1,2-dichloroethane removal by *Bruguiera gymnorrhiza* (L.) Lamk and *Pseudomonas putida* BCC 23535

Sawitree Saiyood, Duangrat Inthorn, Alisa Vangnai and Paitip Thiravetyan

**ABSTRACT**

The purpose of this work was to investigate the ability of *Bruguiera gymnorrhiza* (L.) Lamk and *Pseudomonas putida* BCC 23535 to remove 1,2-dichloroethane (1,2-DCA) in contaminated water under hydroponic conditions. *B. gymnorrhiza* (L.) Lamk is a representative mangrove plant that can tolerate high levels of 1,2-DCA with a lethal dose 50 (LD50) of 34.67 mM. A concentration of 10 mM 1,2-DCA was chosen in the present study because it had no adverse effect on the plant. Using *B. gymnorrhiza* (L.) Lamk alone could completely remove 1,2-DCA over four cycles of 1,2-DCA exposure. *P. putida* BCC 23535 alone could also remove 1,2-DCA but the efficiency was lower than *B. gymnorrhiza* (L.) Lamk. The combination of *B. gymnorrhiza* (L.) Lamk and *P. putida* BCC 23535 could completely remove 1,2-DCA within 6 days, which was more effective than the individual plants alone. *P. putida* BCC 23535 can be applied in 1,2-DCA contaminated water in groundwater which *B. gymnorrhiza* (L.) Lamk can be used in above ground contaminated environments. Therefore, this study suggests that both *B. gymnorrhiza* (L.) Lamk and *P. putida* BCC 23535 are alternative ways to treat 1,2-DCA in contaminated environments.

**Key words** | 1,2-dichloroethane, bioaugmentation, *Bruguiera gymnorrhiza* (L.) Lamk, mangrove plant, *Pseudomonas putida*

**INTRODUCTION**

One of the most widely produced chlorinated solvents in industrial processes is 1,2-dichloroethane (1,2-DCA), which is used as a solvent for resins, oils, waxes, gums, paints, and finish removers. (HSDB 2009). Hughes *et al.* (1994) reported that it may be harmful to the kidneys, liver, and nervous system in humans and is a suspected carcinogen. Environmental pollution by 1,2-DCA is mainly due to poor disposal practices, accidental spillage, and illegal dumping (Janssen *et al.* 1985; Pham *et al.* 2009), which results in soil contamination and leaching into groundwater, as 1,2-DCA can dissolve in water up to 8.600 mg/l (86 mM) (Lawrence 2006). There are many reports on contamination of aquifers with various concentrations of 1,2-DCA, ranging from 10 mg/l (0.1 mM) to 2,500 mg/l (25 mM) (Klecka *et al.* 1998; Dyer *et al.* 2000; Nobre & Nobre 2004; Hunkeler *et al.* 2005). Therefore, 1,2-DCA that has contaminated soil and groundwater should be removed to prevent this compound from accumulating in aquifers (Mena-Benitez *et al.* 2008). The United States Environmental Agency (USEPA) regulates 1,2-DCA in drinking water at levels not higher than 0.005 mg/l to protect public health (USEPA 2009).

Several studies have reported on the degradation of 1,2-DCA using mainly microorganisms. Both aerobic and anaerobic bacteria are able to degrade 1,2-DCA and use it as a sole carbon and energy source (Janssen *et al.* 1985; van den Wijngaard *et al.* 1999). Various microorganisms that can completely degrade 1,2-DCA under aerobic conditions have been found, belonging to the genera *Xanthobacter*, *Pseudomonas*, and *Ancylobacter* (Janssen
et al. 1985; van den Wijngaard et al. 1992; Hage & Hartmans 1999). Moreover, P. putida is a rod-shaped, flagellated, Gram-negative bacterium that is found in most soil and water habitats. It is considered to be one of the best pollutant-degrading bacteria for both aromatic compounds and halogenated organic compounds (Chaudhry & Chapalamadugu 1991; Movahedyan et al. 2009).

However, one of the most promising technologies for remediation of 1,2-DCA and other chlorinated solvents in the environment is phytoremediation (O’Neill & Nzengung 2004). Phytoremediation has the potential to remove a variety of contaminants, is easy to maintain, and is a cost-effective removal method for both soil and groundwater (O’Neill & Nzengung 2004). Several higher plants, especially hybrid poplars, have been successfully used for remediation of trichloroethylene-contaminated soil and groundwater (Gordon et al. 1998; Orchard et al. 2000). Mena-Benitez et al. (2008) inserted two genes (DhlA and DhlB) for the degradation of 1,2-DCA in tobacco plants. This experiment was conducted under a hydroponic system. Plants can uptake 1,2-DCA via the roots, stems, and transfer to the leaves. 1,2-DCA was degraded in the leaves by DhlA and DhlB enzymes (Mena-Benitez et al. 2008). On the other hand, Naeasted et al. found several plants such as Arabidopsis, oil seed rape, rice, tobacco, and maize that have limited ability to degrade 1,2-DCA. These plants have no detected haloalkane dehalogenase activity; this is an indication that dehalogenase is involved in 1,2-DCA degradation (Naeasted et al. 1999).

In this research, we selected an evergreen mangrove plant, Bruguiera gymnorrhiza (L.) Lamk, which is distributed in the intertidal areas of tropical countries (Rahman et al. 2011). There are no previous reports on the use of this plant for 1,2-DCA remediation. This plant can grow in brackish water and there is no need to harvest frequency. In addition, Pseudomonas putida BCC 23535 was selected to remove 1,2-DCA. P. putida is a bacterium that is able to degrade both aromatic compounds and halogenated organic compounds (Chaudhry & Chapalamadugu 1991; Movahedyan et al. 2009) in the environment. Therefore, B. gymnorrhiza and P. putida BCC 23535 were applied, individually and in combination, to remove 1,2-DCA from water. The toxicity of 1,2-DCA in B. gymnorrhiza (L.) Lamk was also investigated.

### METHODS

#### Chemicals and reagents

1,2-DCA (99% purity) was purchased from Merck (Germany), and other chemicals, all of analytical grade, were purchased from Carlo Erba (Italy).

#### Plant culture conditions

All B. gymnorrhiza (L.) Lamk plants selected were at the same growth stage (around 30–50 g fresh weight, 50 cm height) and were purchased from a local plant nursery (Jatujak market in Bangkok, Thailand). The plants were carefully washed with tap water to remove dirt before being used in the experiment. B. gymnorrhiza plants were pre-cultured in tap water in a greenhouse at 35°C with average photosynthetically active radiation 920 μmol m⁻² s⁻¹ (12-h day/12-h night cycles).

#### Determination of 1,2-DCA toxicity in B. gymnorrhiza (L.) Lamk

B. gymnorrhiza (L.) Lamk plants were cultured in 500 ml of distilled water supplemented with 0, 10, 20, 40, and 60 mM 1,2-DCA in glass bottles. Each concentration of 1,2-DCA was carried out with 10 replicates. The glass bottles were wrapped with an aluminum foil sheet to protect against illumination of plant roots and algae growth. The mouths of the bottles were also wrapped with aluminum foil and sealed with paraffin film to prevent evaporation. The characteristics of the plants were observed during exposure to 1,2-DCA at all concentrations. Symptoms such as wilting, necrosis, and chlorosis were clearly visible on the leaves. In addition, probit analysis (Finney 1971) was used for determination of the lethal dose 50 (LD₅₀) values using StatPlus 2009 software. The concentration of 1,2-DCA that had no harmful effects on the plants (10 mM) was selected for further study.

#### Ability of B. gymnorrhiza (L.) Lamk to remove 1,2-DCA

Each whole plant was washed with distilled water and transferred to a 1 l glass bottle containing 500 ml of distilled water.
water supplemented with 1,2-DCA at the selected concentration (10 mM). Distilled water containing 10 mM of 1,2-DCA without a plant was used as the control. The plants were grown in hydroponic solution. Water samples were collected every 2 days and kept in a refrigerator at 4°C for 1,2-DCA concentration and pH analysis. The pH was determined using a pH meter (Mittler Toledo, USA). This experiment was carried out over four treatment cycles.

**Ability of *P. putida* BCC 23535 to remove 1,2-DCA**

*P. putida* BCC 23535 bacteria was purchased from the National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand. *P. putida* BCC 23535 was placed in a 250 ml flask supplemented with 100 ml of sterile LB (Luria broth) medium and cultured on a rotary shaker at 200 rpm and 30 °C overnight, until the desired cellular concentration (O.D. of 0.8) was obtained. *P. putida* BCC 23535 (3% v/v) was inoculated into a 250 ml flasks containing sterile distilled water supplemented with 100 ml of 10 mM 1,2-DCA. In this experiment, 1,2-DCA was used as the carbon source for bacteria. In addition, sterile distilled water containing 10 mM of 1,2-DCA was used as a control. The 1,2-DCA was filtered through a sterile filter of 0.45 μm pore size (Minisart, Germany). Both treated and control samples were shaken in a rotary shaker (Weiss Gallenkamp, UK) at 200 rpm and 30 °C for 6 days. The experiment was carried out using flasks closed with rubber bungs and sealed with paraffin film. Water samples were prepared as described above before analysis by gas chromatography (GC).

**Ability of *B. gymnorrhiza* (L.) Lamk and *P. putida* BCC 23535 to remove 1,2-DCA**

Prior to treatment, the whole plant was washed with sterile distilled water. Then, the plants were soaked in 1.5% (v/v) active chloride solution combined with 0.04% (v/v) Tween 80 for 5 min (Weyens *et al.* 2009) and washed several times with sterile distilled water. Sterile *B. gymnorrhiza* (L.) Lamk plants, and/or *P. putida* BCC 23535 (3% v/v inoculum), were transferred to 1 liter sterile glass bottles supplemented with 500 ml of sterile 10 mM 1,2-DCA. The experimental conditions were: (1) plant + *P. putida* BCC 23535 + 1,2-DCA; (2) plant +1,2-DCA; (3) *P. putida* BCC 23535 + 1,2-DCA; and (4) a control of 1,2-DCA alone. Water samples were collected every 2 days until the end of the experiment and kept in a refrigerator at 4 °C prior to 1,2-DCA concentration analysis.

**Determination of 1,2-DCA concentration by gas chromatography**

1,2-DCA was analyzed by a GC (GC-17A; Shimadzu, Japan) equipped with a flame ionization detector and an auto injector (AOC-20i, Shimadzu). The GC conditions were as follows: 30 m × 0.32 mm capillary column 0.25 μm thickness (Agilent J&W capillary column); and column oven, injector, and detector temperatures set at 60 °C, 140 °C, and 260 °C, respectively. Helium was used as the carrier gas at a flow rate of 25 ml min⁻¹. Before GC analysis, the water samples were centrifuged at 10,000 × g for 10 min using a microcentrifuge (Microfuge® 16; Beckman Coulter, USA), and then filtered through 0.2 μm cellulose acetate syringe filter (Verti Pure™ CA Syringe Filter, Thailand). A total of 1 μl of each filtrated water sample was injected.

**RESULTS AND DISCUSSION**

**Toxicity of 1,2-DCA in *B. gymnorrhiza* (L.) Lamk**

The results for the toxicity of 1,2-DCA in *B. gymnorrhiza* (L.) Lamk at various concentrations are presented in Table 1. When the plants were exposed to 10 mM of 1,2-DCA, they retained their green leaves, were healthy, and had a 100% survival rate. These results indicated that 10 mM 1,2-DCA had no adverse effect on plant leaves. However, at higher concentrations (20, 40, and 60 mM 1,2-DCA), plant leaves exhibited toxic symptoms such as wilting, chlorosis, and necrosis, as shown in Table 1. At 60 mM 1,2-DCA, the plants could not tolerate the toxicity and all plants died after 10 days. Therefore, 10 mM 1,2-DCA was selected for further study.

These results are in agreement with research by Mena-Benitez *et al.* (2008), who inserted bacterial dhlA and dhlB genes into tobacco plants to remediate 1,2-DCA from the culture media. They reported the plants remained healthy at 1,2-DCA concentration of 10 mM (Mena-Benitez *et al.*
In addition, the LD50 of 1,2-DCA toxicity to this plant was statistically evaluated at about 34.67 mM for this tested condition. However, this concentration was found in contaminated aquifers (Klecčka et al. 1998; Dyer et al. 2000; Nobre & Nobre 2004; Hunkeler et al. 2005). These results suggested that this plant may be used for 1,2-DCA removal from contaminated groundwater.

### Ability of B. gymnorrhiza (L.) Lamk to remove 1,2-DCA

*B. gymnorrhiza* (L.) Lamk plants were cultured in a hydroponic system at an initial concentration of 10 mM 1,2-DCA (one plant per pot). 1,2-DCA was completely removed after 5 days in the treatment conditions (plant + 1,2-DCA) in the first cycle. At the second, third, and fourth cycles, *B. gymnorrhiza* could completely remove 1,2-DCA from the system after 8, 8, and 6 days, respectively (Figure 1(a)). The removal rate decreased after the first cycle. This was due to the prolonged exposure of the plant to 1,2-DCA.

The plant leaves remained green, appeared healthy, and exhibited no symptoms during the first, second, and third cycles. In contrast, plants showing some yellow leaves (chlorosis) were observed at the fourth cycle in comparison with the plant control (without 1,2-DCA), but they still survived. The plant tolerates a total concentration of 40 mM, when given in four sequential doses of 10 mM each, but if the concentration is given in just one dose it does not (result observed in the toxicity experiment). In the control (without the plant), 1,2-DCA decreased to about 21%, 17%, 20%, and 28%, respectively, of the initial concentration at the end of the first, second, third, and fourth cycles of experiment. The decrease in concentration of the controls can be explained by oxidation (Figure 1(a)) (Stucki & Thuer 1995). The result of the first cycle of treatment showed a complete removal of 1,2-DCA at a rate of 2.09 mM day⁻¹, which was more than the other cycles. This phenomenon was probably caused by the concentration of 1,2-DCA at the first cycle t having no adverse effects on the plant. The second, third, and fourth cycles all exhibited a gradual increase in toxicity to the

### Table 1 | Toxicity of 1,2-DCA concentrations on *B. gymnorrhiza* (L.) Lamk

<table>
<thead>
<tr>
<th>1,2-DCA concentration (mM)</th>
<th>Total number of plants</th>
<th>Total number of surviving plants</th>
<th>Survival rate (%)</th>
<th>LD50 (mM)</th>
<th>Number of plants with appearance symptoms</th>
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Figure 1 | Ability of *B. gymnorrhiza* (L.) Lamk to remove 1,2-DCA in a hydroponic system over four treatment cycles: (a) remaining 1,2-DCA after treatment with *B. gymnorrhiza* (L.) Lamk; and (b) pH change in the system. Symbols: closed circles (blue) represent *B. gymnorrhiza* (L.) Lamk plant + 1,2-DCA and closed squares (red) represent 1,2-DCA without plant. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/ws.2016.060.
plants; this was especially evident in the case of chlorosis of plant leaves during the fourth cycle. The initial pH of the solutions with and without the plant in the first, second, third, and fourth cycles ranged from 6.88 to 7.31. During treatment, the pH in the treatment solutions (with plant) changed only slightly, ranging from 6.43 to 7.31, which are suitable conditions for plant growth. The changes of pH in the control (without the plant) ranged from 6.44 to 7.31 for all cycles over the experiment (Figure 1(b)). In case of real application, the system should contain soil as a supporter and nutrients for plant growth, which also reduced toxicity in the plants.

**Ability of *P. putida* BCC 23535 to remove 1,2-DCA**

In the experiment, *P. putida* BCC 23535 used 1,2-DCA as carbon source (at 10 mM 1,2-DCA). This bacteria was able to reduce the 1,2-DCA levels in the solution to approximately 2.56 ± 0.08 mM (reduced to 75% of the initial 1,2-DCA concentration) after 6 days (Figure 2).

This result relates to various reports where several microorganisms (such as *Xanthobacter*, *Ancylobacter*, and *Pseudomonas*) can easily degrade 1,2-DCA by the process of aerobic degradation and can use 1,2-DCA as a substrate for their growth, then degrade this compound until it enters the central metabolic pathway (Janssen et al. 1985; van den Wijngaard et al. 1992; Hage & Hartmans 1999; Nobre & Nobre 2004).

*P. putida* BCC 23535 might be suitable to apply in 1,2-DCA contaminated groundwater by pumping the microorganism and air in the groundwater. *P. putida* BCC 23535 enhanced 1,2-DCA removal in a shorter time more efficiently than only natural air pumping conditions.

**Ability of *B. gymnorrhiza* (L.) Lamk and *P. putida* BCC 23535 to remove 1,2-DCA**

In the treatment containing *B. gymnorrhiza* (L.) Lamk + 1,2-DCA, 1,2-DCA was completely removed from initial 10 mM 1,2-DCA within 6 days (Figure 3). In the system containing only *P. putida* BCC 23535, 1,2-DCA can only be removed up to 76% within 10 days. In addition, both *B. gymnorrhiza* (L.) Lamk + *P. putida* BCC 23535 can remove 1,2-DCA in a shorter time, within 5 days (Figure 3). The result confirmed that both *B. gymnorrhiza* (L.) Lamk and *P. putida* BCC 23535 enhanced 1,2-DCA removal. It was able to remove 1,2-DCA faster than the system containing only the plant or the bacteria alone. The control system (only 1,2-DCA) was able to remove 26% within 10 days due to natural aerobic oxidation.
CONCLUSIONS

The LD₅₀ of 1,2-DCA toxicity for B. gymnorrhiza (L.) Lamk was 34.67 mM. The result showed that B. gymnorrhiza (L.) Lamk was able to remove 10 mM of 1,2-DCA at the first, second, third, and fourth cycles. However, 1,2-DCA removal was faster in the first cycle; this may be caused by the concentration of 1,2-DCA in this cycle having no adverse effects on the plant. After that, 1,2-DCA gradually exhibited toxicity to the plants, especially chlorosis of the plant leaves at the fourth cycle. P. putida BCC 23535 alone was able to remove 10 mM 1,2-DCA up to 75% at 6 days. In addition, the combination of plant and organisms enhanced 1,2-DCA removal in a shorter time. In the case of using plants to treat 1,2-DCA, it might be suitable for use in above ground contaminated water. In addition, P. putida BCC 23535 can be applied to underground contaminated water and the combination of B. gymnorrhiza and P. putida can therefore be applied to treat 1,2-DCA in both above ground and underground contaminated water. Therefore, both B. gymnorrhiza (L.) Lamk and P. putida BCC 23535 can be used to treat 1,2-DCA in contaminated environments.

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