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# Using *Cyperus alternifolius* for treating ink factory wastewater: effect of microbial communities in the system

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**ABSTRACT**

In this study, the performance of phytoremediation by *Cyperus alternifolius* on treating ink factory wastewater is investigated. The wastewater from ink factory showed high levels of colorants, vehicles, solvents, and additives that affected to high total dissolved solids (TDS), chemical oxygen demand (COD), biochemical oxygen demand (BOD), and total Kjeldahl nitrogen (TKN) that are approximately 6,426, 987, 258, and 156 mg $L^{-1}$, respectively. Phytoremediation of wastewater by *C. alternifolius* is an effective method to remove pigments, organic carbon and nitrogen compounds. Plant absorption, soil adsorption, including microbial activities played important roles for cleanup of wastewater as final step for removing TDS, COD, BOD, ammonium, nitrate, and colors. Using denaturing gradient gel electrophoresis technique and then sequencing of partial 16S rDNA revealed that the microbial community was aerobic and facultative-anaerobic groups. The major group affiliated with Proteobacteria was *Pseudomonas*, *Diaphorobacter*, *Sulfurospirillum*, and especially *Azospirillum*, as a dominant group. The result confirmed that the efficient process of plant and microbe cooperation for the treatment of wastewater was a suitable and sustainable technology. In addition, the data of microbial diversity can be useful in understanding plant–microbe interactions for improving system performance.

*Keywords: Cyperus alternifolius*; Ink factory wastewater; Phytoremediation; Microbial community

## 1. Introduction

The ink factory wastewater contains high number of colorants, auxiliary solvents, and additives [1] that results in high concentrations of suspended solids (SS), chemical oxygen demand (COD), and nitrogen compounds, and dark black color. Discharges of this wastewater directly affect the environment and these toxic substances may cause many operational problems in biological wastewater treatment systems [2]. Treatment of wastewater has to be used, and there are many methods for COD, nitrogen compounds, and color removal, which affect cost.

There are many methods of wastewater treatment. Some examples include coagulation [3], precipitation [4], electrochemical oxidation [5], and the adsorption method with zeolite [6] or activated carbon [7]. Although these methods could reduce the color and turbidity of wastewater, the color, COD, and nitrogen compound concentrations still remained in the system. Biological processes

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have been developed based on biological nitrification and denitrification. However, it was difficult to control the systems' levels of dissolved oxygen (DO), pH, substrates, temperature, sludge age, etc. Moreover, pigments have difficulty in biodegradation [8,9]. Hence, there is a need for a treatment process to remove color, SS, COD, and nitrogen compounds from this wastewater in a short time and low cost.

Thus, in this study, the ultimate goals of a complete treatment of ink factory wastewater are simplicity, efficiency, short time, and low cost. To reduce the pigments, COD, biochemical oxygen demand (BOD), total dissolved solids (TDS), ammonium, and nitrate values in wastewater, phytoremediation was applied for the cleanup wastewater by *Cyperus alternifolius*. While pH, DO, and oxidation reduction potential (ORP), as parameters for observed information about the optimization of biological process and improved biological nitrogen removal efficiency. In addition, the diversity of microbial communities was studied using the polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) analysis of 16S rDNA fragments to determine the relation of micro-organisms in the system. The identification of groups of microbes could explain numerically dominant organisms, which may be of critical importance to system performance.

## 2. Materials and methods

### 2.1. Ink factory wastewater and chemical pretreatments

Ink factory wastewater used in the experiment was collected from a factory that produced ink for the packaging of flexography printing (Thailand). The major compositions were pigments, acrylic resins, monoethanolamine, $NH_4OH$, and additives that had high levels of COD, total Kjeldahl nitrogen (TKN), SS, TDS, and high strong color and odor as shown in Table 1. Treating wastewater before discharging into the environment is crucial. The original wastewater was pretreated with sulfuric acid in order to precipitate pigments and ink. The original wastewater was poured into a plastic container. Sulfuric acid was added directly into the original wastewater and stirred continuously until flocculation was appeared (system pH of solution about 3), and then, the solution was allowed to settle. After precipitation, the supernatant was collected to adjust the pH to a neutral pH with $Ca(OH)_2$. Thereafter, *C. alternifolius* was used to treat the pretreated wastewater because it was still contained high COD, BOD, TDS, and color.

### 2.2. Phytoremediation

*C. alternifolius*, about 2–3 months old, was obtained from a garden market, Bangkok, Thailand. Plants were washed carefully with tap water to remove soil and any contaminated substances from their roots. After that, they were acclimatized in tap water for one week without addition of any nutrient. Plants of similar sizes and similar fresh weights were used to the following experiments. Clay soil obtained from a garden market was used to support the plant roots. Soil samples were air-dried and ground, then, mixed for homogeneous soil. The soil properties are shown in Table 2.

The experiments were conducted in glass containers (17 cm in diameter and 28 cm in height). Under soil-containing pretreated wastewater, 300 g of soil was placed in each pot. While about 300 g each of healthy plants and soil were used under plant grown in soil-containing pretreated wastewater. After that, 2 L of pretreated wastewater was added in the pots. In addition, 2 L of wastewater was used as control conditions (no plant and soil). The solution levels in each container were marked in order to adjust to the same levels due to evaporation and plant respiration with tap water before sampling the solution. One hundred and fifty milliliters of wastewater was taken from the pot for analysis. All experiments were performed in three replicates in each treatment under natural sunlight conditions and in ambient temperature (30°C ± 2°C). This experiment was operated in a batch experiment, no recirculation of the wastewater. The sample solution was analyzed for organic carbon and nitrogen compounds at day 1, 3, 5, and 7. The results showed that organic carbon and nitrogen compounds concentrations were rapidly reduced and lower than the effluent standards at day 7. Therefore, 7 d for wastewater treatment was the shortest time in order for the effluents to pass the standards. In a real system, a constructed wetland will be performed in a continuous system and recirculated of the wastewater in case of the effluents did not pass the effluent standards.

Table 1
Characteristics of ink factory wastewater before and after treatment by chemical substances

| Parameter | Original wastewater | Pretreated wastewater | Industrial effluent standards[a] |
|---|---|---|---|
| Color and odor | Strong color and odor | Transparent orange red color | Not objectionable |
| COD (mg $L^{-1}$) | 12,601 ± 151 | 987 ± 86 | ≤400 |
| pH | 8.45 ± 0.01 | 7.05 ± 0.01 | 5.5–9 |
| TKN (mg $L^{-1}$) | 165 ± 26 | 156 ± 4 | ≤200 |
| $NH_4^+$–N (mg $L^{-1}$) | – | 145.57 ± 2.03 | – |
| $NO_2^-$–N (mg $L^{-1}$) | – | 0.0135 ± 0.001 | – |
| $NO_3^-$–N (mg $L^{-1}$) | – | 27.54 ± 0.07 | – |
| MEA (mg $L^{-1}$) | 173 ± 21 | 39 ± 0.39 | – |
| SS (mg $L^{-1}$) | 1,176 ± 13 | 30 ± 6 | ≤150 |
| TDS (mg $L^{-1}$) | 6,781 ± 34 | 6,426 ± 187 | ≤5,000 |
| BOD (mg $L^{-1}$) | – | 258 ± 11 | ≤60 |

[a]Industrial Effluent Standard Notification from the Ministry of Science, Technology and Environment, 1996, Thailand.

Table 2
Chemical properties of soil

| | |
|---|---|
| pH | 4.3 |
| Sand (%) | 16 |
| Silt (%) | 16 |
| Clay (%) | 68 |
| Organic carbon (%) | 1.78 |
| P (mg $kg^{-1}$) | 6 |
| K (mg $kg^{-1}$) | 169 |
| Ca (mg $kg^{-1}$) | 3,501 |
| Mg (mg $kg^{-1}$) | 395 |
| Cation exchange capacity (CEC) (cmol $kg^{-1}$) | 30.20 |

### 2.3. Analytical methods

#### 2.3.1. Organic carbon and nitrogen analysis

Sample solution was sampled from glass containers and then rapidly filtered with filter paper (Whatman GF/A) to remove solids. The concentrations of COD, TKN, $NH_4^+$ – N, $NO_3^-$ – N, BOD, and TDS were analyzed periodically starting from 0 h and completely investigated within 7 d according to standard methods [10]. COD was measured by the reactor digestion method using COD digestion vials (HACH Odyssey DR/2500 spectrophotometer, USA). TKN was measured by the digestion–titration method using an analyzer (Kjeldatherm, Gerhardt, Germany). Ammonium was measured by the titration method using a rapid distillation system (Vapodesk 20, Gerhardt, Germany), and nitrate was measured using HACH methods by the cadmium reduction method using NitraVer 5 reagent.

#### 2.3.2. DGGE analysis and sequencing

Wastewater solution samples under pretreated wastewater at day 0, pretreated wastewater day 7, and soil-containing pretreated wastewater at day 7, and plant grown in soil-containing pretreated wastewater at day 7 conditions were collected for genomic deoxyribonucleic acid (DNA) extraction. Collected samples were centrifuged at 4,000 rpm for 20 min, and then, the pellets were collected. The pellet samples were stored at –20°C for the preparation of cell lysis. Samples were suspended in 10% CTAB (hexadecyltrimethylammonium bromide) lysis buffer, 1 M Tris-HCl pH 8.0, 5 M NaCl, and 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8.0. Lysozyme and protinase K were added to the final concentration of 10 mg/mL and mixed gently. Tubes were incubated at 37°C for 2 h and vortexed every 10 min. The supernatant was collected and transferred to a fresh microfuge tube after centrifugation at 4,000 rpm for 15 min. Then, samples were extracted, a 1 volume chloroform:isoamyl alcohol (24:1) was added to the mixture when it cooled, and then mixed for 30 s. A supernatant was collected and transferred to a fresh microfuge tube after centrifugation at 14,000 rpm for 5 min. A 0.6 volume of isopropanol was added, and the mixture was incubated at room temperature for 1 h. DNA was pelleted by centrifugation for 20 min at 14,000 rpm, isopropanol was decanted, and the pellet was left to dry. The dried pellet was resuspended in 1 mL 100% ethanol and sediment at 14,000 rpm for 5 min. The supernatant was discarded, and the pellet was dried. The pellet was resuspended in 50 μL of sterile distilled water.

In order to increase the sensitivity and to facilitate the DGGE by analyzing fragments of the same length, a nested PCR technique was applied. In the first round, 16S rRNA genes were amplified using forward primer 8F (5′ATRGTTTGATCCTGGCTCA3′) and reverse primer 1492R (5′CGGCTACCTTGTTACGACT3′) to obtain approximately 1,500 bp PCR products. The PCR reaction mixture contained 1 μL of template DNA (50 ng $\mu L^{-1}$), 2.5 μL of 10× buffer (contains 15 mM $MgCl_2$), 1.5 μL of 25 mM $MgCl_2$, 0.25 μL of 10 mM dNTPs, 0.25 μL of 10 pM of each primer, 0.2 μL of *Taq* polymerase (Qiagen, USA), and sterile water to reach a final volume of 25 μL. The PCR was performed on an Eppendorf® Mastercycler Gradient (Eppendorf, Germany). The PCR amplifications were carried out using the following program: 1 denaturation cycle of 5 min at 95°C; 24 denaturation–annealing–extension cycles of 50 s at 95°C, 30 s at 55°C, and 2 min at 72°C; and 1 final extension cycle of 7 min at 72°C. The presence of PCR products was confirmed by analyzing 5 μL of product on a 0.8% agarose gel stained with ethidium bromide.

A nearly complete 16S rRNA gene fragment from the first step was used as a template for a second amplification with 16S rDNA-V3 region primers: 338F-GC (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACG GGGGGACTCCTACGGGAGGCA) and 518R (ATTACCGC GGCTGCTGG) to produce PCR products of approximately 200 bp. The PCR amplifications were carried out using the following program: 1 denaturation cycle of 5 min at 95°C; 29 denaturation–annealing–extension cycles of 50 s at 95°C, 30 s at 60 °C and 50 s at 72°C; and 1 final extension cycle of 7 min at 72°C. The presence of PCR products was confirmed by analyzing 5 μL of product on a 1% agarose gel stained with ethidium bromide.

DGGE was performed using a DGGE-2000 system apparatus (CBS Scientific Company, Del Mar, CA). The PCR products generated by the 338F-GC and 518R primers were loaded onto 7% (wt/vol) polyacrylamide gels with a denaturing gradient ranging from 30% to 60% denaturants (100% denaturant contains 7 M urea and 40% (vol/vol) formamide in 1X TAE). Electrophoresis was performed at 60°C for 5 h at 200 V. After that, the gel was stained with SYBR Gold nucleic acid stain (Invitrogen, USA) for 30 min. The images were visualized on a UV transilluminator and captured using Biovision CN 1000/26M (Vilber Lourmat, France). To identify DGGE bands, individual DGGE bands were cut from gels using a sterile scalpel in a new eppendorf and dissolved in 20 μL of DNase/RNase free distilled water (Invitrogen, USA). The eluted DNA was used as a DNA template following PCR amplification with primers 338F (devoid of a GC clamp) and P518R under the same PCR conditions. PCR products were purified using Gel/PCR DNA fragment extraction kits (Geneaid, Taiwan) before sequencing by 1st BASE (Malaysia). After that, the sequences were compared with the available databases using the Geneious program to determine their approximate phylogenetic affiliations.

### 2.4. Statistical analysis

The data were analyzed statistically using the SPSS statistics software to perform a one-way analysis of variance.

Significantly different means were assessed by a Duncan's multiple-range test ($p < 0.05$).

## 3. Results and discussion

### 3.1. Color and TDS removal from pretreated wastewater

TDS is an important problem for treatment process of wastewater. Original wastewater is highly color by the pigments and acrylic resin that impact on increasing of TDS content. Although, original wastewater was pretreated by chemical substances, the remaining TDS concentration of pretreated wastewater was still high approximately 6,426 mg $L^{-1}$ (Table 1).

Phytoremediation of pretreated ink factory wastewater by *C. alternifolius* could reduce color and TDS removal from wastewater. The results indicated that plant grown in soil-containing pretreatment wastewater had the capacity to reduce TDS from 6,426 to approximately 1,147 mg $L^{-1}$ at day 7 which was less than the effluent standard of Thailand (Fig. 1). Moreover, the color of wastewater was changed from a clear orange-red color to clear color and a mild odor to no odor under plant grown in soil-containing pretreatment wastewater, respectively. However, under soil-containing pretreatment wastewater conditions can also adsorb pigments and other pollutants. It was appeared clear color at day 7. Soil contained an acidic soil (pH 4.3) and had highly clay fraction (Table 2). Moreover, CEC values (30.20 cmol $kg^{-1}$) showed strong adsorption. Clay soil is an effective adsorbent, exchangeable cations on its surface can adsorb and store cations [11]. Therefore, it was possible that soil had a great capacity to attract and hold cation because of its chemical structure. In addition, the color of pretreated wastewater conditions still appeared orange-red color. The results indicated that nature microbes in pretreated wastewater could not remove the color of pigments and other substances within 7 d.

### 3.2. Treatment of organic carbon and nitrogen compounds from pretreated wastewater

The phytoremediation experiment had the highest efficiency and good performance in organic carbon removal. COD concentrations were rapidly decreased from 987 to 171 mg $L^{-1}$ under plant grown in soil-containing pretreated wastewater conditions, which was equal to 83% COD removal (Fig. 2). While under control conditions and soil-containing pretreated wastewater conditions, the remaining COD was 510 and 350 mg $L^{-1}$, which equaled to 48% and 65% COD removal (Fig. 2). Moreover, the remaining BOD under plant grown in soil-containing pretreated wastewater reached a concentration of 7 mg $L^{-1}$ from the beginning 258 mg $L^{-1}$. Meanwhile, under control conditions and soil-containing pretreated wastewater conditions, BOD concentration was 93 and 28 mg $L^{-1}$, respectively (Fig. 3). The results confirmed that

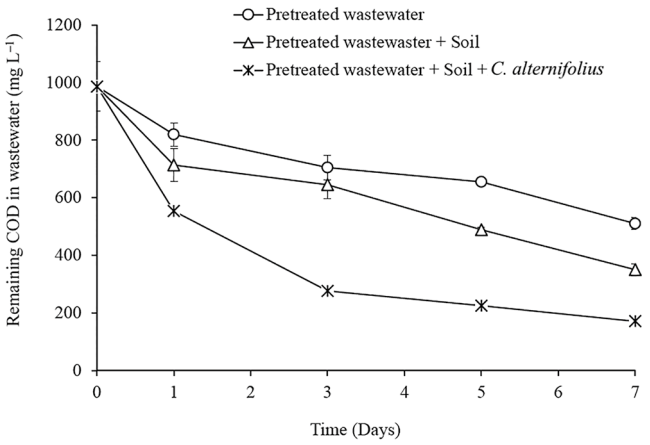


Fig. 2. Remaining COD in pretreated wastewater, soil-containing pretreated wastewater, and plant grown in soil-containing pretreated wastewater conditions.

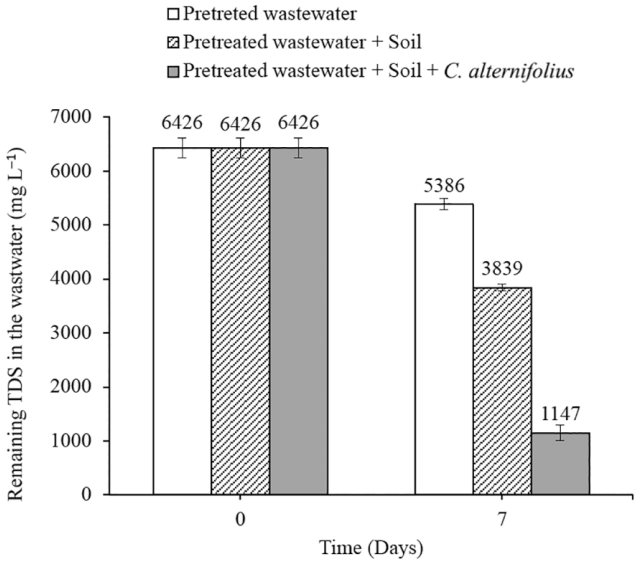


Fig. 1. Remaining TDS in pretreated wastewater, soil-containing pretreated wastewater, and plant grown in soil-containing pretreated wastewater conditions after treatment for 7 days.

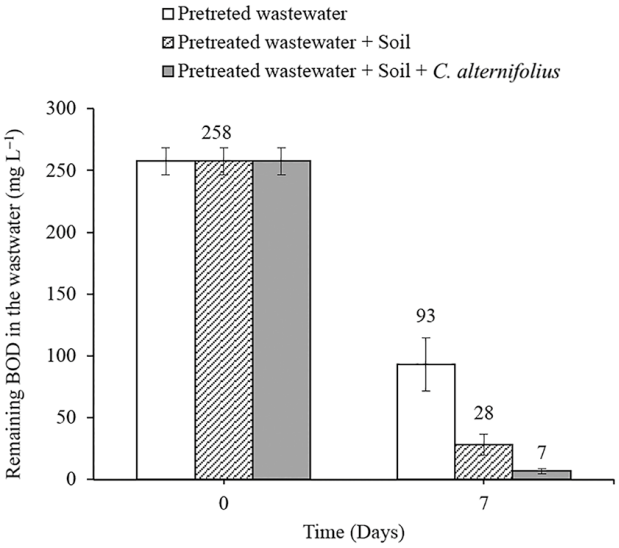


Fig. 3. Remaining BOD in pretreated wastewater, soil-containing pretreated wastewater, and plant grown in soil-containing pretreated wastewater conditions after treatment for 7 d.

micro-organisms, soil and plant involved in reducing organic carbon.

Nitrogen compounds are one of a major problem associated in wastewater. It can increase the eutrophication of aquatic ecosystems and can reach toxic levels to aquatic animals to survive, grow, and reproduce. Moreover, inorganic nitrogen pollution in water can also induce adverse effects on human health and economy [12]. TKN, ammonium, and nitrate concentrations in pretreated wastewater were approximately 156, 146, and 28 mg $L^{-1}$, respectively. Plant grown in soil-containing pretreated wastewater conditions had the highest efficiency in TKN, $NH_4^+$ – N, and $NO_3^-$ – N removal that were 45, 39, and 0.05 mg $L^{-1}$, respectively at day 7. While, soil-containing pretreated wastewater conditions had capacity for TKN, $NH_4^+$ – N, and $NO_3^-$ – N treatment that were 73, 54, and 5 mg $L^{-1}$, respectively. Meanwhile, under control conditions, TKN, $NH_4^+$ – N, and $NO_3^-$ – N were reduced to 93, 78, and 12 mg $L^{-1}$, respectively (Fig. 4). This information indicates that microbial activity, soil adsorption, and ability of plants uptake nutrient affected on nitrogen removal. In phytoremediation system, microbial activity could efficiently degrade organic nitrogen to ammonium, and plants took up both $NH_4^+$ – N and $NO_3^-$ – N forms of nitrogen during their growth. Phytoremediation was clearly confirmed that plant enhanced ammonium and nitrate removal faster than microbial activity and soil adsorption only.

### 3.3. Relation of organic carbon, nitrogen compounds, and the profiles of pH, ORP, and DO

In this study, pH, DO, and ORP were investigated to determine the biological nutrient removal from ink factory wastewater process because of different species of bacteria have widely differing carbon and nitrogen need. The ORP profile is very effective for anoxic phase control and it also reflects the concentration of DO [13]. The pH demonstrates the characteristics of microbial reactions, increasing of pH indicate that ammonification and denitrification while decreasing of pH was nitrification [13,14].

At the beginning of experiment, DO and ORP of pretreated wastewater showed aerobic conditions were approximately 2.71 mg $L^{-1}$ and 91 mV, respectively. The results of DO curves revealed that all the wastewater samples were decreased from 2.71 to 0 mg $L^{-1}$ and then, slowly increasing levels when increasing the period of time (Fig. 5(a)). Meanwhile, the ORP values rapidly declined, lower than –50 mV, followed by a sudden increase (Fig. 5(b)). Interestingly, plant grown in soil-containing pretreated wastewater conditions showed DO and ORP curves sharply decreased to 0 mg $L^{-1}$ and –113 mV at day 1. Meanwhile, DO curves of pretreated wastewater and soil-containing pretreated wastewater conditions were slowly decreased at day 3 and ORP curves of both conditions were decreased to –116 and –173 mV at day 5 and day 3, respectively. These results related to the highest efficiency removal of organic carbon and nitrogen compounds. It indicated that the concentration of carbon and nitrogen of plant grown in soil-containing pretreated wastewater conditions were rapidly reduced, as substrate was consumed, a large amount of DO was demanded for microbial activity that uses organic matters and nitrogen compounds for building cell tissue, and then, anoxic phase was occurred. The hydrolysis of organic matters generated alkalinity, causing the increasing of pH (Fig. 5(c)).

### 3.4. Microbial communities

In this study, PCR-DGGE technique and the sequencing of 16S rDNA were observed the numerically dominant organisms of pretreatment wastewater at day 0 and day 7, soil-containing pretreated wastewater at day 7, and plant grown in soil-containing pretreated wastewater at day 7

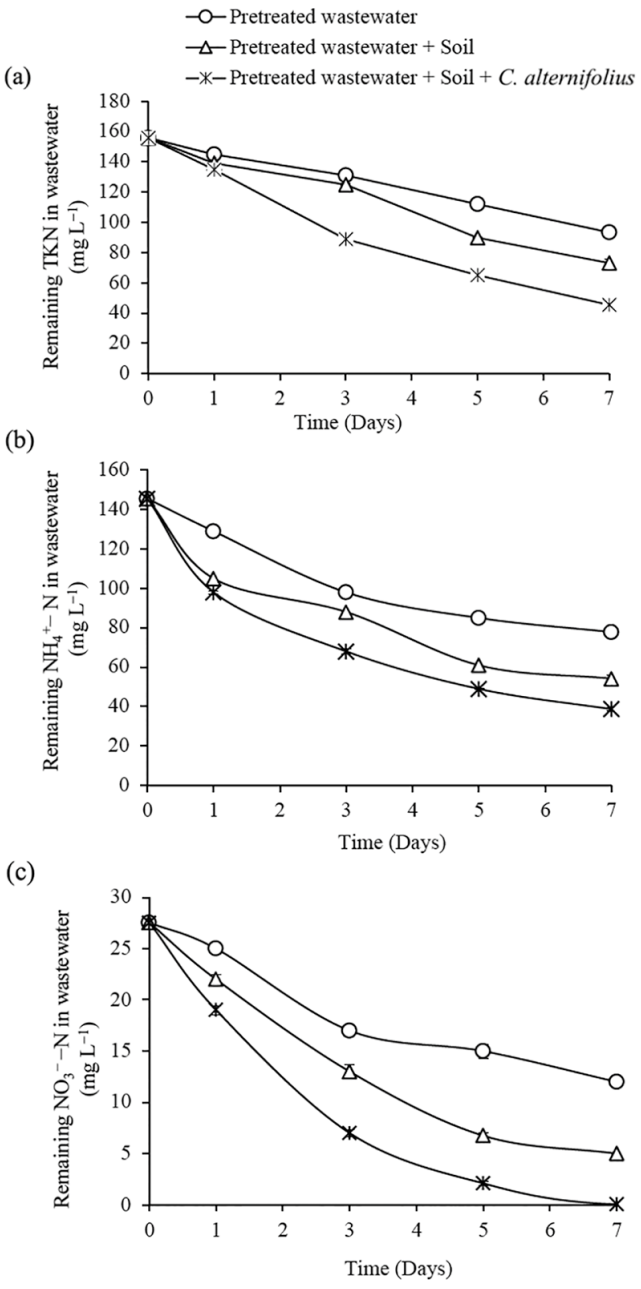


Fig. 4. Concentrations of TKN (a), $NH_4^+$ – N (b), and $NO_3^-$ – N (c) during the time of pretreated wastewater conditions, soil-containing pretreated wastewater conditions, and plant grown in soil-containing pretreated wastewater conditions.

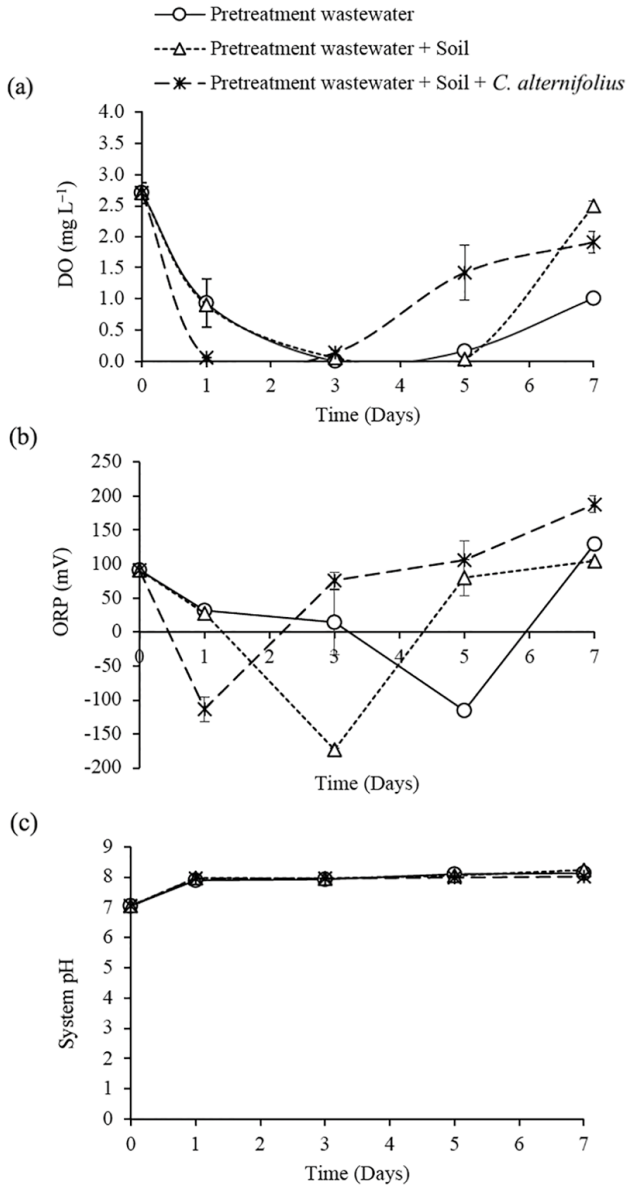


Fig. 5. The variations of DO (a), ORP (b), and system pH (c) during the time of pretreated wastewater conditions, soil-containing pretreated wastewater conditions, and plant grown in soil-containing pretreated wastewater conditions.

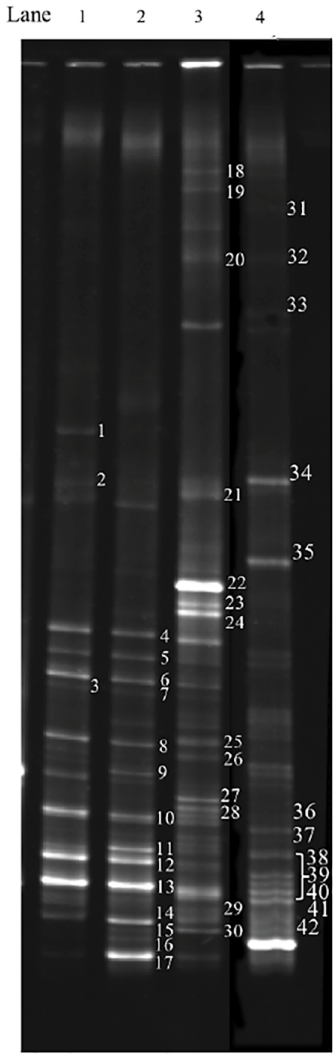


Fig. 6. PCR-DGGE pattern of pretreated wastewater at day 0 (lane 1) and day 7 (lane 2), soil-containing pretreated wastewater at day 7 (lane 3), and plant grown in soil-containing pretreated wastewater conditions at day 7 (lane 4).

(Fig. 6). The result indicated that each condition had different microbial groups and very complex. It showed that under pretreated wastewater conditions at day 0 and day 7, the dominant band of the DGGE pattern was closely related to *Enterobacter* species as shown in Table 3. This species was reported as a polysaccharide-producing bacterium and a proportion of the nitrate-reducing bacteria of the denitrifying process [15,16]. The result was confirmed with the appearance of mucilage in the solution under pretreated wastewater conditions at day 7, which was possibly polysaccharide. Besides *Enterobacter*, the predominant bacteria in pretreated wastewater at day 0 and day 7 the other group was identified as being affiliated with the *Pseudomonas* species. *Pseudomonas* species could reduce nitrate, especially *Pseudomonas aeruginosa*, which has been extensively studied genetically with regard to denitrification [17–19]. Meanwhile, *Pseudomonas putida* demonstrated a very diverse metabolism, including the ability to degrade organic solvents and it is a strain reported that it is heterotrophic nitrification and aerobic denitrification for ammonium, nitrate, and nitrite removal [20,21]. In addition, it was found *Diaphorobacter nitroreducens* that had the ability to perform simultaneous nitrification and denitrification under both aerobic and anaerobic conditions [22]. This micro-organism was an important role for reducing nitrate.

Under soil-containing pretreated wastewater conditions, two major groups are shown to belong to genera *Firmicutes* and *Proteobacter*. Classification from Proteobacteria was closely related to *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Diaphorobacter nitroreducens*. Meanwhile, a major bacterium of the *Firmicutes* group was *Lysinibacillus* sp. Li and co-worker reported that *Firmicutes*-related Fe(III)-reducing

Table 3
The partial 16S rRNA gene sequences of bacteria from the DGGE gels with the best-matching sequences determined by BLAST searches

| Band no. | Closest relative | Phylogenetic affiliation | % similarity | Samples | | | |
|---|---|---|---|---|---|---|---|
| | | | | $W_0$ | $W_7$ | $WS_7$ | $WSP_7$ |
| 1 | Gamma proteobacterium SCGC AAA024-E17 | γ-Proteobacteria | 89 | + | − | − | − |
| 2 | *Enterobacter* sp. N0-20R2A | γ-Proteobacteria | 92 | + | − | − | − |
| 3 | *Pseudomonas* sp. 3CB6 | γ-Proteobacteria | 92 | + | − | − | − |
| 4 | *Pseudomonas aeruginosa* strain A12 | γ-Proteobacteria | 98 | + | + | + | − |
| 5 | *Pseudomonas putida* strain GNA5 | γ-Proteobacteria | 94 | + | + | + | − |
| 6 | *Diaphorobacter nitroreducens* strain AW3 | β-Proteobacteria | 100 | + | + | + | − |
| 7 | *Methylobacterium populi* strain L3-774 | α-Proteobacteria | 82 | − | + | + | − |
| 8 | *Acidovorax delafieldii* strain NBGD35 | β-Proteobacteria | 100 | + | + | − | − |
| 9 | *Enterobacter* sp. AJAR-A2 | γ-Proteobacteria | 94 | − | + | − | − |
| 10 | *Stenotrophomonas maltophilia* strain DTQ-CRS31 | γ-Proteobacteria | 94 | + | + | − | − |
| 11 | *Flexibacteraceae bacterium* CH30#7 | Bacteroidetes | 100 | + | + | − | − |
| 12 | *Enterobacter sakazakii* strain z759 | γ-Proteobacteria | 100 | + | + | − | − |
| 13 | *Enterobacter* sp. AJAR-A3 | γ-Proteobacteria | 100 | + | + | − | − |
| 14 | *Azospirillum* sp. Z012 | α-Proteobacteria | 100 | + | + | − | − |
| 15 | *Dechloromonas denitrificans* strain ED1 | β-Proteobacteria | 100 | − | + | − | − |
| 16 | *Azospirillum irakense* strain L-6 | α-Proteobacteria | 100 | − | + | − | − |
| 17 | *Candidatus Azospirillum massiliensis* strain URAM1 | α-Proteobacteria | 100 | − | + | − | − |
| 18 | *Lysinibacillus fusiformis* | Firmicutes | 96 | − | + | − | − |
| 19 | *Lysinibacillus sphaericus* strain T12-16 | Firmicutes | 100 | − | − | + | − |
| 20 | *Lysinibacillus* sp. CH-N5 | Firmicutes | 96 | − | − | + | − |
| 21 | *Lysinibacillus sphaericus* strain P16 | Firmicutes | 94 | − | − | + | − |
| 22 | *Lysinibacillus sphaericus* strain VB7 | Firmicutes | 100 | − | − | + | − |
| 23 | *Lysinibacillus sphaericus* strain AIMST Ehe33 | Firmicutes | 100 | − | − | + | − |
| 24 | *Bacillus cereus strain* Q-hrb05 | Firmicutes | 99 | − | − | + | − |
| 25 | *Acinetobacter calcoaceticus* strain MCMB868 | γ-Proteobacteria | 99 | − | − | + | − |
| 26 | *Geobacter uraniireducens* Rf4 | δ-Proteobacteria | 100 | − | − | + | − |
| 27 | *Bacillus azotoformans* strain NBRC 15712 | Firmicutes | 99 | − | − | + | − |
| 28 | *Desulfitobacterium metallireducens* strain 853-15 | Firmicutes | 93 | − | − | + | − |
| 29 | *Bacillus pocheonensis* | Firmicutes | 100 | − | − | + | − |
| 30 | *Clostridium* sp. 'HY-129-11 T' | Firmicutes | 98 | − | − | + | − |
| 31 | *Azospirillum amazonense* | α-Proteobacteria | 100 | − | − | − | + |
| 32 | *Diaphorobacter nitroreducens* strain AW3 | β-Proteobacteria | 100 | − | − | − | + |
| 33 | *Pseudomonas putida* 5IIANH | γ-Proteobacteria | 99 | − | − | − | + |
| 34 | *Bacteroides graminisolvens* strain XDT-1 | Bacteroidetes | 95 | − | − | − | + |
| 35 | *Sulfurospirillum deleyianum* DSM 6946 | ε-Proteobacteria | 97 | − | − | − | + |
| 36 | *Azospirillum* sp. 21R | α-Proteobacteria | 98 | − | − | − | + |
| 37 | *Azospirillum* sp. 2456 | α-Proteobacteria | 100 | − | − | − | + |
| 38 | *Azospirillum* sp. YC6995 | α-Proteobacteria | 100 | − | − | − | + |
| 39 | *Azospirillum amazonense* | α-Proteobacteria | 100 | − | − | − | + |
| 40 | *Azospirillum amazonense* | α-Proteobacteria | 100 | − | − | − | + |
| 41 | *Azospirillum* sp. 12812 | α-Proteobacteria | 96 | − | − | − | + |
| 42 | *Azospirillum amazonense* | α-Proteobacteria | 98 | − | − | − | + |

Note: + = clearly band, − = no band, $W_0$ = pretreated wastewater at day 0, $W_7$ = pretreated wastewater at day 7, $WS_7$ = soil-containing pretreated wastewater at day 7, $WSP_7$ = plant grown in soil-containing pretreated wastewater at day 7.

bacteria might also be an important group of Fe(III) reducers besides the well-known *Geobacter* species [23]. Micro-organisms could oxidize organic compounds with Fe(III) serving as the electron acceptor as well as degrade organic contaminants [24,25]. These micro-organisms were related to the presence of a rust-colored iron oxide scum on surface of wastewater, which was possible due to clay soils being contaminated with iron. Meanwhile, *Desulfitobacterium metallireducens*, an obligate anaerobe that can reduce Fe(III), may have a competitive advantage in anaerobic subsurface environments in which Fe(III) is obtained abundantly, but often lacks significant quantities of the other electron acceptors such as nitrate, thiosulfate and sulfite [26]. It is suggested that Fe(III)-reducing micro-organisms may be beneficial to reduce nitrate under soil-containing pretreated wastewater condition.

Interestingly, the bacterial identity abundance under plant grown in soil-containing pretreated wastewater conditions showed the highest microbe group was *Azospirillum* species. Rare sequences identified as *Diaphorobacter nitroreducens*, *Pseudomonas putida*, and *Sulfurospirillum deleyianum* were also found. Bacteria belonging to *Pseudomonas* genera are known to be denitrifiers. The genus *Azospirillum* are highlighted as plant growth promoting rhizobacteria (PGPR) and N2-fixers bacteria, which can help the plants grow better [27]. Moreover, under anaerobic conditions, *Azospirillum* utilizes nitrate as a respiratory electron acceptor and reduces it to molecular nitrogen via nitrite and nitrous oxide [28]. The information indicated that microbial activity had ability for organic carbon and nitrogen removal in phytoremediation system. Microbial diversity in the system showed very complex of microbial groups. Different species of bacteria has widely different carbon and nitrogenous need including organic carbon, ammonium, and nitrate. *Azospirillum*, PGPR, nitrogen fixing bacteria, a major micro-organism in plant grown in soil-containing pretreated wastewater conditions (Table 3). Plants and microbes could enhance the sustainable of phytoremediation in the system (Fig. 6).

## 4. Conclusion

Phytoremediation of wastewater from ink factory by *C. alternifolius* was considered as a key point for reducing TDS, COD, BOD, TKN, $NH_4^+$ – N, $NO_3^-$ – N, and change the orange color to colorless within 7 d. The result indicated that the highest efficiency for substances removal involved micro-organism activities, soil adsorption, including plants absorption. Plants promoted the removal of pollutants, including uptake, transformation of pollutants, and rhizosphere degradation, in which plants enhanced the bacterial growth underground in the root zone to break down the pollutants. Using PCR-DGGE method indicates the structure of the microbial community in the system. Based on the results, we strongly recommended that *Azospirillum* detected in association with plant roots that represents the best-characterized genus should be highlighted as a natural habitat, plant root interaction, nitrogen fixation, and denitrification that had an essential role in wastewater treatment. Therefore, this study was insightful for the operations and maintenance of the treatment process to maximize organic carbon and nitrogen compound removal efficiency.

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