Original Article

Field Test of On-Site Treatment of 1,4-Dioxane-Contaminated Groundwater Using *Pseudonocardia* sp. D17

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ABSTRACT

Contamination of groundwater by 1,4-dioxane has been detected in many regions worldwide, especially in illegal industrial waste dumping sites. As 1,4-dioxane is a potential human carcinogen that is highly resistant to most conventional biological and physicochemical treatments, the development of a practical application method for treating 1,4-dioxane-contaminated groundwater is strongly desirable. Therefore, this study aimed to develop an on-site treatment system for 1,4-dioxane-contaminated groundwater using *Pseudonocardia* sp. D17, and assess the feasibility of the developed system for long-term treatment. This bacterial strain is capable of constitutively degrading 1,4-dioxane and using it as a source of carbon and energy. The on-site treatment of 1,4-dioxane-contaminated groundwater was performed in a 450-L continuous-flow bioreactor using strain D17 with a hydraulic retention time of 12 – 18 h. To maintain a sufficiently high microbial concentration, strain D17 was monitored by qPCR, targeting the *thmC* gene associated with 1,4-dioxane degradation, and inoculated when needed. 1,4-Dioxane removal to meet the Japanese environmental quality standard for groundwater pollution (0.05 mg/L) was achieved for nearly 3 months by the additional inoculation of strain D17. These results suggest that on-site treatment using strain D17 could provide a practical solution for the treatment of 1,4-dioxane-contaminated groundwater.

Keywords: 1,4-dioxane, groundwater contamination, bioremediation, on-site treatment, *Pseudonocardia* sp. D17.

INTRODUCTION

1,4-Dioxane is an emerging contaminant that is becoming a great concern. The compound is industrially important and is used as a solvent in chemical industries. It is also an unexpected and inevitable by-product of the production of ethylene oxide, ethylene glycol, and detergents such as alkyl ether sulfate. In recent decades, 1,4-dioxane contamination has become widespread in aquatic environments, especially in groundwater, in developed countries including Japan [1–4]. The main causes are illegal dumping of industrial waste and the incomplete treatment and leakage of landfill leachate and industrial wastewater. Owing to its physicochemical stability [2,5–7], 1,4-dioxane can persist for long periods of time once it is released into the environment. Because 1,4-dioxane is a

group 2B human carcinogen [8], the removal of 1,4-dioxane-contaminated water is of great importance.

Because of its stability, most conventional physical and chemical remediation methods are not effective for the decomposition of 1,4-dioxane [9]. Advanced oxidation processes (AOPs) with the combined use of ozone, hydrogen peroxide, and/or ultraviolet treatments have been shown to efficiently decompose 1,4-dioxane [9–12]. However, the use of AOPs involves high energy consumption, high operational cost, and a reduction of reaction efficiency by turbidity and coexisting reductive compound that scavenge hydroxyl radicals [9,13,14]. As such, their application for groundwater remediation is not practical.

By contrast, in parallel with the findings of bacterial strains capable of degrading 1,4-dioxane in different environments

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over the last two decades [3,15–17], previous studies have assessed the feasibility of several bioremediation strategies to treat 1,4-dioxane-contaminated groundwater, including natural attenuation, biostimulation, and bioaugmentation. The occurrence of natural attenuation, which depends on the intrinsic degrading abilities of indigenous microorganisms, has been verified in lab-scale microcosm studies [18–20] and in field surveys [21,22], indicating monitored natural attenuation as a potential remedial strategy. However, 1,4-dioxane degradation by natural attenuation, if any, only takes place after a very long period of time (*i.e.*, years) [22]. Biostimulation, which activates the degradative ability of indigenous microorganisms by supplementing auxiliary substrates, has been attempted with the substrates that induce 1,4-dioxane catabolic enzymes (*e.g.*, tetrahydrofuran [20]) or that do not induce enzyme expression but support growth (*e.g.*, 1-butanol [18,20]). Although biostimulation has been found effective in removing 1,4-dioxane compared to natural attenuation, identifying stimulating agents that enable continuous 1,4-dioxane degradation is still a challenge. Efficient 1,4-dioxane removal within much shorter periods than in natural attenuation and biostimulation is possible in bioaugmentation with an effective degrading strain [18]. Lippincott *et al.* [23] demonstrated at a field level that bioaugmentation with a propanotroph, *Rhodococcus ruber* ENV425, in combination with propane sparging, can be used for the *in situ* treatment of 1,4-dioxane-contaminated aquifer. However, the applicability of bioaugmentation in the natural environment is limited due to the rapid population decline of the exogenously introduced strain, as discussed previously [24–26]. In addition, most of the bacterial strains that are currently known to degrade 1,4-dioxane are actinomycetes that form filaments or mycelia. Such characteristics make it difficult to transport the bioaugmented strain into the contaminated aquifer in *in situ* bioaugmentation.

Pump-and-treat remediation, which treats the contaminated groundwater in an on-site bioreactor using effective 1,4-dioxane-degrading bacteria, can be proposed as an alternative strategy for the bioremediation of 1,4-dioxane-contaminated groundwater. This strategy, in principle, allows for the efficient and stable removal of 1,4-dioxane by controlling preferred conditions for degrading bacteria. Therefore, this study aimed to develop an on-site treatment system for 1,4-dioxane-contaminated groundwater using an effective 1,4-dioxane-degrading strain, *Pseudonocardia* sp. strain D17 (strain D17), and assess the feasibility of the developed system for long-term treatment. The on-site bioremediation was carried out in an illegal industrial waste dumping site

lying across Iwate and Aomori Prefectures in Japan where 1,4-dioxane had been detected in groundwater over the Japanese groundwater quality standard (0.05 mg/L). A specific quantitative PCR method was developed to monitor the fate of strain D17 during treatment. A series of lab experiments and field tests, including feasibility test to judge the applicability of strain D17 to 1,4-dioxane treatment in groundwater, short-term on-site treatments in batch and continuous-flow modes, and long-term demonstrative on-site treatment for nearly 3 months, were performed to demonstrate the effectiveness of the proposed 1,4-dioxane-contaminated groundwater treatment system for the first time.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Pseudonocardia sp. strain D17 [7] was used for the remediation of 1,4-dioxane-contaminated groundwater in this study. Strain D17 is capable of constitutively degrading 1,4-dioxane by using it as a carbon and energy source. Thus, 1,4-dioxane degradation by the strain does not require the addition of primary substrate that is needed to induce the degradation enzymes by co-metabolic 1,4-dioxane-degrading bacteria. In addition, it occurs even under low 1,4-dioxane concentrations that are often encountered in groundwater and prevent the induction of the degradation enzymes by inductive 1,4-dioxane-degrading bacteria. Further, strain D17 had the highest 1,4-dioxane ability among constitutive 1,4-dioxane-degrading strains, which were reported before 2013, in regard to maximum specific degradation rate and cell yield. Therefore, strain D17 would be a promising 1,4-dioxane-degrading strain to be applied to 1,4-dioxane treatment in groundwater. The strain was routinely cultivated in MGY medium (malt extract 10 g/L, D (+)-glucose 4 g/L, yeast extract 4 g/L) [15] or basal salt medium (BSM) [17] containing 1,4-dioxane as the sole carbon source in the laboratory. BSM supplemented with supplemented with corn steep liquor at 5 g/L and 1,4-dioxane at 3 g/L (BSM-CD medium) was also used for precultivation of strain D17 with a high cell yield. The initial pH of the medium was adjusted to 7.0 – 7.3, and the culture was incubated at 28°C.

Real-time PCR quantification of strain D17

Design of PCR primers

The genes found to be associated with the initial oxidation of 1,4-dioxane in strain D17 in a previous study [27] were selected for the design of PCR primers. The nucleotide sequences of the genes were extracted from the draft genome

data of strain D17. Candidate primers were designed with the Primer3 program [28,29], and their specificity was assessed with the sequences registered in GenBank using the Primer-Blast program [30]. Both programs were used with the default settings.

Real-time PCR conditions and standard DNA preparation

Real-time PCR quantifications using the optimum primers, thm-F (5'-TGA TTA TGT GGG GCT GGT TAT G-3') and thm-R (5'-CGA GGA AAG TTG TGT TCG TGA TG-3'), were carried out on a PikoReal real-time PCR system (Thermo Scientific, Waltham, USA). The conditions of real-time PCR were optimized in regard to primer concentration, 2-step or 3-step reactions, and annealing temperature. The optimized reaction mixture (20 μ L) contained 1 $\times$ GeneAce SYBR qPCR Mix α , 0.5 μ M of each primer, and 2 μ L of DNA template. The optimized thermal cycling process consisted of an initial activation at 95°C for 1 min, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s.

Standard DNA used for real-time PCR quantification was prepared as follows: PCR amplification products obtained from the genome DNA of *Pseudonocardia* sp. D17 with primers thm-F and thm-R were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), after which they were cloned using a DynaExpress TA PCR Cloning kit (pTAC-1) with a Jet Competent Cell (BioDynamics Laboratory, Tokyo, Japan). After cultivation of the recombinants, plasmids with cloned sequences were extracted and purified with a FastGene Plasmid Mini kit (Nippon Genetics, Tokyo, Japan). A serial dilution of the purified plasmids was used to generate a standard curve for real-time PCR quantification of the samples.

Evaluation of correlation with other cell density measurements

The correlation between the quantification results by the developed real-time PCR and by other cell density measurements, namely the measurement of optical density at 600 nm (OD_{600}) and acridine orange direct count (AODC) method, was assessed. The cells of strain D17 cultivated in MGY medium for 2 weeks were harvested and washed twice with sterilized saline. The washed cells were resuspended in phosphate buffered saline (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.44 g/L, KH_2PO_4 0.24 g/L, pH 7.4) containing 0.5% Tween 80, and the flocs were disrupted by sonicating on ice using an ultrasonic disruptor UD-201 (Tomy, Tokyo, Japan)

for 30 s with the condition of output 1 and duty 50. Then, the cultures showing different OD_{600} values were prepared by resuspending the washed cells in sterilized saline. AODC was performed as follows: the culture (0.01 mL) was mixed with 2.29 mL of a solution (NaCl 1.75%, AO 0.005%, and glutaraldehyde 0.87%) and reacted for 5 min. After filtration through a Nucleopore polycarbonate membrane (pore size 0.2 μ m; Advantec, Tokyo, Japan), the stained cells were counted with a fluorescence microscope (DX-50, Olympus, Tokyo, Japan). For real-time PCR quantification, the cells were harvested by centrifuging (8,500 $\times$ g, 4°C, 5 min) the culture and discarding the supernatant. DNA was extracted using the Cica Geneus DNA extraction kit ST (Kanto Chemical, Tokyo, Japan) and used for real-time PCR quantification with the above conditions.

On-site treatment of 1,4-dioxane-contaminated groundwater

Site description

On-site field tests were carried out at a 27-ha illegal dumping site across the boundary of the Iwate and Aomori Prefectures. At this site, illegal dumping of large amounts of industrial waste was found in 1999, and thereafter, the contamination of soil and groundwater by a variety of organic and inorganic compounds such as dioxins, oil, volatile organic compounds (VOCs), and heavy metals [31,32] was detected. 1,4-Dioxane contamination of groundwater was first detected in 2010, a year after the enforcement of the Japanese environmental quality standard for groundwater pollution of 1,4-dioxane (0.05 mg/L). The illegally dumped wastes had been subsequently completely removed from the site. Regarding the contaminated groundwater, in addition to the installation of impermeable walls, pump-and-treat remediation using a combination of physicochemical methods such as sparging, coagulation-sedimentation, activated carbon adsorption, and AOPs, was applied on the site to remove VOCs, heavy metals, and 1,4-dioxane in each prefecture. However, the level of 1,4-dioxane contamination in groundwater is still over the Japanese environmental quality standard. This study aimed to remove 1,4-dioxane in groundwater through the existing on-site groundwater treatment facility located in Iwate Prefecture.

Groundwater samples were collected for measurements of organic, nitrogen and phosphorus compounds in 2013 and 2014.

On-site biological treatment demonstration facility

The demonstration facility used for on-site treatment of

Fig. 1 Configuration of on-site biological treatment system used in this study.

1,4-dioxane-contaminated groundwater is depicted in **Fig. 1**. The contaminated groundwater flowed from the existing on-site groundwater treatment facility, which was equal to the influent without any treatment in the existing on-site groundwater treatment facility, was stored in the influent tank, and flowed into the biological treatment tank (450 L) with a working volume of 300 L. The effluent after biological treatment was filtered through the membrane separation units (2 × 4 modules; PTFE, pore size 0.1 μm , membrane area 0.1 m^2/module ; Sumitomo Electric Industries, Osaka, Japan), and flowed into the effluent tank. A disinfection tank containing sodium hypochlorite was installed to disinfect from any strain D17 that unexpectedly permeated through the membrane filtration, although disinfection was not needed throughout our study.

Preparation of strain D17 cells

For the flask test to examine the applicability of strain D17 for the treatment of 1,4-dioxane-contaminated groundwater, the cells of strain D17 were precultivated in MGY medium, harvested, and washed twice, as described above. For the other studies, the culture of strain D17 was prepared as follows. Strain D17 precultivated in MGY medium was inoculated in a 10-L bioreactor containing 7.5 L BSM-CD medium. The culture was then incubated at 28°C with a controlled pH of 7.0 ± 0.2 and continuous aeration at 2.5 L/min. Additional 1,4-dioxane was added whenever its levels fell below 50 mg/L. After 10–14 days, the cells were harvested

by centrifugation ($2,700 \times g$, 20 min), washed twice with sterilized saline, and applied for on-site treatment.

Preliminary batch studies

At first, the applicability of strain D17 for the treatment of 1,4-dioxane-contaminated groundwater was examined. The cells of precultivated strain D17 were inoculated into 300-mL Erlenmeyer flasks containing 100 mL of actual contaminated groundwater at an OD_{600} of 0.03, which corresponded to approximately 10 mg-dry cells/L. Inorganic constituents in BSM were supplemented at quarter strength, and the pH was adjusted to 7.3. Incubations were carried out at 28°C on a rotary shaker at 120 rpm, and temporal variations of 1,4-dioxane concentration were monitored.

The effect of scale-up on 1,4-dioxane degradation by strain D17 was examined by 1,4-dioxane degradation tests at laboratory and on-site scales. The groundwater used in the tests was supplemented with 1,4-dioxane (final concentration 6 mg/L), ammonium sulfate, and potassium hydrogen phosphate (C:N:P = 10:2:0.1). In the laboratory scale test, 0.65 L of the prepared groundwater was added to a 1-L bioreactor, and the cells of strain D17 prepared as above were inoculated at 24 mg-dry cells/L. Cultivation was carried out at 25°C with the aeration at 0.15 vvm. In the on-site scale test, 200 L of the prepared groundwater was added to a 450-L biological treatment tank, and strain D17 was inoculated at 26 mg-dry cells/L. Cultivation was carried out at 25°C with the aeration at 0.1 vvm. The 1,4-dioxane degradation profiles

at two scales were compared. To enable the comparison of the results in different scale tests, almost the same cultivation conditions were used. The aeration was conducted to stably maintain over 7 mg/L of dissolved oxygen (DO) concentration. In the laboratory scale test, the aeration at 0.1 vvm was not sufficient to meet the aim, and thus the aeration was conducted at 0.15 vvm.

Short-term continuous-flow treatment

To start the on-site short-term treatment, the cells of strain D17 prepared as above were inoculated in the biological treatment tank containing groundwater (200 L) supplemented with 1,4-dioxane (final concentration 20–50 mg/L), ammonium sulfate, and potassium hydrogen phosphate (C:N:P = 10:2:0.5), then cultivated as described above. The concentration of phosphorus, which was the potential limiting factor in the treatment, was increased compared to that used in preliminary studies to avoid unexpected depletion. 1,4-Dioxane, ammonium sulfate, and potassium hydrogen phosphate were supplemented additionally five times. 1,4-Dioxane and cell dry weight concentrations were monitored temporally.

After the start-up period, 100 L of groundwater was additionally pumped in the biological treatment tank. Then, continuous-flow treatment was initiated by continuously inflowing the influent groundwater and drawing the effluent through the membrane separation under varying hydraulic retention times (HRT) (12 or 24 h) and temperature (20°C or 25°C). During the operation of continuous-flow treatment, aeration was fixed at 0.07 vvm, and potassium hydrogen phosphate was supplemented to obtain a phosphorus concentration in the biological treatment tank of 0.5 mg-P/L. The concentration of 1,4-dioxane in the influent and effluent was monitored temporally.

Long-term continuous-flow treatment

Long-term continuous-flow treatment was initiated by flowing 200 L of influent groundwater and inoculating 86 mg-dry cells/L of strain D17 prepared as above in the biological treatment tank, after which influent groundwater was continuously inflowed. Continuous-flow treatment was operated for 85 d under the following conditions: temperature at $25 \pm 0.3^\circ\text{C}$, pH at 7.3 ± 0.2 , aeration at 0.07 vvm, and HRT at 12 or 18 h. The culture of strain D17 precultured as described above was additionally inoculated three times during the treatment period, with cell amounts of 14.2, 6.25, and 18.4 g for the 1st, 2nd, and 3rd additional inoculations, respectively. A shorter precultivation period (10 d) than the others (14 d) resulted in smaller cell amount in the 2nd

additional inoculation compared with those in the 1st and 3rd additional inoculations. To increase the 1,4-dioxane concentration in influent, 1,4-dioxane was artificially added to make the influent 1,4-dioxane concentration over 0.5 mg/L after 15 d. 1,4-Dioxane concentrations in the influent and effluent were monitored temporally. Additionally, strain D17 in the biological treatment tank was monitored using the developed real-time PCR method. DNA extracted from the samples as described above was used without dilutions to obtain the results of real-time PCR quantification because no inhibitory effect by environmental matrices was observed.

Analytical procedures

Samples for water quality measurements, except for the analysis of biochemical oxygen demand (BOD), were filtered through a cellulose acetate filter (pore size 0.45 μm ; Advantec Toyo Kaisha, Tokyo, Japan). Dissolved organic carbon (DOC) and dissolved total nitrogen (DTN) concentrations were measured using the total organic carbon analyzer multi N/C 2100 (Analytik Jena AG, Jena, Germany). Analysis of BOD was entrusted to the Environmental Control Center Co. Ltd. (Tokyo, Japan). Chemical oxygen demand (COD) and phosphate concentrations were measured using a DR2700 portable spectrophotometer (Hach, Loveland, USA) with the corresponding reagents and methods (Hach methods 8000 and 8048, respectively). The concentrations of ammonium and nitrate ions were determined using an Alliance 2695 ion chromatography system (Waters, Milford USA) equipped with a Waters 432 conductivity detector, as described previously [33]. The concentration of 1,4-dioxane was determined by headspace-gas chromatography-mass spectrometry using a GCMS-QP2010 plus (Shimadzu, Kyoto, Japan) combined with a TurboMatrix HS40 headspace sampler (PerkinElmer, Waltham, USA) and equipped with a Rtx-624 capillary column (60 m $\times$ 0.32 mm i.d.; film thickness 1.8 μm ; Restek, Bellefonte, USA), as described previously [17]. The dry cell weight concentration was measured by the method described previously [17].

RESULTS AND DISCUSSION

Design of real-time PCR method to monitor *Pseudonocardia* sp. D17

In our previous study, transcriptomic analysis was performed using whole genome microarray for strain D17 where oligonucleotide probes targeting a total of 7,511 genes that covered almost all the genome of the strain, which identified potential genes associated with the initial oxidation of

1,4-dioxane, the *thmADBC* genes, in the genome of strain D17 [27]. The *thmADBC* genes were found to catalyze the initial 1,4-dioxane oxidation by *Pseudonocardia dioxanivorans* CB1190 [34]. Therefore, several primers were designed using the sequences of *thmADBC* genes. An experimental assessment by conventional PCR found that the primers targeting the *thmC* gene, which was predicted to encode the monooxygenase component MmoB/DmpM, produced a 107-bp PCR product the most specifically and sensitively among the designed primers. The primers were thus designated as *thm*-F and *thm*-R and applied for the development of a real-time PCR method. It should be noted that *in silico* analysis showed that the primers can generate PCR products with the same length not only from strain D17 but also from strain CB1190 and tetrahydrofuran-degrading bacteria, *Pseudonocardia* sp. ENV478, *Pseudonocardia* sp. K1, and *Rhodococcus* sp. YYL due to very high sequence similarities of *thm* genes in these strains. This indicates the importance of prior assessment of the background level to apply the primers for specific monitoring of strain D17.

The conditions of real-time PCR using the primers *thm*-F and *thm*-R were experimentally optimized as described in the Materials and Methods section. Real-time PCR of the standard DNA with the optimized conditions generated a linear regression curve ($r^2 = 0.994$ to 0.999) over 6 orders of magnitude with PCR efficiency of 94.6% to 104.7% ($n = 4$). Furthermore, the results of real-time PCR quantification for strain D17 cultures correlated well with both OD_{600} values and AODC results (Fig. 2). These results indicated that the developed real-time PCR method can be applied for monitoring the fate of strain D17.

On-site treatment of 1,4-dioxane-contaminated groundwater using strain D17

Applicability of strain D17 to treat 1,4-dioxane-contaminated groundwater

The water quality parameters for the 1,4-dioxane-contaminated groundwater used during our study period in 2013 and 2014 are summarized in Table 1. The groundwater contained less than 0.3 mg/L of 1,4-dioxane. The concentration of readily-utilizable organic compounds expressed as BOD was relatively low (2.3–4.1 mg/L). Phosphate concentrations were low compared with nitrogen concentrations, which suggests that phosphate might be a limiting factor in biological 1,4-dioxane treatment and thus should be supplemented for efficient 1,4-dioxane treatment.

Preliminary studies on 1,4-dioxane degradation in actual contaminated groundwater using strain D17 performed at the

Fig. 2 Correlations of the *thmC* gene concentrations determined by the developed real-time PCR method with OD_{600} values (A) and cell concentrations determined by AODC (B).

flask level revealed that approximately 0.17 mg/L of 1,4-dioxane could be degraded below 0.05 mg/L within 15 h (Fig. 3A). In addition, 1,4-dioxane-contaminated groundwater treatment experiments at laboratory (1-L bioreactor) and on-site scales (450-L biological treatment tank) revealed that there was no noticeable difference in 1,4-dioxane degradation in terms of reactor scale when sufficient aeration and nutrient supplementation were carried out (Fig. 3B). These results clearly suggest the applicability of strain D17 to 1,4-dioxane degradation and removal from the contaminated groundwater at the study site.

Previous studies have reported that the co-presence of VOCs, such as trichloroethene (TCE) and 1,1-dichloroethene, diminished 1,4-dioxane degradation by several 1,4-dioxane-degrading bacteria by (1) competition between 1,4-dioxane and VOCs on monooxygenases that catalyze the initial oxidation of 1,4-dioxane and also the oxidation of VOCs, (2) repression of degradation gene expression, and (3) toxic effects

Table 1 Quality of 1,4-dioxane-contaminated groundwater at the study site*

Parameter (unit)	value
pH	7.51 – 7.85
1,4-Dioxane (mg/L)	0.0540.281
DOC (mg/L)	8.2 – 11.0
BOD (mg/L)	2.3 – 4.1
COD (mg/L)	26 – 33
DTN (mg-N/L)	18.5 – 36.5
NH ₄ -N (mg-N/L)	6.0 – 10.8
NO ₃ -N (mg-N/L)	3.4 – 4.9
PO ₄ -P (mg-P/L)	0.08 – 0.35

* 1,4-Dioxane concentration was determined four times in 2013 and five times in 2014, while the other parameters were measured twice in 2013.

Fig. 3 (A) Removal of 1,4-dioxane in actual contaminated groundwater using strain D17 at the flask level. Error bars indicate the data range in duplicate experiments. (B) Comparison of 1,4-dioxane removal performance in 1-L bioreactor (lab scale) and 450-L biological treatment tank (on-site scale). Experiments were conducted without repetition.

of themselves or their degradation products [35,36]. It has also been reported that some metals have inhibitory effects on 1,4-dioxane degradation by *P. dioxanivorans* CB1190 [37]. By contrast, 1,4-dioxane degradation rate by strain D17 observed in the contaminated groundwater (**Fig. 3**) was equivalent to that in BSM-based artificial groundwater (data not shown), which suggested a minute inhibition, if any, of 1,4-dioxane degradation by short-term exposure to the contaminated groundwater. Although detailed measurements of contaminants such as VOCs and metals were not performed in this study, their existence has been proven [31,32]. Thus, the results of this study may suggest that the concentrations of VOCs and metals with inhibitory effects in groundwater were below their effective levels on 1,4-dioxane biodegradation or that 1,4-dioxane degradation by strain D17 is less affected by VOCs and metals compared with that by other 1,4-dioxane-degrading bacteria to which negative effects by VOCs and metals were found, although further study is needed to clarify the reason. In addition, the possibility of inhibitory effects on 1,4-dioxane degradation of strain D17 by long-term continuous exposure of VOCs and metals is an important issue to be clarified.

Short-term continuous-flow treatment

The start-up of on-site 1,4-dioxane-contaminated groundwater treatment employing strain D17 was conducted in batch mode with a total of 6 sequential amendments of 1,4-dioxane and nitrogen and phosphorus sources. 1,4-Dioxane, which was sequentially added, was degraded to below 0.05 mg/L within 24 h, irrespective of the 1,4-dioxane concentration added (**Fig. 4**). In GC/MS analysis, the accumulation of any 1,4-dioxane degradation intermediates was not detected (data not shown). In addition, the concentration of DOC declined

Fig. 4 Performance of on-site 1,4-dioxane-contaminated groundwater treatment system during the start-up period operated with a batch mode. The arrows over the figure indicate the time when 1,4-dioxane was added. The treatment was conducted without repetition. The error bars for dry cell weight concentrations denote the data range in duplicate measurements.

concomitantly with 1,4-dioxane degradation. Although the remaining DOC concentration increased between the first and fifth batches, the cell concentrations in the biological tank declined in parallel, indicating that the increased TOC could be derived from the dead cells of strain D17. This suggests that 1,4-dioxane could be mineralized rapidly without detectable accumulation of intermediates in the on-site biological treatment system inoculated with strain D17. Given the low cellular yield of strain D17 when grown on 1,4-dioxane [7] and the limitation of readily-degradable organic compounds in groundwater (**Table 1**), a possible reason for the decline of the strain D17 cell concentration could be the limitation of carbon sources to support the growth of strain D17. In addition, strain D17 can form aggregates during cultivation in liquid media, as reported for other *Pseudonocardia* strains [17]. The adhesion of some cell aggregates to the observation window of biological treatment tank was observed (data not shown). Thus, the formation of aggregates might lead to the underestimation of cell concentration.

After the aforementioned start-up period, the operation of the on-site treatment system was changed to a continuous-flow mode for approximately 4 d under varying HRT (12 or 24 h) and temperature conditions (20°C or 25°C). In the

short-term trial, 1,4-dioxane treatment consistently met the Japanese environmental quality standard irrespective of the HRT and temperature conditions (**Fig. 5**). This confirmed the feasibility of continuous-flow treatment of 1,4-dioxane-contaminated groundwater in an on-site treatment system employing strain D17.

Long-term continuous-flow treatment

Following a series of preliminary studies and short-term trials, our demonstrative operation of a continuous-flow treatment system was carried out from September to November 2014 with the aim of verifying the long-term performance of the treatment system in continuous-flow mode. The variations of 1,4-dioxane concentration in influent and effluent and of the *thmC* gene concentration in the biological treatment tank during continuous-flow treatment of 1,4-dioxane-contaminated groundwater for nearly 3 months are shown in **Fig. 6**. Prior to the demonstrative study, the *thmC* genes measured by the developed real-time PCR method were confirmed to be below the detectable level in the contaminated groundwater used as the influent. A level of 1,4-dioxane below the quantification limit (0.005 mg/L) was achieved during the initial 15 d, under an HRT of 12 h.

Fig. 5 Performance of on-site 1,4-dioxane-contaminated groundwater treatment system during the short-term continuous-flow operation, after the start-up shown in Fig. 4, under different HRT and temperature conditions. The treatment was conducted without repetition.

Because the 1,4-dioxane concentration in influent during the demonstrative operation period was low compared to that in 2013, 1,4-dioxane was artificially added to the influent to make the influent 1,4-dioxane concentration over 0.5 mg/L after 15 d. Subsequently, the effluent 1,4-dioxane concentration increased gradually and over 0.05 mg/L of 1,4-dioxane remained after biological treatment, albeit for the stable cell concentrations of strain D17 at around 10^6 copies/mL (Fig. 6) and sufficient nitrogen and phosphorus compounds (data not shown). In addition, the elongation of HRT from 12 h to 18 h after 32 d did not achieve successful 1,4-dioxane removal. Therefore, the culture of strain D17, which was freshly cultivated, was additionally inoculated after 34 d, and this restored 1,4-dioxane treatment performance. However, the treatment performance slightly worsened again. Therefore, artificial 1,4-dioxane addition was ceased after 46 d, and the operation of on-site treatment system was continued for nearly 40 d. The cease of artificial 1,4-dioxane addition and the 2nd additional inoculation of fresh culture of strain D17 led the decline of effluent 1,4-dioxane concentration. However, a gradual increase of the 1,4-dioxane concentration in the effluent, although not exceeding 0.05 mg/L, was

Fig. 6 Performance of on-site 1,4-dioxane-contaminated groundwater treatment system during the long-term continuous-flow operation. 1,4-Dioxane was artificially added to the influent from 15 d to 45 d. Strain D17 was additionally inoculated three times as indicated by the downward-pointing arrows over the figure. The treatment was conducted without repetition. The gray dashed line denotes the Japanese environmental quality standard for groundwater pollution of 1,4-dioxane (0.05 mg/L).

observed with the decline of strain D17 concentration from 57 d. This worsening of the 1,4-dioxane treatment was restored again by additional inoculation of fresh strain D17 cultures. The decline of strain D17 concentration after the additional inoculations would be partially attributable to the adhesion of cell aggregates to the inner wall of biological treatment tank as mentioned above, although the cell death due to limited organic compounds was also affected. Even the adhesion of the cell aggregates to the tank wall reduced the contact to 1,4-dioxane, which resulted in the decline of 1,4-dioxane treatment performance. The results of these demonstrative operations, over a period of nearly 3 months, confirmed that 1,4-dioxane treatment of contaminated groundwater can meet the Japanese environmental quality standard using an on-site biological treatment system employing strain D17 under the continuous-flow mode with HRT of 12 – 18 h by successive inoculations. Because the influent groundwater was not sterilized, our results demonstrated that sufficient 1,4-dioxane treatment can be achieved in the presence of groundwater-derived microorganisms in the proposed system. In addition, the restoration of 1,4-dioxane treatment performance by additional inoculation of fresh culture of strain D17 three times emphasized the importance of active cells at certain density for successful treatment of 1,4-dioxane-contaminated groundwater.

Pseudonocardia sp. D17, which was employed in the on-site treatment of 1,4-dioxane-contaminated groundwater, has been previously studied for the treatment of model industrial wastewater containing 5 – 50 mg/L of 1,4-dioxane in a 1-L lab-scale bioreactor [38]. This earlier study immobilized strain D17 with a gel-entrapment technique to prevent washout of cells from the bioreactor and achieved the removal of 1,4-dioxane to meet the Japanese effluent standard (0.5 mg/L) in a continuous-flow mode under short HRTs of 3.4–20 h. Although this study did not apply the immobilization technique, 1,4-dioxane removal to meet the Japanese environmental quality standard (0.05 mg/L) was achieved in a continuous-flow mode by preventing cell washout by effluent separation using membrane filtration and by successively inoculating strain D17. It is worth noting that the treatment process applied here can mitigate the labor and cost for preparing gel-entrapped cells. However, this process required successive inoculations to maintain a sufficiently high performance of 1,4-dioxane removal. In particular, 1,4-dioxane treatment worsened after 15 d in the long-term demonstrative study, despite the stability of strain D17 concentrations which were monitored by the developed real-time PCR method. A reason for this phenomenon was

the repression of physiological activities, including 1,4-dioxane-degradation activity, of strain D17 by environmental and operational factors such as the limitation of readily-utilizable organic compounds (**Table 1**) and other trace elements in groundwater and/or the endogenous respiration caused by excess aeration. Previous studies have reported the possibility of the repression of 1,4-dioxane biodegradation by catabolite repression and/or metabolic flux dilution [20,39]. Given the low 1,4-dioxane concentration in the presence of other utilizable organic compounds (*i.e.*, BOD) in the contaminated groundwater, the 1,4-dioxane degradation ability of strain D17 might be repressed by catabolite repression and/or metabolic flux dilution. The mechanism of repressing the 1,4-dioxane degradation ability during the groundwater treatment should be investigated in detail to improve the stability of the performance of 1,4-dioxane treatment and to reduce the repetitive inoculations, which enhances the practical usefulness of the proposed treatment process. In addition, to establish an economically- and environmentally-friendly on-site 1,4-dioxane-contaminated groundwater treatment system employing strain D17, the followings still need to be studied further: (1) the exploration of substrates to specifically support the growth and degradative activity of strain D17, (2) the optimization of aeration strength for 1,4-dioxane mineralization in groundwater by strain D17, and (3) the development of a monitoring system (possibly real-time reverse-transcription method) of active strain D17 cells to appropriately judge the timing of additional inoculation. Further, the improvement of the treatment tank to diminish the adhesion of cell aggregates would be another issue for prolonged stable 1,4-dioxane treatment performance by strain D17.

CONCLUSIONS

This study developed a specific quantitative PCR method to monitor the fate of strain D17 and conducted a series of lab experiments and field tests to demonstrate the effectiveness of on-site treatment of actual 1,4-dioxane-contaminated groundwater at an illegal industrial waste dumping site. This demonstrative study revealed that 1,4-dioxane removal to meet the Japanese environmental quality standard for groundwater pollution (0.05 mg/L) can be achieved by the additional inoculation of strain D17 during the removal process. Although further studies to improve the treatment performance and economical and environmental friendliness are needed, our results suggested for the first time that on-site treatment using strain D17 could be a practical option

for the treatment of 1,4-dioxane-contaminated groundwater.

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