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Diethylene glycol removal by *Echinodorus cordifolius* (L.): The role of plant–microbe interactions

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ABSTRACT

This work presents the use of the plant *Echinodorus cordifolius* for remediating diethylene glycol (DEG) contaminated waters. The potential of this plant for treating DEG wastewater in a remediation system was observed. We found that *E. cordifolius* was able to remove DEG from wastewater, decrease the pH to neutral and remove approximately 95% of the chemical oxygen demand within 12 days. The plants can grow well in DEG wastewater, as indicated by their root and leaf biomass, which was found to be statistically similar to control. Wilting, chlorosis and necrosis were observed in DEG-treated plants, but the relative water content was not significantly different between control and treated plants, suggesting that the plants were able to take up and tolerate DEG present in the wastewater. Plant roots changed to black colour during experimental period. The fluorescence in situ hybridisation and bacterial enrichment confirmed that 4.30×10^5 cells/g of sulphate reducing bacteria and 9.30×10^8 cells/g of acid-producing bacteria were found associated with the plant roots. Furthermore, volatile fatty acids were found in non-sterile soil treatments, indicating that soil microorganisms are associated with DEG remediation. These results demonstrated that plants and bacteria have the ability to form a relationship to remove the organic contaminant DEG.

1. Introduction

Diethylene glycol (DEG; CAS number 111-46-6) is an organic compound prepared by heating ethylene oxide and glycol to form an ether bond joining two ethylene glycol molecules. The compound is used as a coolant, as a building block in organic synthesis and as a solvent in many industries [1,2]. Environmental contamination by DEG is of worldwide concern because of its toxicity to living organisms, for example, mice and humans [3–6]. DEG environmental contamination has been reported, especially in water [7].

The conventional method that is mostly used for treating DEG wastewater is chemical precipitation. However, this method is not efficient enough since the chemical oxygen demand (COD) of treated wastewater still remains higher than the acceptable standard of around 120 mg l^{-1}. It might be due to high solubility of DEG in water that makes it difficult to precipitate or eliminate from wastewater. The other methods for treating DEG such as nanofiltration, biodegradation, chemical oxidation with ozone and modified Fenton processes have been reported in many literatures [8–10]. Although, these methods are effective but they need specific operational conditions and the intermediate products may occur. Under such problems, phytoremediation could be an alternative method for treatment of DEG contaminated wastewater. The use of phytoremediation to remove organic contaminant has been reported in several studies [11–13] with the goal of completely mineralising the contaminants into relatively non-toxic constituents, such as carbon dioxide, nitrate and ammonia [14]. For example, *Jatropha curcas*, grasses, legumes and their associated bacteria have been studied for treatment of organic contaminant [15,16]. However, the use of phytoremediation in the field is still limited by our incomplete knowledge of the biological processes involved in plants, microbes and soil. Thus, a better understanding of the basic biological mechanisms would lend more efficiency to the management of phytoremediation.

Echinodorus cordifolius, or burhead, is an aquatic plant with a fibrous root system that is easily cultivated and requires low growth maintenance. It has a high growth rate and can grow in a wide range of environmental conditions. It also consumes a large quantity of water in a short time [17], which makes it a good choice for phytoremediation. Furthermore, this plant has root nodules that might be associated with rhizosphere bacteria, which has never

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been reported in a phytoremediation system. Therefore, this work presents the potential treatment of DEG-contaminated wastewater by the plant *E. cordifolius*, and the bacterial communities associated with its roots were studied to better understand the use of this plant in remediation systems.

2. Materials and methods

2.1. Plant culture conditions

E. cordifolius was grown in a greenhouse at the Remediation Laboratory of King Mongkut's University of Technology Thonburi (KMUTT), Bangkhuntien campus. Plants at the same growth stage (8 leaves, 500 g of fresh weight) were selected and cultured in glass cylinders (30 cm × 50 cm) containing Hoagland's solution [18] for a week prior to the start of the experiments.

2.2. Experimental design

DEG wastewater was obtained from the effluent of a plasticisers and stationary materials factory at Samut Prakan, Thailand. The wastewater had been pre-treated by coagulation, but it was still high in COD content because DEG remained in the solution.

The experimental design was random with six treatments to study the efficiency of each factor in the treatment system: three experimental groups, three control groups and an additional control of DEG wastewater, without plants, to evaluate DEG photodegradation and water evaporation. The three experimental groups were DEG wastewater + *E. cordifolius* plants, DEG wastewater + *E. cordifolius* plants + non-sterile soil (1000 g) and DEG wastewater + non-sterile soil (1000 g). The three control groups were distilled water + *E. cordifolius* plants, distilled water + *E. cordifolius* plants + non-sterile soil (1000 g) and distilled water + non-sterile soil (1000 g).

All treatments were done in triplicate in separate glass cylinders under a static system, in which the 3000 ml of DEG wastewater was not refreshed during the 20-day experimental period, but water was added to maintain a constant volume despite evaporation. In addition, sterile soil was used to study the effects of soil microbes in the remediation system. The experiment was conducted at an average temperature of 32 ± 5 °C, with 60.51 ± 8% relative humidity and 12-h light/dark cycles.

2.3. Water sample analysis

Water samples of 30 ml each were taken by autopipette from different depths and points in the glass cylinders over a period of 20 days. The system pH, total phosphorus (P) and COD were analysed following standard methods [19]. The DEG concentration was analysed by capillary gas chromatography (GC). The DEG removal efficiency was calculated by the following equation:

$$\text{DEG removal efficiency } (\%) = \frac{(C_0 - C_t)}{C_0} \times 100 \quad (1)$$

where C_0 is the initial DEG concentration (mg l^{-1}) and C_t is the DEG concentration at the time indicated (mg l^{-1}).

2.4. Plant sample analysis

Tissue samples of *E. cordifolius*, including roots and leaves, were taken. The plant tissue samples were immediately frozen in liquid nitrogen and stored at −20 °C for subsequent analysis. One gram of sample was ground and DEG was extracted by MilliQ water. Then the DEG was quantified by GC.

Signs of stress, such as leaf and root appearance, were observed, the number of roots and leaves were counted for dry weight and the relative water content (RWC) was calculated to determine the toxicity of DEG to the plant. The RWC calculation was performed as described in Barr and Weatherley [20].

2.5. DEG analysis using GC

The DEG concentration was analysed by GC using the following parameters: inlet split/splitless (split mode), flame ionisation detector (FID) and 30 m × 0.32 mm, Rtx-200 capillary column (internal diameter 0.32 μm). The experimental conditions for GC–FID were 1 μl injection volume, 250 °C inlet temperature, 250 °C column oven temperature and 280 °C detector temperature.

For analysis, the external standard technique was used. Stock standards were prepared by diluting DEG (GC grade, Sigma–Aldrich) in distilled water at a concentration of 3000 mg l^{-1}. DEG concentrations in the standard solutions for the calibration curve were 0, 100, 500, 1000, 1500, 2000, 2500, and 3000 mg l^{-1}.

2.6. Fluorescence in situ hybridisation (FISH)

The FISH technique enables both the isolation and determination of specific bacteria by specific probes, and FISH has been widely applied in the analysis of SRB communities [21]. FISH technique includes four steps: the fixation and permeabilisation of samples, hybridisation, washing away the unbound probe and the detection of labelled cells by microscopy [22]. *E. cordifolius* roots were washed with tap water and rinsed three or four times with sterile distilled water to remove the dirt. Then, the roots were ground and extracted by sterile distilled water for bacterial analysis.

The bacteria in *E. cordifolius* roots were fixed with 1 ml of 4% paraformaldehyde at 4 °C. All fixation steps were done overnight, which ensured that the gram-positive bacteria could also become permeable to the oligonucleotide probe. Subsequently, the samples were washed 3 times with 750 μl of phosphate-buffered saline (PBS) (10 mM sodium phosphate buffer, 130 mM sodium chloride; pH 7.2) to remove the fixative and then kept at 4 °C in a mixture of 1:1 ethanol and PBS.

The following Cy3-labelled oligonucleotide probes were used: EUB338 for the domain Eubacteria and SRB385 for most SRB. In addition, *Escherichia coli* and *Desulfovibrio desulfuricans* strain ESSEX were used as positive controls for EUB338-Cy3 and SRB385-Cy3, respectively. The formamide concentrations used for optimum stringency are shown in Table 1. In situ hybridisation was performed at 46 °C for 1.5 h. After washing away the excess oligonucleotide probes, the slides were stained with 4′,6-diamidino-2-phenylindole (DAPI) (6.26 μg ml^{-1} in 0.1 M Tris–HCl and 0.9 M NaCl, pH 7.2) for 5 min and rinsed with distilled water. The slides were mounted with an anti-fade agent (molecular

Table 1
Oligonucleotide probes used in this study with target groups and optimized formamide concentrations.

Probe	Specificity	Position[a]	Probe sequence (from 5′ to 3′)	Formamide[b] (%)
EUB338 (S-D-Bact-0338-a-A-18)	Most bacteria	338–355	GCTGCCTCCCGTAGGAGT	15
SRB385 (S-*-Srb-0385-a-A-18)	SRB of the δ-proteobacteria	385–402	CGGCGTCGCTGCGTCAGG	35

[a] Position in the 16S rRNA of *Escherichia coli* [23].
[b] Formamide concentration in the hybridization buffer [24,25].

Table 2
Summary means value (and standard deviation) of DEG wastewater treatment by *E. cordifolius* plant for 12 days under various conditions.

Parameter	pH	COD (mg l^{-1})	DEG (mg l^{-1})	P (mg l^{-1})
Control DEG wastewater	9.68 ± 0.78^{A}	2224.68 ± 48.36^{A}	2241.44 ± 16.12^{A}	3.54 ± 0.12^{A}
Plant + DEG wastewater	7.48 ± 0.36^{C}	512.48 ± 20.88^{C}	185.22 ± 0.02^{C}	0.12 ± 0.06^{B}
Plant + soil + DEG watewater	7.75 ± 0.16^{BC}	127.07 ± 16.12^{D}	0^{aD}	0.10 ± 0.02^{B}
Soil + DEG watewater	8.00 ± 0.14^{B}	672.17 ± 27.02^{B}	1038.63 ± 0.30^{B}	0.03 ± 0.02^{C}

Values in the same column with the same letter are not significantly different ($\alpha = 0.05$).
[a] Not found peak area.

probes) and the hybridised cells were photographed under an epifluorescence microscope.

2.7. Bacterial enrichment

Bacterial enrichment cultures showed that the bacteria associated with the roots are acid-producing bacteria (APB) and SRB. *E. cordifolius* roots were thoroughly cleaned under gentle running tap water to remove dirt, adhering algae and insect larva and rinsed three to four times in sterile distilled water. The roots were ground and extracted by sterile distilled water for bacterial analysis. The APB and SRB in *E. cordifolius* roots were enumerated using the most probable number (MPN) techniques. The MPN technique using tenfold dilutions in three parallel tubes was used for the bacterial enrichment. Glucose, lactose and sucrose were added to a concentration of 5 g l^{-1} as the substrates in synthetic media modified from Zhang and Noike [26] for enumeration of the APB population. Postgate's medium C was used to study the SRB population [27]. The presence of SRB was indicated by APB medium changed from red to yellow and the formation of a black FeS precipitate on Postgate's medium C.

2.8. Statistical analysis

A completely randomised design was used for the experiments. The data were statistically analysed using the Minitab 14 software to perform a one way analysis of variance (ANOVA), and significantly different means were assessed by a Tukey comparison test ($p < 0.05$).

3. Results and discussion

3.1. DEG removal efficiency of E. cordifolius

The remediation of DEG by *E. cordifolius* was studied. The experiment found that plants growing in soil (plant + soil + DEG wastewater) showed the best efficiency of DEG removal. Under this condition, approximately 95% of the COD could be removed, and no DEG remained in the solution by 12 days. However, in the plant + DEG wastewater and the soil + DEG wastewater conditions, DEG remained in the solution. The use of only plants or only soil for treating contaminated wastewater could remove approximately 77% and 70% of the COD, respectively (Table 2).

The average system pH of all treatments tended to decrease towards neutral (Table 2), perhaps because the volatile fatty acids such as acetic acid, propionic acid and butyric acid were produced by plant and microorganism during the experimental period (Fig. 1). Furthermore, the plant ability in order to control pH surrounding their roots by released H^+ and OH^- has been reviewed [28].

3.2. Toxicity of DEG on E. cordifolius

Symptoms of DEG toxicity to *E. cordifolius* were observed. Control plants looked healthy (Fig. 2A and C), whereas the DEG-treated plants showed wilting, chlorosis and necrosis of leaves caused by the toxicity of DEG (Fig. 2B). These symptoms were evident in plants grown under the soilless condition. After four days of DEG exposure, we found much black precipitate in the wastewater, and the plant roots had become black (Fig. 2D).

The treated plants decreased their water uptake, possibly to decrease DEG uptake into their cells, suggesting that DEG might have an effect on the water potential of plant cells, as plants try to recover the water balance by decreased uptake of the contaminant. However, the RWC of control plants was not significantly different with that of DEG-treated plants, indicating that perhaps this concentration of DEG was not toxic and, thus, did not inhibit the growth of the plant. The RWC was 93.90 ± 5.27% in control plants, 92.46 ± 5.21% for plants grown in the plant + soil + DEG wastewater condition and 89.80 ± 4.83% for plants grown in the plant + DEG wastewater condition.

The plants can grow well in DEG wastewater, as indicated by their root and leaf biomass, which was found to be statistically similar to control (Table 3). However, the plant growing under soil conditions can produce the weight higher than the plant under soilless conditions, indicating that elements in the soil might be enhancing the growth of plant. These results suggest that *E. cordifolius* was able to take up and tolerate the DEG present in the wastewater.

3.3. E. cordifolius root-associated microbes

Our experiment showed that this plant could remove DEG from contaminated wastewater. In addition, the colour of the plant roots was changed to black, indicating that sulphate-reducing bacteria (SRB) might be associated with the plant roots in the treatment system. In order to detect and characterise the microbial community associated with *E. cordifolius* roots, the FISH technique was applied. FISH analysis showed that the dominant organisms in the plant root were in the domain bacteria (Fig. 3B), and SRB hybridisation signals were detected in the plant root (Fig. 3D). Nonetheless, the hybridisation signalling showed that SRB (SRB385) is not the only bacterial species present, indicating that *E. cordifolius* roots possibly have associations with SRB and other bacterial species. The epifluorescence micrographs of *E. cordifolius* roots with bacteria stained with DAPI and with the probes EUB338 and SRB385 are shown in Fig. 3.

SRB are among the most common organisms in wetland treatment systems. They could contribute to removal of COD in the wetland treatment system [29]. The ability of SRB to degrade some glycols has been examined [30–32]; for example, *Desulfovibrio alco-*

Table 3
Biomass of *E. cordifolius* plant after cultures under various conditions for 20 days.

Treatments	Biomass (g dry weight plant^{-1})
Plant + distilled water (control)	114.55 ± 0.82^{b}
Plant + DEG wastewater	115.76 ± 0.50^{b}
Plant + soil + distilled water (control)	115.83 ± 1.48^{ab}
Plant + soil + DEG wastewater	117.03 ± 0.32^{a}

The data are presented as the means ± SD of three individual experiments. Values in the same column with the same letter are not significantly different ($\alpha = 0.05$).

holovorans can oxidise ethylene glycol and propylene glycol to acetate and/or propionate [33]. SRB are also present in anaerobic wastewater digestion systems with several types of bacteria. Among these bacteria, APB have a role in the degradation of macro organic compounds to hydrogen, ethanol and volatile fatty acids, which are later utilised by SRB to reduce sulphate. The symbiotic relationship of SRB and APB has been examined [34]. Previous reports have suggested that APB are very likely to be significant partners of SRB and possibly are associated with roots. Hence, bacterial enrichment of APB and SRB was observed. The positive tubes of the APB medium changed from red to yellow, and the formation of a black FeS precipitate on Postgate's medium C indicated that APB and SRB were found in the root sample. The plants and bacteria might have a specific mechanism to reduce DEG from wastewater. The numbers of APB and SRB in plant roots were approximately 9.30×10^8 cells/g and 4.30×10^5 cells/g, respectively.

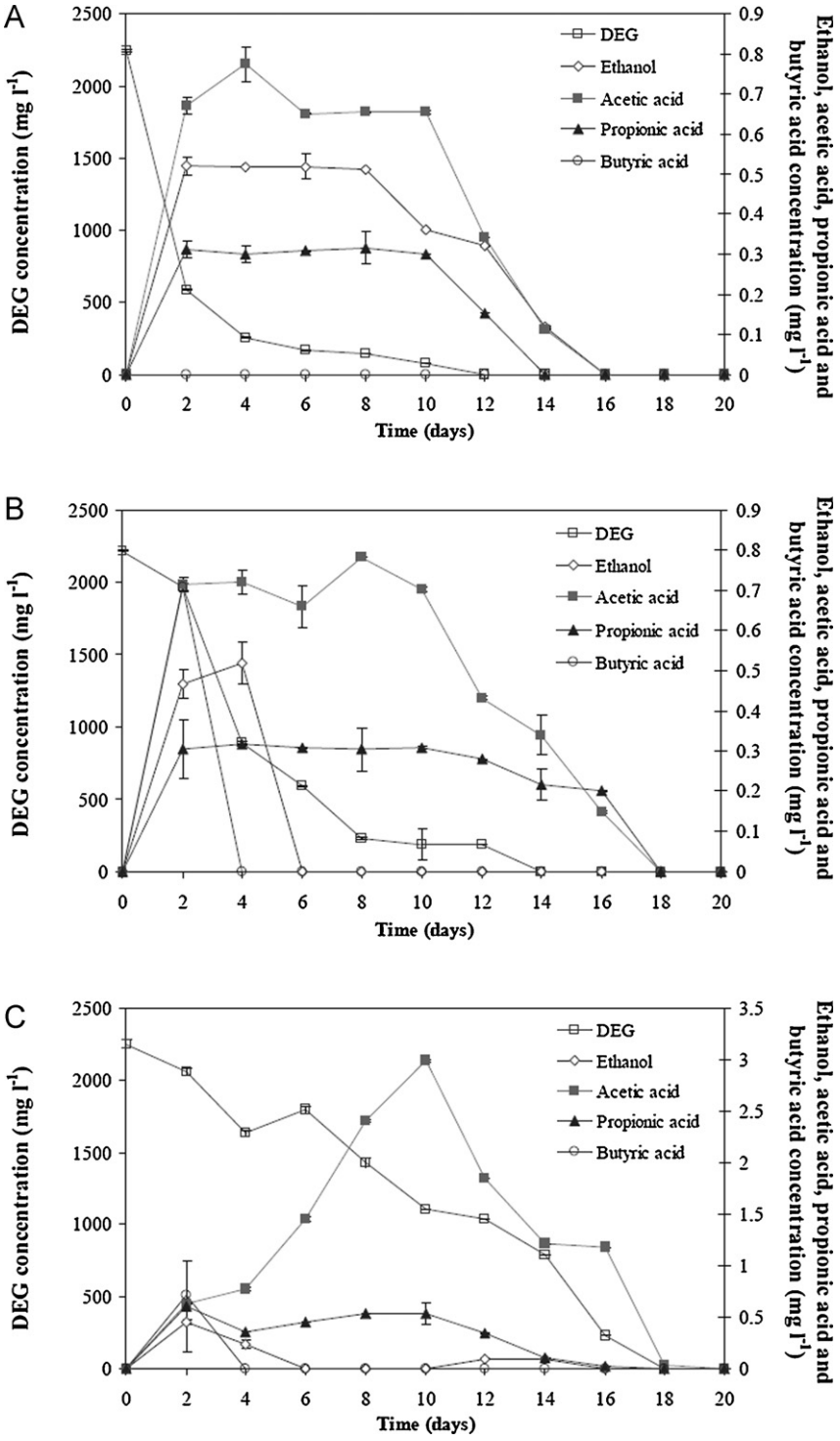

Fig. 1. Acid production during remediation of DEG-contaminated wastewater by *E. cordifolius* under various conditions; (A) plant + soil + DEG wastewater, (B) plant + DEG wastewater and (C) soil + DEG wastewater. Values are means ± standard deviations.

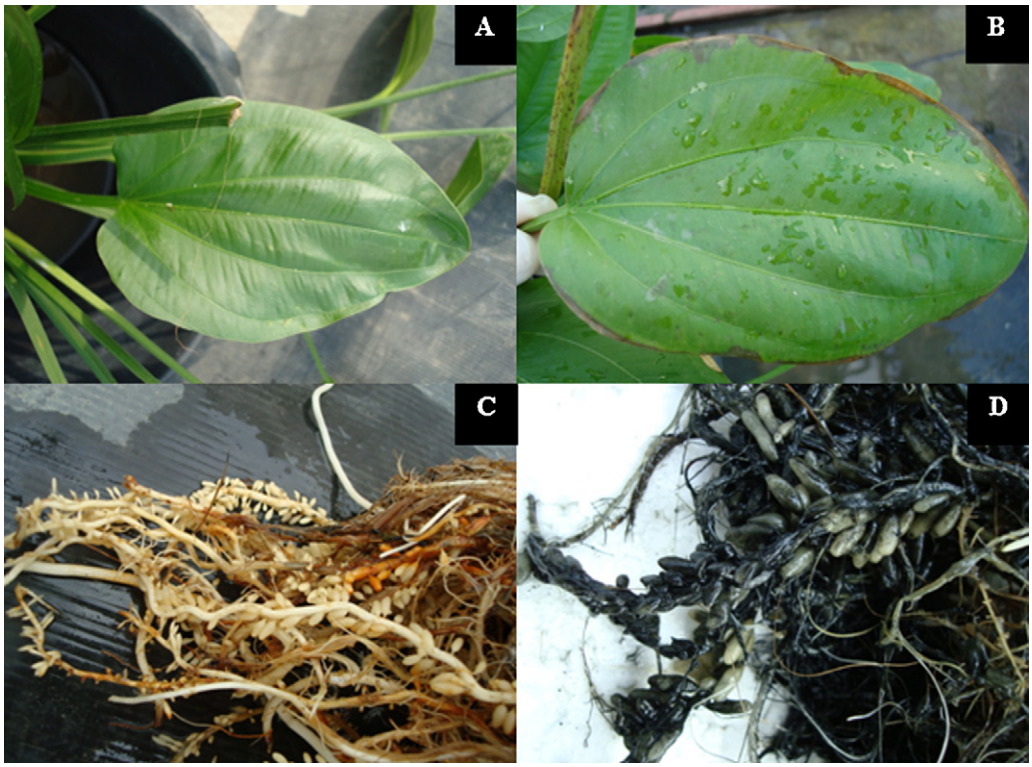

Fig. 2. Chlorosis, marginal leaf necrosis and black roots of *E. cordifolius* plants grown in DEG-contaminated wastewater for four days under soilless conditions. (A) Control leaf, (B) DEG-contaminated wastewater leaf, (C) control root and (D) DEG-contaminated wastewater root.

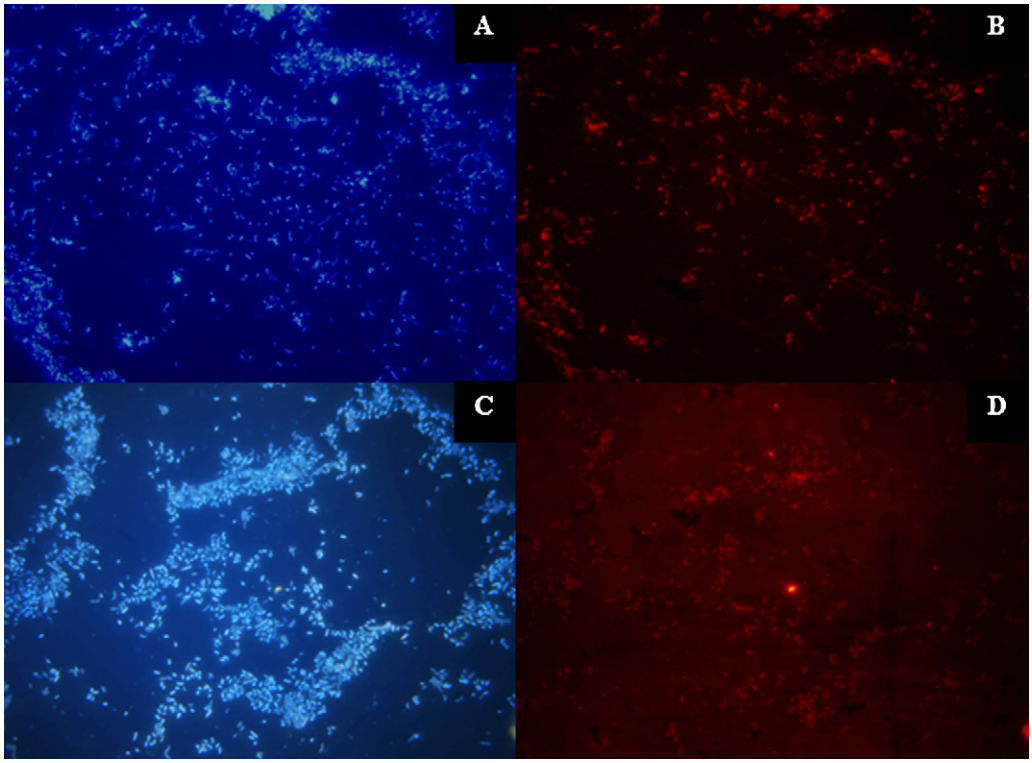

Fig. 3. Epifluorescence micrographs of bacteria. (A) DAPI staining of cells, (B) epifluorescence micrograph of microbial cells in an *E. cordifolius* root hybridised with the Cy3-labelled EUB338 probe for most bacteria (same microscopic field as (A)), (C) DAPI staining cells, (D) epifluorescence micrograph of microbial cells in *E. cordifolius* root hybridized with Cy3-labeled SRB385 probe (same microscopic field as (C)).

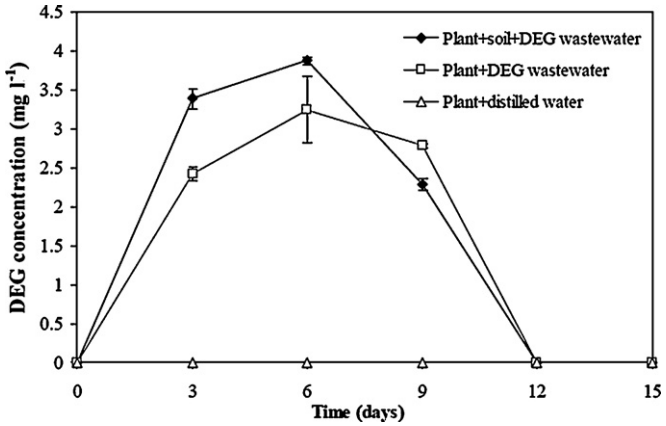

Fig. 4. DEG in leaves of *E. cordifolius* grown in DEG-contaminated wastewater. Values are means ± standard deviations.

3.4. DEG removal and biotransformation in phytoremediation

To confirm that DEG was taken up and translocated into *E. cordifolius* the plant tissues were analysed. The GC analyses of *E. cordifolius* leaves showed a peak in DEG uptake from the wastewater during nine days of experiment. No DEG was found in the plant tissue on day 12 (Fig. 4), but unknown peaks were found instead. The results indicated that a portion of the DEG in the wastewater was removed by root uptake of DEG into the cells and transport to the leaves and that DEG may be transformed into other forms. However, the unknown peaks in DEG-treated plants do not exactly match with GC–MS library data. Therefore, further study of the metabolic breakdown of DEG in *E. cordifolius* is needed.

In addition, while the DEG concentration in wastewater was decreased, ethanol, acetic acid and propionic acid were found in the wastewater (Fig. 1). These compounds were not found in the control sterile-soil condition. Thus, the breakdown products of DEG when degraded by soil microorganisms are propionic acid, ethanol and acetic acid. These breakdown products could be completely degraded aerobically via the tri carboxylic acid cycle or anaerobically via methanogenesis [35]. Therefore, soil microorganisms are also associated with this remediation system.

The total P in wastewater was decreased from about 3.54 mg l^{-1} to around 0.03 mg l^{-1} within 3 days by plant uptake and soil adsorption. However, after 12 days this content then increased to about 0.1 mg l^{-1} in plant growing under either soil or soilless treatments. Table 2 shows statistically similar P content for plant growing under either soil or soilless treatments. The control is indicated as 'a' and the remaining three treatments are indicated as 'b' and 'c'. The results indicated the P contents were released by the plants. The loss of P from plant roots is the subject of varied discussion; for example, Bieleski and Ferguson [36] suggested that P efflux is at least partially under metabolic control and is a component of the mechanism whereby P balance is maintained in plants. Sakano [37] presented data suggesting that plant roots could release phosphate to outside cells for balancing the pH level for survival and growth. The organic degradation and removal of phosphate by bacteria has been reported [38]. Moreover, the biodegradation rates of glycols were increased by one order of magnitude after phosphate addition [39].

From our experiment, when new DEG wastewater was added under each condition, in each case the concentration of DEG decreased faster than in the first cycle of the experiment (data not shown). However, *E. cordifolius* growing with soil still showed the highest efficiency of DEG wastewater treatment. Therefore, the occurrence of phosphate in the wastewater during our experiment might have enhanced the growth of microorganisms and resulted in DEG degradation. These results indicated that the plant *E. cordifolius* and bacteria show the ability to remove DEG from contaminated wastewater. Based on our results, we suggest that phytodegradation or phytotransformation and microbial degradation were involved in this remediation system.

4. Conclusions

Our overall results suggest that *E. cordifolius* can potentially be used for the remediation of DEG-contaminated wastewater. The plant can take up and transport DEG, and it may transform DEG into other forms. The occurrence of ethanol, acetic acid and propionic acid while the DEG concentration decreased in the wastewater suggests that soil microbes are associated with this DEG-remediation system. FISH and bacterial enrichment analyses confirm that SRB and APB are associated with the plant roots. The plants could decrease the DEG concentration and pH stress by releasing phosphate to outside cell. The appearance of phosphate in plants growing under both the soil and soilless conditions may enhance the growth of microorganisms and result in DEG degradation. Plants growing with soil showed the best efficiency of DEG removal. Therefore, the *E. cordifolius* plant, root-associated bacteria and soil bacteria are all involved in DEG remediation. However, the relationship of this plant with the microorganisms merits further study. Understanding the biochemical mechanism of DEG degradation by the plant and microorganisms might help us understand how the bacteria are associated with plant in the system. This knowledge can also be applied in the removal of other organic contaminants from wastewater.

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