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Phytoremediation potential of *Landoltia punctata* on petroleum hydrocarbons

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Abstract: In this study, the potential of *Landoltia punctata* for petroleum phytoremediation was evaluated. *L. punctata* was treated with different concentrations of Batman crude oil (BCO). The plants were grown in nutrient solutions containing 0.5%, 1%, 2%, and 3% BCO by volume in a growth chamber for 7 days. The fresh weight of the plants in the presence of BCO at a concentration of 0.5% v/v increased by 60% relative to the initial fresh weight. Furthermore, in comparison to the control, plant growth was retarded by 72% and 91% at concentrations of 2% and 3%, respectively. When plants were grown in media containing 0.5% BCO, more than 90% of C_{15}–C_{25} n-alkane compounds were removed compared to the unplanted control (medium plus 0.5% BCO without plants). Pristane (Pr) and phytane (Ph) were both present in all samples, except in the experimental medium of 0.5% BCO application. On the other hand, Pr/Ph values obtained from all oil applications were not statistically different from those of the control samples. As a result, *L. punctata* could be used for the phytoremediation of fresh water resources contaminated with up to 1% crude oil.

Key words: Crude oil, alkanes, biodegradation, freshwater

1. Introduction

The ever-increasing release of industrial waste into the environment causes the contamination of scarce freshwater resources and arable land with organic and inorganic pollutants, risking irrecoverable devastation of these vital resources. These environmental catastrophes can be caused by oil pipeline leaks, accidents during transportation, or ruptures in oil storage tanks (Mrayyan and Battikhi, 2005). Although there has been intensive research into alternative energy sources, petroleum oil is still the mainstay energy source (Okoh, 2006). Petroleum is composed of many toxic, mutagenic, or carcinogenic compounds, e.g., n-alkanes, aromatics, resins, and asphaltenes (Kalf et al., 1997).

Bioremediation, or the use of organisms for remediation, has been a promising approach for the removal of environmental contaminants (Kigigha and Underwood, 2009; Pandey and Fulekar, 2012). Phytoremediation is a type of bioremediation where plants are used for clean-up processes (Yang, 2008; Vangronsveld et al., 2009). There is an increasing number of studies that evaluate the potential use of plants in petroleum phytoremediation (Mohsenzade et al., 2009; Wu et al., 2009). These studies have been conducted with different plant species such as *Juncus roemerianus* (Lin and Mendelssohn, 2009), *Phragmites australis* (Ghobrial, 2008), and *Vetiveria zizanioides* (Brandt et al., 2006).

Landoltia punctata, formerly known as *Spirodela punctata*, was identified as a separate genus (Les and Crawford, 1999). Currently, there are several studies suggesting the capacity of these plants for the removal of organic contaminants such as petroleum hydrocarbons and transforming or sequestering them in nonphytotoxic forms (Macek et al., 2000).

The purpose of this study was to assess the potential use of *L. punctata* for phytoremediation of freshwater resources polluted with oil hydrocarbons.

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2. Materials and methods

2.1. Plant material and experimental design

L. punctata plants were obtained from the İstanbul University Botanical Garden. The plants were grown in Hoagland solution at 23 ± 2 °C, under 16/8-h light/dark periods with 65% humidity and 8000 lx light intensity. Batman crude oil (BCO) was obtained from the Batman Refinery (Batman, Turkey). Growth media containing 0.5%, 1%, 2%, and 3% v/v BCO were stored for 24 h for better homogenization. Six grams of *L. punctata* was used for each treatment. The plants were then allowed to grow for 7 days. Containers containing different amount of crude oil (0.5%, 1%, 2%, and 3% v/v) within Hoagland nutrient solution were used as unplanted controls. At the end of 7 days of application, the plants were weighed and stored at –80 °C until analysis. Eighty mL samples taken from the control and the experimental growth media were stored at 4 °C until extraction. The details of the experimental design can be seen in Figure 1.

2.2. Growth parameters

The effect of BCO on the growth of *L. punctata* was measured by comparing the weight of the plants before and after the application of different concentrations of BCO. *L. punctata* relative growth rate (RGR) was calculated according to Hunt's equation:

$$RGR = (\ln W2 - \ln W1) / (t2 - t1),$$

where RGR is the relative growth rate (g day^{-1}); W1 and W2 are the initial and final fresh weights, respectively; and (t2 – t1) is the experimental period (Hunt, 1978).

2.3. Sample extraction and cleanup

The growth media samples were extracted by using dichloromethane (DCM, Merck) with a separatory funnel by following the liquid–liquid extraction protocol described in US EPA method 3510C (http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/3510c.pdf). The plant samples were extracted using DCM with an accelerated automated Soxhlet (Velp Scientifica SER148), following a slightly modified version of US EPA method 3541 (http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/3541.pdf).

Total extracts were fractioned with adsorption chromatography with a Florisil column and topped with sodium sulfate. The extracts were carefully added to the columns and the fractions were collected using 10 mL of hexane (aliphatic hydrocarbons) and 10 mL of 1:1 DCM and hexane (aromatic compounds) based on a modified version of US EPA method 3600C (http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/3600c.pdf). All of the extracts were concentrated by gentle N_2 blow-down.

2.4. Analysis of petroleum hydrocarbons

Gas chromatography (GC) and mass spectrometry (MS) analyses were performed with a PerkinElmer Thermo DSQ Turbo MSD system. The extracts were analyzed in splitless

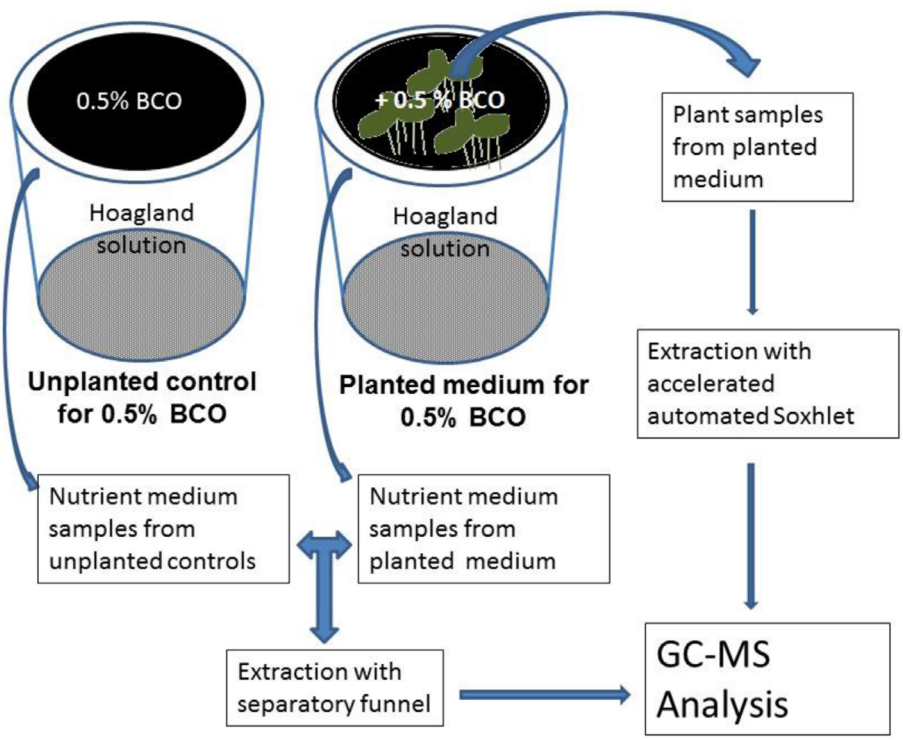

Figure 1. The experimental design and the analysis methods.

mode using a fused silica capillary column HP-5 MS (30 m × 0.25 mm i.d. × 0.25 μm film thicknesses) and helium gas (1 mL min^{-1}) as the carrier. The front inlet temperature was set to 280 °C and the injector was set for splitless injection. The GC oven temperature was programmed from 50 °C (held for 1 min) to 320 °C at a rate of 10 °C min^{-1} and maintained at 320 °C for 5 min. The MS temperature program for transfer line was 220 °C. The MS was operated in EI mode (70 eV) scanning from 50 to 600 amu. The library search was carried out using the Wiley and NIST GC-MS libraries. GC-MS was calibrated for aliphatic hydrocarbons (n-alkane calibration mixture purchased from Dr Ehrenstorfer, Augsburg, Germany) from nC_{10} to nC_{35} by using the internal standard calibration procedure as described in US EPA methods 8000 (http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/8000b.pdf) and 8015 (http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/8015c.pdf), respectively. Retention indices and the mass spectra of the primary ion were detected using GC-MS analysis. Isoprenoid ratios [C_{17}/pristane (Pr), C_{18}/phytane (Ph), and Pr/Ph] were detected using the peak areas and the secondary intensity measures. All of the solvents were of HPLC grade.

2.5. Quality assurance/quality control

Laboratory quality control procedures were considered by the analyses of blanks, use of reference material, and spiked samples. The reference material used for the quality control was 2-fluorobiphenyl. Instrument stability and response was checked by using NIST standard solutions. GC-MS was calibrated for alkanes (nC_{10}–nC_{35}, Dr Ehrenstorfer) by using the internal standard calibration procedure as described in US EPA method 8015. Major characteristic ions of each compound were used for the quantification. The detection limits of the methods were in the range of 1 ng/g for alkanes. The library search was carried out with the Wiley and NIST GC-MS libraries.

2.6. Statistical analysis

All the experimental data were obtained in 3 replicates. The experimental results were expressed as mean ± standard deviation of the triplicate measurements and analyzed by GraphPad Prism version 5.2 for Windows (GraphPad Software, San Diego, CA, USA). Significant differences between the means were determined by the post hoc Bonferroni test and Tukey's multiple comparison test.

3. Results

3.1. Plant growth

The plants survived at all concentrations of BCO applications. After a weekly cultivation period, the weight of plants in the control medium increased by 128% relative to the initial fresh weight. BCO concentrations of 0.5%, 1%, 2%, and 3% stunted plant growth in comparison to the control by 53%, 60%, 72%, and 91%, respectively (Figure 2A). Relative growth rate of *L. punctata* decreased in the presence of BCO in a concentration-dependent manner (Figure 2B).

3.2. Chemical analysis of hydrocarbons

The chromatograms of total extracts indicated an unresolved complex mixture (UCM) of hydrocarbons, which is a typical result for fossil fuels. A UCM was also observed in control media samples. However, for the experimental media, UCMs completely disappeared in 0.5% BCO applications (Figure 3).

n-Alkane (C_{10}–C_{35}) concentrations were obtained from the GC-MS analysis of aliphatic fractions. The changes in the amount of the most abundant alkanes compared with the control groups are shown in Figure 4. As can be seen, *L. punctata* was effective at substantially reducing the amount of n-alkanes with 0.5% BCO application (Figure 4A). As oil concentration increased in the media, the portion of petroleum hydrocarbons removed from the media also decreased. For example, with 0.5% BCO treatment, the amount of the most common alkanes (C_{15}–C_{25}) was reduced by more than 90% with the application of *L. punctata* compared to the control (medium plus 0.5% BCO without plants). However, with 1%, 2%, and 3% BCO

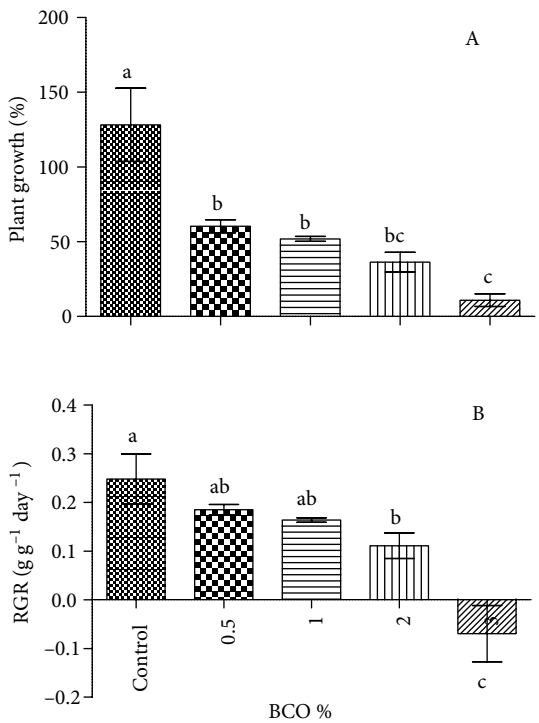

Figure 2. The effect of BCO on the plant growth of *L. punctata* at day 7 of crude oil application **(A)**. Relative growth rates of *L. punctata* treated with BCO **(B)**. The bars represent the standard deviation. Significant differences determined by Tukey's multiple comparison test ($P < 0.05$) are indicated by different letters.

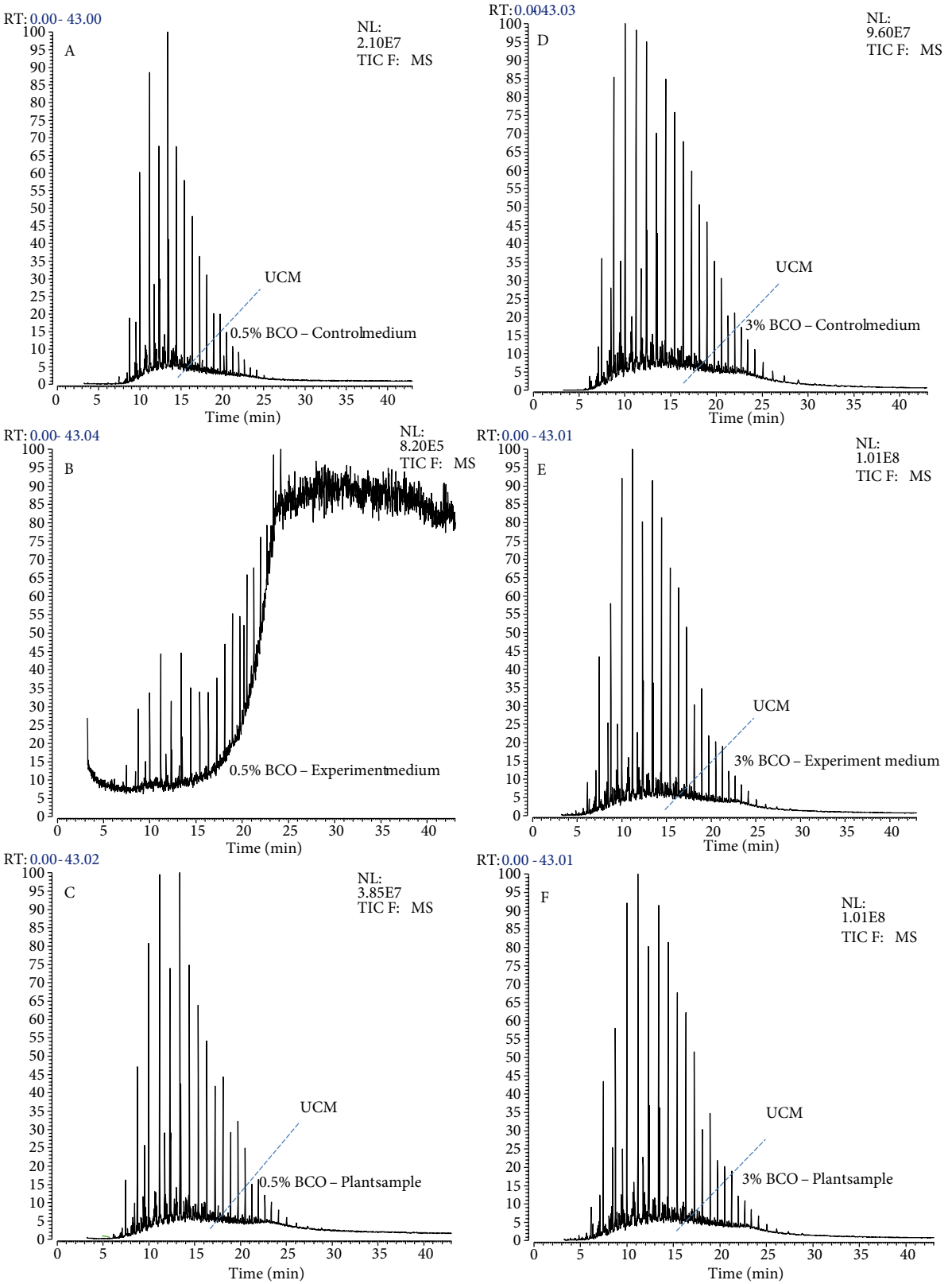

Figure 3. Total ion chromatograms of control and experimental media and *L. punctata* plant samples from 0.5% and 3% BCO applications. **A**- 0.5% BCO control medium. **B**- 0.5% BCO experiment medium. **C**- 0.5% BCO plant sample. **D**- 3% BCO control medium. **E**- 3% BCO experiment medium. **F**- 3% BCO plant sample.

applications, 70–85, 50–60, and 40–50% decreases in the amount of the most abundant n-alkanes were observed, respectively (Figures 4B–4D).

In order to determine the petroleum hydrocarbon degradation capacity of *L. punctata*, isoprenoid ratios (C_{17}/Pr), C_{18}/Ph), and Pr/Ph) for each oil application are given in the Table. Even though C_{17}/Pr and C_{18}/Ph ratios decreased, especially within the range of 0.5% to 1% BCO concentrations, the difference was not statistically meaningful from the control at P > 0.05 (Table). Pr and Ph

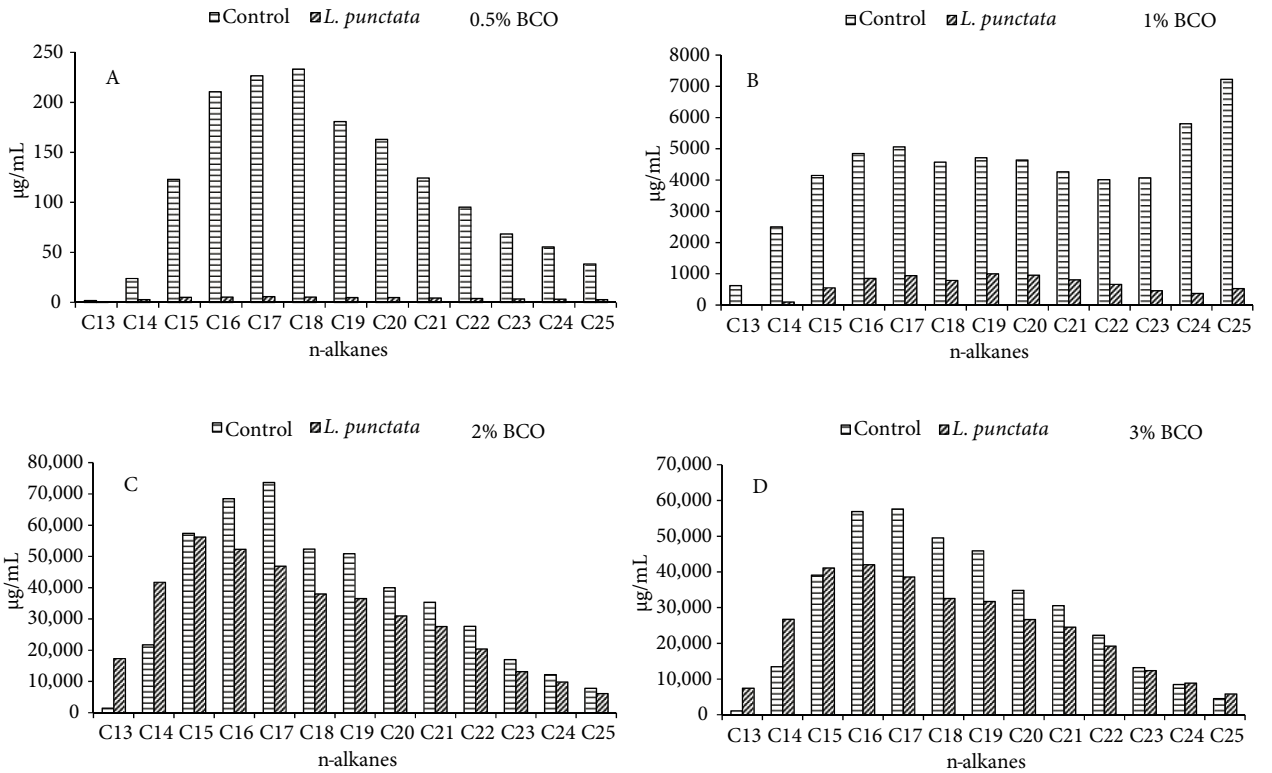

Figure 4. Aliphatic hydrocarbons determined from the plant and control media samples for the BCO applications at the concentrations of 0.5% **(A)**, 1% **(B)**, 2% **(C)**, and 3% **(D)** at the end of day 7.

Table. Isoprenoid ratios (C_{17}/Pr, C_{18}/Ph, Pr/Ph) obtained from the control and experimental media and the plant samples at BCO concentrations of 0.5%–3% at the end of day 7 of the applications. "±" indicates standard deviation. '*, **, ***' indicate significant difference from control at P < 0.05, P < 0.01, and P < 0.001, respectively. Significant differences were determined by the post hoc Bonferroni test.

	nC_{17}/Pr	nC_{18}/Ph	Pr/Ph
0.5% BCO Control medium	5.54 ± 0.02	3.52 ± 0.30	0.67 ± 0.05
0.5% BCO Experimental medium	nd	nd	nd
0.5% BCO Plant	5.13 ± 2.07	2.98 ± 0.94	0.66 ± 0.06
1% BCO Control medium	6.78 ± 0.19	3.90 ± 0.27	0.65 ± 0.04
1% BCO Experimental medium	5.22 ± 0.19 ***	3.59 ± 0.45	0.64 ± 0.02
1% BCO Plant	4.90 ± 0.89	2.75 ± 0.37 *	0.66 ± 0.05
2% BCO Control medium	3.96 ± 0.19	2.82 ± 0.21	0.76 ± 0.04
2% BCO Experimental medium	6.59 ± 0.20 ***	3.96 ± 0.63 **	0.71 ± 0.10
2% BCO Plant	4.79 ± 0.46 **	2.76 ± 0.32 *	0.67 ± 0.04
3% BCO Control medium	4.42 ± 0.19	2.63 ± 0.29	0.69 ± 0.04
3% BCO Experimental medium	5.54 ± 0.05 ***	3.21 ± 0.23	0.63 ± 0.02
3% BCO Plant	6.54 ± 0.80 *	3.72 ± 0.51 *	0.66 ± 0.01

nd: Not determined.

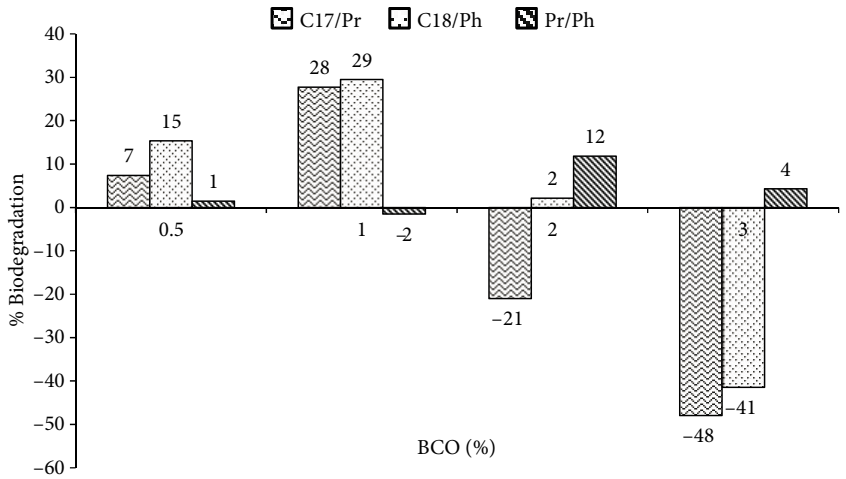

Figure 5. Biodegradation percentages of isoprenoids (C_{17}/Pr, C_{18}/Ph, Pr/Ph) in the plant samples relative to control samples at BCO concentrations of 0.5%–3% at the end of day 7 of the applications.

were both present in all samples, except in the experimental medium of 0.5% BCO application (Table). Relative to the control samples, the biodegradation percentage of the C_{17}/Pr ratios of the plant samples was 7% at 0.5% oil concentration (Figure 5).

4. Discussion

Crude oil contamination significantly hinders plant growth and can cause complete mortality at high concentrations (Lin et al., 2002). In this study, different crude oil concentrations were tested for their effects on the growth of *Landoltia punctata*. We observed that the presence of 0.5% BCO did not noticeably reduce the growth of *L. punctata*. Furthermore, plant growth was almost completely inhibited in the media containing more than 2% crude oil. The hindering effect of oil on plant growth is likely to be a result of the toxic effects of low-molecular-weight hydrocarbons in petroleum (Zand et al., 2010).

UCM could be observed in total ion chromatograms of the control and experimental media and plant samples (Figure 3). On the other hand, for the experimental media at the 0.5% oil concentration, UCM completely disappeared. In general, the presence of UCM in aliphatic hydrocarbon chromatograms is considered to be associated with degraded or weathered petroleum residues (Readman et al., 2002). UCMs are frequently observed in petroleum and hydrocarbon extracts that have undergone biodegradation. UCMs of biodegraded oils are typically characterized with a low abundance or complete lack of aliphatic compounds (Ventura et al., 2008).

Günther et al. (1996) reported that the loss rate of hydrocarbons is much higher than in unplanted soil and that aliphatic hydrocarbons (C_{10}–C_{24}) disappeared faster. The present study also showed that the effect of *L. punctata* on the loss of n-alkanes was significant, especially for C_{15}–C_{25} alkanes at lower oil contaminations, i.e. 0.5% crude oil by volume.

The ratio of unbranched heptadecane (C_{17}) and octadecane (C_{18}) to isoprenoids Pr and Ph, respectively, can be used as an indicator to measure the level of alkane degradation. The relative abundances of these 2 isoprenoids (Pr/Ph) are used as an indicator to monitor the biogenic effect on degradation (Readman et al., 2002). In this study, the effect of *L. punctata* on the biodegradation of crude oil was evaluated together with the plant growth, and it could be concluded that *L. punctata* may have some effect on the biodegradation rate of crude oil contamination of up to 1% (Table; Figures 2 and 5).

In conclusion, the present findings suggest that crude oil adversely affects the growth of *L. punctata*. Our results also indicated that *L. punctata* plants were not particularly tolerant to the presence of oil hydrocarbons in the growth media. Thus, it can be suggested that *L. punctata* could be a good candidate for phytoremediation of freshwater resources contaminated with low amounts of petroleum hydrocarbons.

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