copy numbers were calculated based on the equation below.

$$\frac{\text{gene copies}}{\mu\text{L}} = \left(\frac{\frac{\mu\text{g of DNA}}{\mu\text{L}}}{\frac{7.44\,\text{Mb}}{\text{genome}}}\right)\left(\frac{9.124 \times 10^{14}\,\text{bp}}{\mu\text{g of DNA}}\right)\left(\frac{\text{gene copies}}{\text{genome}}\right)$$

Analytical procedures

Groundwater samples were withdrawn from column's lateral sampling ports using a 1-mL glass type syringe (Hamilton). Dioxane was measured using an Agilent 7820A gas chromatograph (GC) equipped with a 5977E mass spectrum detector (MSD) after filtration through 0.22 μm syringe filters and extraction by a liquid/liquid frozen microextraction method with dichloromethane as the solvent (Li et al. 2011). GC operational temperatures were set at 175 °C for the injector, 250 °C for the detector, and 150 °C for the oven. Detection limit for dioxane was 5 μg/L. The concentration of dissolved oxygen was measured directly from the columns sampling ports using a micro dissolved oxygen probe (Microsensor PreSens, PM-PSt1). Bromide used as a tracer (1 g/L added in the groundwater feeding solution) was measured over time in the effluent of the columns by conductivity (Orion Versastar Pro, Thermo scientific). All chemicals used were analytical grade (> 99.8 purity or higher).

Statistical differences between two data sets were determined by comparison of means (obtained from triplicate samples) using two-tailed *t* test at the 95% confidence level ($p < 0.05$; Microsoft Excel 2016 version 1910).

Results and discussion

Low (ppb) dioxane levels may not sustain CB1190, hindering bioaugmentation efficacy

Figure 1 shows dioxane concentration profiles in the natural attenuation alone and bioaugmented columns (columns 1 and 2, respectively, Table 1) fed synthetic

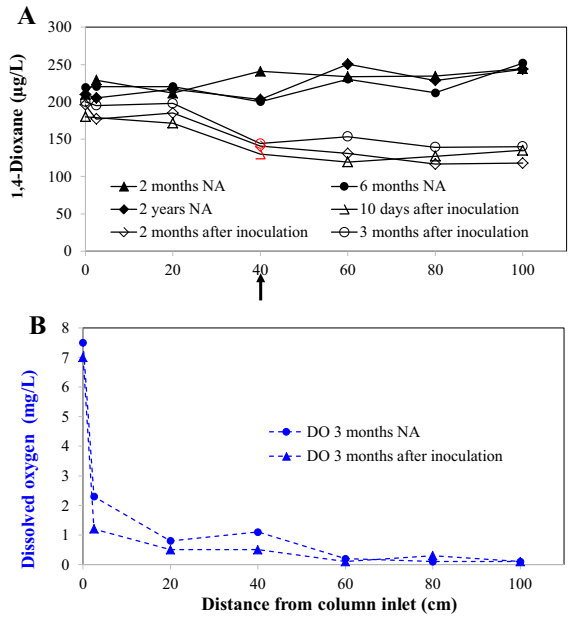

Fig. 1 Dioxane and dissolved oxygen concentration profiles. **a** shows dioxane concentrations along the control column (not bioaugmented, column 1) under natural attenuation (NA) conditions (closed symbols) and along the column inoculated with *Pseudonocardia dioxanivorans* CB1190 (column 2, open symbols). Dioxane was not biodegraded in the control column, even after 2 years of acclimation. Bacteria were inoculated at sampling port vial located 40 cm from the column 2 inlet (indicated by arrow). **b** depicts DO concentration profiles

groundwater amended with 200 µg/L dioxane. Dioxane was not removed in the control column even after 2 years of acclimation (146 pore volumes exchanged), implying the absence of dioxane degradation potential. Whereas some studies successfully demonstrated biodegradation in microcosms (He et al. 2018; Sei et al. 2013) and small flow through columns (Zhao et al. 2018), epidemiological analyses of plume data have shown dioxane to be persistent in many contaminated sites (Adamson et al. 2015). This implies that indigenous microorganisms may not always degrade this contaminant and that bioaugmentation may be justified to enhance bioremediation.

The observed recalcitrance of dioxane (Fig. 1) motivated us to investigate whether bioaugmentation with the metabolic dioxane degrader CB1190 could enhance sustained biodegradation of dioxane present at low (µg/L) levels. Bioaugmentation has a great probability of success when the added microorganism can fill a metabolic niche that is not being exploited by the indigenous microflora (Hurst et al. 2007). Thus, after 6 months, one of the acclimated columns (column 2, Table 1) was inoculated with CB1190 at the sampling port located 40 cm from the column's inlet. The effects of bioaugmentation was then measured by comparing the dioxane concentration profiles between the inoculated and unamended control columns.

In the bioaugmented column 2, dioxane decreased approximately 34% ± 5.6% after 10 days (~ 2 pore volumes exchanged) and for up to 3 months (~ 18 pore volumes exchanged) following inoculation, suggesting the resiliency of bioaugmentation and its potential to offer long-term enhanced biodegradation capabilities. Despite the observed decrease in concentration, however, dioxane concentrations downgradient from the inoculation point remained above established regulatory drinking water levels that range from 0.3 to 85 µg/L depending on the state (U.S.EPA 2014). Biodegradation of dioxane at µg/L levels seems difficult to achieve, apparently because of insufficient substrate concentration needed to induce enzyme activity and/or sustain bacterial growth. For instance, recent studies reported the low affinity of tetrahydrofuran monooxygenase (THM) in CB1190 for dioxane, with a half-saturation coefficient (Km = 235.8 ± 61.6 mg/L) that is four times higher than that of propane monooxygenase (PRM) from another dioxane degrading bacterium, *Mycobacterium smegmatis* mc2-155 (Km = 53.0 ± 13.1 mg/L) (Li et al. 2020). Furthermore, at low dioxane concentrations (250 ppb), THM had lower activity than PRM (i.e., 0.04 ± 0.01 versus 0.11 ± 0.01 µg dioxane/h/mg protein). Thus, CB1190 may not be as well suited as PRM-expressing bacteria for bioaugmentation of sites contaminated with low (ppb) levels of dioxane.

Other studies reported that complete degradation of dioxane at ≤ 1 mg/L may still be possible in the presence of additional co-substrates that support dioxane cometabolism (Barajas-Rodriguez and Freedman 2018). For instance, bioaugmentation studies conducted at the field-scale using the propanotroph *Rhodococcus ruber* ENV425a showed dioxane degradation to < 2 µg/L in the presence of a co-substrate (e.g., propane) (Lippincott et al. 2015). Nonetheless, it is very important to select and optimize appropriate biostimulation strategies on a case by case basis scenario. This is because dioxane removal may be hindered by preferential degradation of auxiliary substrates and associated consumption of oxygen and nutrients that would be otherwise used for dioxane degradation. Long-term exposure to co-substrates could also cause the loss of the catabolic plasmid (Li et al. 2017). Moreover, some co-substrates [e.g., 1-butanol and tetrahydrofuran (THF)] may exert competitive inhibition on the required monooxygenases. The adequate in situ distribution of some primary substrate such as propane can also be quite challenging but of paramount importance to significantly enhance dioxane degradation rates.

Bioaugmentation can significantly enhance the removal of higher (source-zone-like) dioxane concentrations

In the bioaugmented column fed 5 mg/L dioxane (column 3, Table 1), biodegradation was not observed within 5 days following inoculation and under continuous flow, indicating that the flowrate used was too high to ensure sufficient contact time for biodegradation to occur. To determine if additional residence time was required due to relatively slow biodegradation kinetics, the groundwater flow of 7.2 mL/h (V = 1.5 cm/h) was temporarily shut off for 2 days before sampling. As a result, approximately 92% of dioxane was removed but only near the inoculation port (Fig. 2a). The measured dioxane removal was significantly higher compared to the experiment with

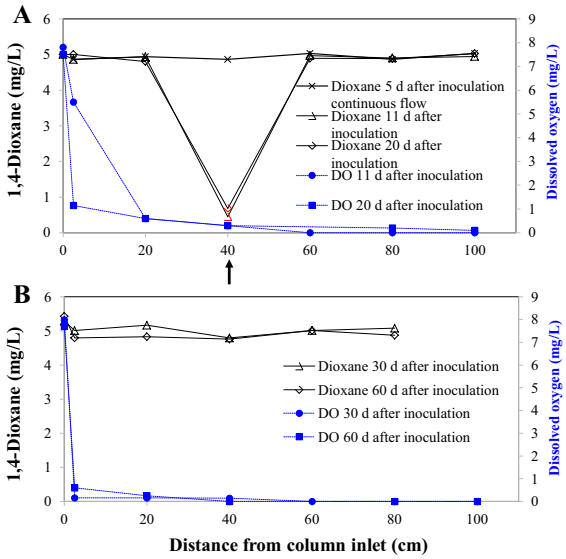

Fig. 2 Dioxane and dissolved oxygen concentration profiles in the bioaugmented column. This column was fed 5 mg/L dioxane. Bacteria was inoculated at sampling port 40 cm from the inlet (indicated by arrow). Negligible dioxane removal was observed 5 days after bioaugmentation at continuous flow rate of 7.2 mL/h (v = 1.5 cm/h). After temporarily stopping the flow for 2 days prior to sampling (i.e., from day 9 to 11, and from day 18 to 20), dioxane biodegradation (92%) was observed but only at the inoculation point (**a**). Decreasing the flow rate from 7.2 to 2 mL/h (v = 0.36 cm/h) after 20 days following inoculation to allow sufficient residence time for biodegradation led to anaerobic conditions and the loss of biodegradation activity (**b**)

dioxane fed at low levels (200 µg/L and 34% removal, Fig. 1). Thus, higher dioxane concentrations were needed to induce and sustain significant bacteria metabolic activity. The observed biodegradation under low DO concentration (~ 0.3 mg/L; Fig. 2), suggest that these oxygenases function at very low dissolved oxygen (DO) concentrations. Studies with the model toluene dioxygenase (TDO) expressing bacteria *Pseudomonas putida* F1, demonstrated that variations in DO (0.7–6.8 mg/L) had negligible effect on TDO activity, and significant degradation of 100 mg/L toluene achieved with 1 mg/L DO. Furthermore, TDO was expressed even at 0.1 mg/L DO (Costura and Alvarez 2000). Thus, dioxane biotransformation may proceed through a wide range of DO concentrations. Subsequently, groundwater flow was resumed at a slower seepage velocity (from 7.2 mL/h, V = 1.5 cm/h to 2 mL/h, V = 0.36 cm/h), but no dioxane removal was observed during the following 40 days under the lower flowrate (Fig. 2b).

CB1190 aggregates, restricting the spread of the catabolic potential beyond injection point

The survival of the added microorganisms and/or low bacterial transport through the aquifer material can pose limitations to bioaugmentation (Gannon et al. 1991). Therefore, DNA analysis of the column's aquifer material was conducted to determine the concentration of the injected bacteria and their potential spread along the column's length. Sediment samples were collected 3 months after the first inoculation (column 3, Table 1) when dioxane biodegradation was no longer observed as result of dissolved oxygen depletion. Distribution of the injected bacteria would be associated with groundwater transport because CB1190 is a non-motile bacterium (Mahendra and Alvarez-Cohen 2005).

The *thmA* gene that encodes tetrahydrofuran monooxygenase that initiates dioxane degradation in CB1190 was used as biomarker (Mengyan Li et al. 2013). The concentration of bacteria in the bioaugmented column 3 was relatively constant along the column's length ($\sim 10^4$ cells g of soil^{-1}), except at the inoculation port (located at 40 cm from the inlet) where the concentration was expectedly higher ($\sim 10^6$ cells g of soil^{-1}) (Fig. 3). The *thmA* gene was only detected at the inoculation port ($\sim 10^6$ cells/ g of soil^{-1}) coinciding with the only location where biodegradation was observed (Fig. 2a). The concentration of CB1190 inferred by quantification of *thmA* copy numbers is in agreement with theoretical

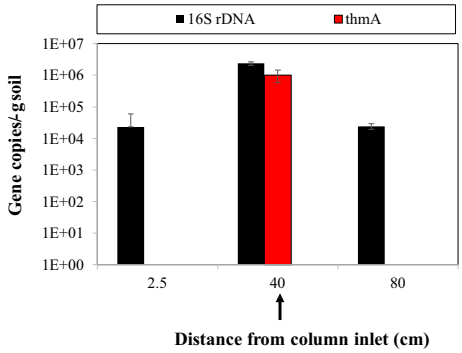

Fig. 3 Total bacteria (16S rRNA) and *Pseudonocardia dioxanivorans* CB1190 (*thmA*) concentration profile along the column's length. Migration of the introduced bacteria and its catabolic gene *thmA* beyond the injection port (arrow) was not observed even after 90 days. Data were presented as average ± standard deviation

estimations of $\sim 7 \times 10^5$ cells/mL assuming CB1190 yield [0.18 mg-cells/mg-dioxane (Li et al. 2010)] the concentration of dioxane consumed ($\sim$ 4 mg/L) (Fig. 2a), and 10^{-9} cells/mg-dry weight. We also tried another set of primers and probe that target *prmA* gene that has been previously encountered in dioxane degraders belonging to *Mycobacteria* genus. However, *prmA* was not detected, corroborating the absence of measurable biodegradation of dioxane by indigenous bacteria in the control column.

Detection of *thmA* in the soil collected 3 months after inoculation suggests the resilience of bioaugmentation and its potential to offer long-term enhanced biodegradation capabilities. Insignificant transport and dispersion of the introduced bacteria beyond the injection port was determined as indicated by the absence of *thmA* used as biomarker to trace distribution of CB1190 along the column's length. This may be attributed to CB1190 tendency to aggregate (Fig. 4) and be filtered by the aquifer material. Mobility and dispersion of bacteria through soil is generally preferable for bioaugmentation (Gannon et al. 1991). Nonetheless, most bacteria, will not easily move or disperse well through aquifer sediments. For instance a wild type bacteria (*Hydrogenophaga flava* ENV735:24) commonly used for bioaugmentation of sites contaminated with methyl tert-butyl ether (MTBE), did not move more than 6 cm through sediments in column studies (Streger et al. 2002). However, while aggregation of cells pose distribution challenges restricting the spread of the catabolic potential beyond the injection well radius of influence, cells adhesion and CB1190′s clumpy characteristic can be useful for the establishment of biobarriers. Application of bioaugmentation as a biobarrier was reported to reduce MTBE downgradient concentrations significantly (99% removal) (Salanitro et al., 2000). A biobarrier inoculated with an anaerobic consortium of dechlorinating bacteria (including *Dehalococcoides* spp.) was also reported effectively treat chlorinated solvents (MacFarlame et al. 2011), which are typically found as co-contaminants in dioxane plumes.

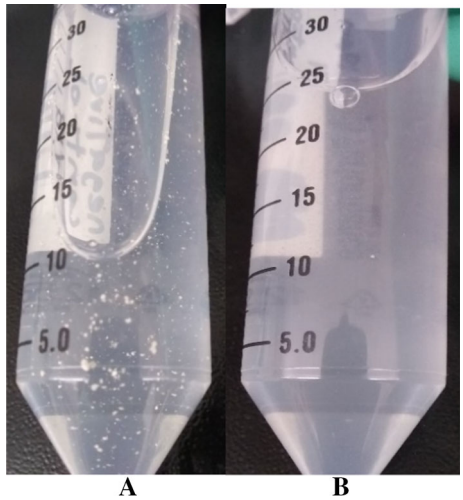

Fig. 4 *Pseudonocardia dioxivorans* CB1190 forms cells aggregates (**a**) that affects dispersion of bacteria and the spread of catabolic potential beyond the injection. Bacteria disaggregation can be achieved after water bath sonication but cells have the tendency to re-aggregate after time (**b**)

Bioaugmentation efficacy with CB1190 depends on dissolved oxygen availability

With the reduction in groundwater flow, oxygen replenishment decreased and so did the concentration of dissolved oxygen (DO) downgradient from the column's inlet, which resulted in anoxic conditions near the inoculation point (at 40 cm from inlet, DO = 0.0 mg/L; Fig. 2b) that hindered dioxane biodegradation. At this point, increasing groundwater flow to previous seepage velocity i.e., V = 1.5 cm/h, neither improved DO concentrations nor recovered biodegradation activity (Fig. 2b). The rapid depletion of DO inside the columns was likely due to the presence of 0.83% organic matter content and associated microbial biodegradation as well as reduced minerals species (e.g., Fe) that that contribute to DO consumption. Soil samples collected along the column's length showed the formation of black precipitates typically associated with precipitation of metals under anaerobic conditions (Hockin and Gadd 2003).

Hence, whether re-inoculation near the column's 3 (at 2.5 cm from inlet, Table 1) inlet where DO was still present could sustain biodegradation activity thus simulating traditional push and pull bioaugmentation strategies was investigated. Following re-inoculation of column 3, dioxane removal (24% $\pm$ 3.5) was consistently observed for over 18 days (Fig. 5). This relatively low removal efficiency was attributed to insufficient concentration of DO at the column inlet (0.17 $\pm$ 0.01 mg/L). To test this hypothesis, influent aeration was conducted (5 mL/h) after 20 days following re-inoculation in an attempt to offset the rapid

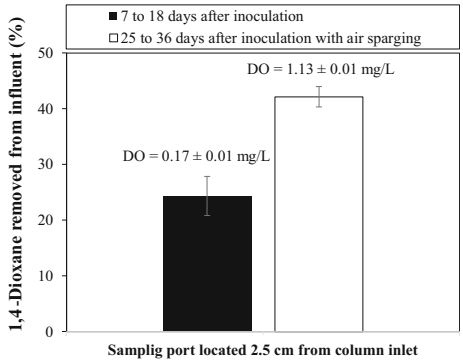

Fig. 5 Dioxane removal in the column's inlet after re-inoculation with CB1190. Influent aeration increased dissolved oxygen concentration significantly ($p < 0.05$), improving dioxane removal. Data presented as average ± standard deviation

depletion of oxygen inside the columns. Prior to influent aeration, hydrogen peroxide was tried alternatively as possible oxygen delivering strategy (aimed at 212 mg/L as H_2O_2); however, degradation of dioxane of approximately 78% was observed in the groundwater stock feeding solution within 24 h. After influent aeration, the concentration of dissolved oxygen increased to 1.13 ± 0.01 mg/L and removal efficiency was significantly ($p < 0.05$) improved to 42% ± 1.8% for the remaining 18 days of monitoring (Fig. 5).

The positive association between DO concentrations and removal efficiency suggests that additional injection of air or oxygen would enhance biodegradation activity and removal rates. However, increasing air flow rate above 5 mL/h could result in hydraulics disruption due to entrapped air bubbles causing groundwater hydraulic fluctuations (Wang and Feyen 1998). Batch simulations showed that dioxane biodegradation can be significantly impaired when DO concentrations are less than 2 mg/L (Barajas Rodriguez 2016). This could explain why dioxane was not completely removed in the bioaugmented column. *Pseudonocardia* spp. has been shown to thrive in microaerophilic conditions (Grostern and Alvarez-Cohen 2013) and survive under anaerobic conditions to degrade dioxane upon subsequent addition of oxygen (Polasko et al. 2018).

Compared to previous columns studies to assess biougmentation for treating high dioxane concentrations (Zhao et al. 2018), this work investigated longer term acclimation and conducted a more comprehensive evaluation of limiting factors such as DO and wider dioxane concentrations (mimicking source zone vs. dilute plume). Moreover, it corroborated limitations in bacteria distribution from the injection point, and offered a feasible approach to exploit CB1190 within these constraints (bioagumented and biostimulated biobarrier).

Conclusions

Bioaugmentation to enhance dioxane biodegradation was investigated in large flow-through aquifer columns inoculated with *Pseudonocardia dioxanivorans* CB1190. Dioxane was only biodegraded in the inoculated column even after 2 years of acclimation, suggesting that bioaugmentation might enhance dioxane removal at sites where intrinsic microbial activity is low or inexistent. However, significant dioxane removal is expected at locations with sufficient concentrations (mg/L levels) to induce and/or sustain the introduced bacteria, such as the vicinity of a source zone. Low dissolved oxygen concentrations commonly encountered in many contaminated sites most likely will hinder the activity of the introduced bacteria. To overcome such challenges, engineered approaches for delivering additional oxygen may be needed.

The transport and dispersion of the introduced bacteria beyond the injection port can benefit the spread of the catabolic potential for successful bioremediation. However, CB1190 forms clumps that limit bacteria mobility through the aquifer material (as demonstrated by the limited spatial distribution of the catabolic gene *thmA*), which restricts the augmented catabolic potential to the immediate vicinity of the inoculation port. Nonetheless, CB1190 can still provide a robust inoculum for bioaugmentation, particularly for biobarrier-based remediation approaches.

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