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Effects of Single and Consortia Inoculants on the Biodegradation Efficiency of Crude Oil in Seawater

Nur Zaida Zahari[1*], Gan Poh Yan[1], Piakong Mohd Tuah[1], Sahibin Abd Rahim[1]

[1]*Faculty of Science & Natural Resources, Universiti Malaysia Sabah, UMS Road, 88400 Kota Kinabalu, Sabah, Malaysia*

Abstract. A bioremediation study was undertaken to assess the biodegradation efficiency of crude oil in seawater using two locally isolated strains namely *Candida tropicalis* RETL-Cr1 and *Pseudomonas aeruginosa* BAS-Cr1. The inoculation was carried out using single strains labelled as T1; *Candida tropicalis* RETL-Cr1, T2; single strain *Pseudomonas aeruginosa* BAS-Cr1 and T3; mixture of both cultures respectively. The biodegradation capability of each strain was examined in a shake-flask culture at 30˚C, agitated at 200 rpm for 28 days. The growth profile was monitored by measuring the optical density (OD600) using spectrophotometry. The biodegradation efficiency of crude oil was quantified by comparing the initial and final crude oil concentrations, whereas the degradation of selected aliphatic hydrocarbons was quantified using gas chromatography-mass spectrometry (GC-MS) by comparing the initial and final area in chromatograms. The present finding showed that in 5% (v/v) of crude oil, consortia cultures had the highest degradation, with 50%, while single cultures of *C. tropicalis* RETL-Cr1 and *P. aeruginosa* BAS-Cr1 achieved 39% and 27%, respectively. The results of biodegradation showed that consortia cultures experienced 1.3-fold higher compared to a single culture of *C. tropicalis* RETL-Cr1 and 2-fold higher compared to a single culture of *P. aeruginosa* BAS-Cr1. Based on GC-MS analysis, the aliphatic hydrocarbons were found degraded through the treatment with the highest degradation recorded in consortia cultures: octadecane (73.93%) > eicosane (73.23%) > nonadecane (70.43) > docosane (67.64%) > heptadecane (66.36%) > heneicosane (65.94%) > tricosane (62.28%). From the results obtained, it can be concluded that the potency of microbes as excellent hydrocarbon degraders is as follows: consortia (mixed of two species) > *C. tropicalis* RETL-Cr1> *P. aeruginosa* BAS-Cr1. This supports the idea that microbial communities, especially in mixtures, have the ability to degrade hydrocarbon contaminants more effectively and can be environmentally friendly due to their specific ability to metabolize hydrocarbons.

Keywords: Biodegradation; Crude oil; Seawater; Single and consortia cultures

1. Introduction

Nowadays, oil spill incidents in the marine environment have become a major threat to ecosystems. It has been reported that marine transportation and activities are the major reasons for petroleum oil tankers bringing oil produced to Northeast Asia (Jaswar & Maimun, 2014). Due to rapid economic development and land-based activities in the marine environment, the release of these complex substances into seawater will immediately be

*Corresponding author's email: zaidazahari@ums.edu.my, Tel.: +60-138988754, Fax: +60-88-43524
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subject to a variety of physical, chemical, and biological weathering processes. This will have a tremendous effect on ecosystems, and the impacts will continue even after the visible oil has been removed or dispersed in the environment. The impacts include obvious immediate consequences; for instance, widespread animal mortality due to toxic effects, and more subtle long-term effects on human health, marine organisms, wetland ecosystems, and coral reefs. Numerous studies have documented that the toxicity of crude oil adversely affects people inhabiting areas affected by oil spills through either direct or indirect contact. This is because the oil can produce compounds with mutagenic and carcinogenic properties. These properties can affect the skin, blood, immune system, and other organs (Ubani et al., 2013). Due to the above-mentioned hazards, it is essential to detoxify or treat such oil-contaminated marine water using various techniques. A number of approaches and technologies have been developed for controlling and clearing oil spills in seawater, including physical, chemical, thermal, and biological remediation technologies (Dave & Ghaly, 2011). The feasibility of these current remediation technologies depends on various elements, such as the type and volume of spilled oil, the temperature of the water body, and the environmental conditions of the contaminated site (Garapati, 2012). Biodegradation, also known as bioremediation, is a very broad field and the most reliable mechanism for eliminating organic and inorganic pollutants from the environment by using microorganisms (Budhijanto et al., 2015; Komala et al., 2013; Thakur & Srivastava, 2011). This method transforms pollutants into harmless metabolites or completely mineralizes them into carbon dioxide and water (Ikhimiukor & Nneji, 2014; Khelil et al., 2014). Scientists have found that crude oil can be degraded faster by using more cost-effective and environmentally friendly treatment technologies for the remediation of hydrocarbons by using hydrocarbon-degrading microorganisms (Garapati, 2012). In the case of marine oil spills, especially in Sabah, Malaysia, there is little information and research on the biodegradation of hydrocarbons. There is also very limited information on identified local species of microorganisms that have the potential to degrade hydrocarbons. Therefore, this paper will emphasize two selected environmentally relevant microorganisms (ERM), namely Candida tropicalis RETL-Cr1 and Pseudomonas aeruginosa BAS-Cr1, in degrading crude oil in seawater environments. The study will focus on the potential of both microorganisms as single and consortia cultures (i.e., mixtures of both strains) on the biodegradation efficiency of different classes of petroleum hydrocarbon compounds. This research also attempts to evaluate the possible improvement capability of biological methods as useful tools in developing different strategies for the removal of hydrocarbons from the marine environment.

2. Methods

2.1. Experimental Design

The biodegradation study was conducted within 28 days on a laboratory scale. The experiment was designed to determine the association between microbe strains in single and consortia cultures to assess their crude oil biodegradation potential in a marine oil spill scenario using a modified shaker flask test. Two strains, *Candida tropicalis* RETL-Cr1 and *Pseudomonas aeruginosa* BAS-Cr1, were used in this study as high-potential hydrocarbon degraders. These two strains (as shown in Figure 1) were previously isolated from polluted sites and were obtained from the Environmental Microbiology Laboratory of Faculty Science and Natural Resource, Universiti Malaysia Sabah.

(a) (b)

Figure 1 Colony morphology of (a) *C. tropicalis* RETL-Cr1 and (b) *P. aeruginosa* BAS-Cr1 observed under a stereo microscope at magnification of ×20

2.2. Culture Medium

Two types of cultural media were prepared: nutrient agar (brand Oxoid) and Ramsay broth (Zahari et al., 2021) Nutrient agar powder was weighed at a total amount of 28 g and dissolved in 1.0 L of distilled water. Both media were sterilized through the autoclave method for 15 min at 121˚C. The liquid agar was poured into a Petri dish and allowed to solidify before being sealed with parafilm. As for Ramsay broth, the media was prepared in liquid medium (broth) for the biodegradation process, as listed in Table 1.

Table 1 Compositions of Ramsay Media (RM)

Component	Composition (gL^{-1})
NH_4NO_3	2.0
KH_2PO_4	0.5
K_2HPO_4	1.0
$MgSO_4.7H_20$	0.5
$CaCL_2.2H_20$	0.01
KCL	0.1
Yeast extract	0.06
Glucose	20.0
Agar (2%)	20.0

2.3. Preparation of Single and Consortia Inoculum

For single and consortia inoculum, a loop full of overnight incubation pure culture (24 h matured *C. tropicalis* RETL-Cr1 and *P. aeruginosa* BAS-Cr1) was inoculated into sterile 50 mL centrifuge tubes (Axygen® 50 mL polypropylene (PP)), which contained sterile Ramsay broth and $MgSO_4.7H_2O$ with the desired carbon source. The tube was then incubated at 30^0C and agitated at 200 rpm in an incubator shaker (Chest-Type GYROMAX™ Refrigerated Incubator Shakers). Alternatively, for consortia inoculum (a mixture of two strains), they were added into sterile centrifuge tubes at once (each culture one loop full) and incubated overnight.

2.4. Sampling of UMS Seawater

The UMS seawater samples were collected from ODEC, UMS (6^0 2.412'N, 115^0 6.236' E) using the YSI 556 MPS Multi Probe System. The seawater was sampled from just below the ocean surface using the Schott bottles. The samples were stored at -4˚C and autoclaved before being used for the biodegradation study. In situ measurements, such as pH, electrical conductivity, salinity, dissolved oxygen, total dissolve solid (TDS), and temperature, were conducted to examine the water quality parameters.

2.5. Biodegradation of Crude Oil

A laboratory simulation for biodegradation studies was carried out for crude oil (carbon source) in a modified shaker flask. The biodegradation study of crude oil (5% v/v) were comprised of four treatment sets as single and consortia cultures (Table 2). The experiment was performed with the working volume preparation in a 250 mL volume of conical flasks. The treatment flasks were then incubated at 30˚C and agitated at 200 rpm for a 28-day incubation period and monitored for microbial growth. The working volume preparation for the biodegradation study was based on formula $M_1V_1=M_2V_2$.

Table 2 Treatment sets for biodegradation study of crude oil in seawater

Treatment	Types of culture	Strains
T1	Single	*C. tropicalis* RETL-Cr1
T2	Single	*P. aeruginosa* BAS-Cr1
T3	Consortia	*C. tropicalis* RETL-Cr1 + *P. aeruginosa* BAS-Cr1
TC	Control	Non-inoculation

2.6. Determination of Biodegradation Efficiency and Biodegradation Ratio of Crude Oil

One mL of crude oil samples from each single and consortia culture were withdrawn at initial and regular intervals. Thereafter, all the samples were stored in Eppendorf centrifuge tubes (1.5 mL Expell Secure Microcentrifuge) at 4˚C prior to gravimetric analysis. The samples were centrifuged at 4000 rpm for 3 min to remove microbial cells. The gravimetric analysis was used through the solvent oil extraction method, with some modification (APHA 5520B). The biodegradation efficiency was determined after measuring the initial and residual crude oil concentrations in the conical flask. For the biodegradation profile, the samples after extraction were dried and underwent a recovery process by adding 1 mL of HPLC-grade hexane (brand Merck) to dissolve the solidified crude oil samples. The freshly prepared samples were sent for gas chromatography analysis using a gas chromatography-mass spectrometer (GC-MS, Thermo Scientific TSQ 8000). From the chromatogram, the biodegradation ratio was interpreted and evaluated. The purpose of the chromatogram acquired from the analysis was to interpret the biodegradation ratio. The index used was C_{17}: Pristane, C_{18}: Phytane and Pristane: Phytane. Both Pristane and Phytane are commonly known as biomarkers (Zhu et al., 2004).

2.7. Data Analysis

Data were statistically analyzed using a data analysis pack from Microsoft Office Excel 2007. Excel was used for the data management and exploratory data analyses. Excel was also used to draw graphs and bar charts regarding microbial growth, TPH reduction, and biodegradation efficiency within 28 days of treatment.

3. Results and Discussion

3.1. Physical and Chemical Characteristics of UMS Seawater

The physical and chemical characteristics of UMS seawater were used to undertake a biodegradation study of crude oil. Six parameters were analyzed: temperature, pH, electrical conductivity, total dissolved solid, salinity, and dissolved oxygen. The physical and chemical properties of seawater vary according to latitude, depth, nearness to land, and input of freshwater. Approximately 3.5% of seawater is composed of dissolved compounds, while the other 96.5% is pure water. The chemical composition of seawater reflects such processes as erosion of rock and sediment, industrial and shipping activity, volcanic activity, gas exchange with the atmosphere, and the metabolic and breakdown products of

organisms and rain. Table 3 shows the data on the physical and chemical properties of UMS seawater.

Table 3 Physical and chemical characteristics of UMS seawater

No.	Parameter	Replicate		Average Reading
		R1	R2	
1.	Temperature	29.04 ˚C	29.60 ˚C	29.32 ˚C
2.	pH	7.8	7.7	7.75
3.	Electrical conductivity	49.74 mS/cm	49.33 mS/cm	49.54 mS/cm
4.	TDS	32.33 g/L	32.07 g/L	32.2 g/L
5.	Salinity	32.44	32.12	32.28
6.	Dissolve oxygen	135.10 %	115.60 %	125.35 %

3.2. Biodegradation Efficiency of Crude Oil in Seawater

The biodegradation efficiency of crude oil was performed using a series of data analyses, which aided in identifying the degradation potency of single and consortia cultures of *C. tropicalis* RETL-Cr1 and *P. aeruginosa* BAS-Cr1. From the results obtained, it was found that the biodegradation of crude oil in seawater was highest in the first week of the treatment, and thereafter, the degradation percentage started to reduce gradually (Figure 2). This was in line with the study of Chorom et al. (2017), who mentioned that the biodegradation rate of crude oil decreased with increasing incubation time. This is due to the microbe's mechanism starting to attack the complex structural petroleum fractions, followed by the complexity of molecular increases; thus, the amount of degradation decreased (Bao et al., 2012). After 28 days of incubation, consortia cultures of both strains exhibited the highest percentage of crude oil degradation (49.49%), followed by single *C. tropicalis* RETL-Cr1 (38.92%) and *P. aeruginosa* BAS-Cr1 (27.04%). This result clearly shows that the complete transformation or mineralization of crude oil could not be accomplished within a 4-week period. A previous study carried out by Morais and Tauk-Tornisielo (2009) indicated a recalcitrant and complex structure of oil components resistant to degradation. To completely degrade these oil components by microbes, a longer incubation period is required. However, the performance of consortia cultures in this study shows evidence that these strains are more efficient hydrocarbon degraders than single cultures. This result can be explained by a previous study done by Malik and Ahmed (2012), which showed that to achieve extensive degradation of crude oil, the assemblage metabolic capacity of both individual microbes was a major required condition. There is no single strain of bacteria with the metabolic capacity to degrade all the components found within crude oil, but undoubtedly only a limited range of hydrocarbons (Al-Wasify & Hamed, 2014).

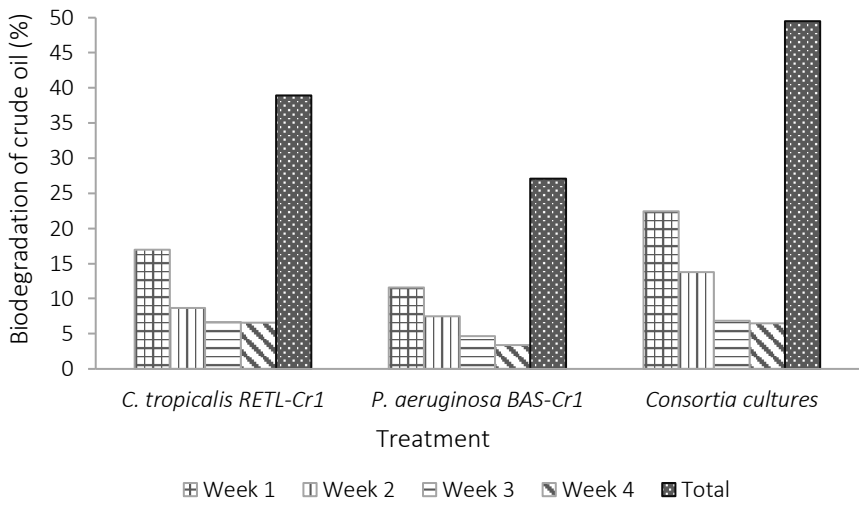
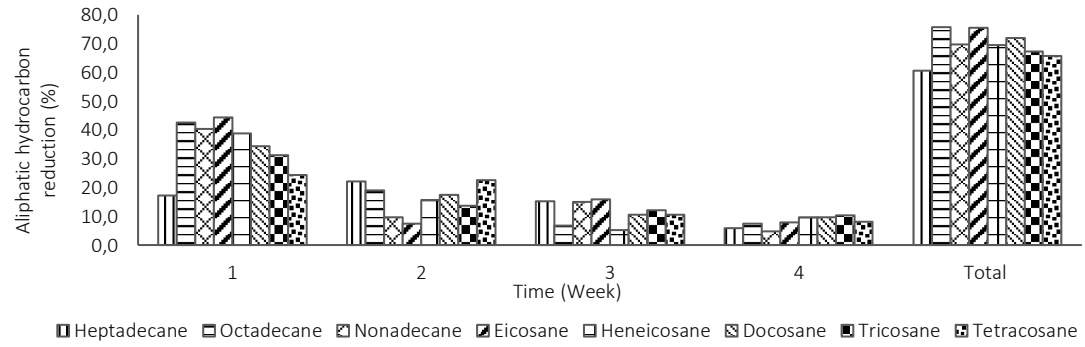


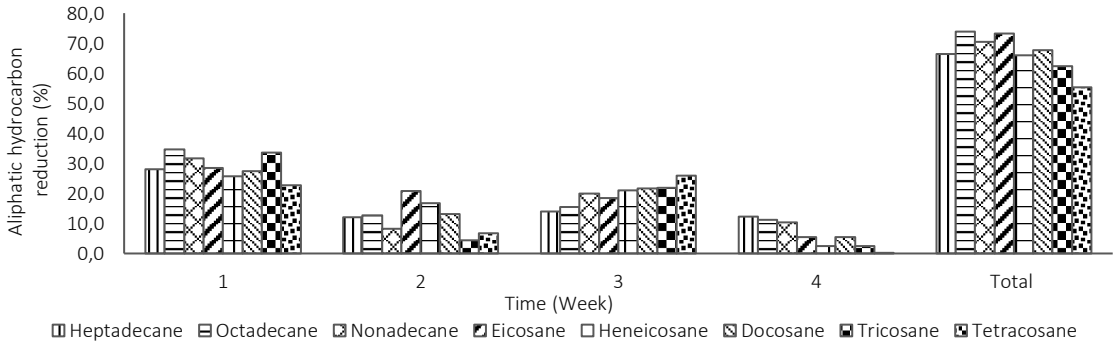
Figure 2 Biodegradation efficiency of crude oil in single and consortia cultures of *C. tropicalis* RETL-Cr1 and *P. aeruginosa* BAS-Cr1

3.3. Biodegradation of Individual Hydrocarbons in Crude Oil

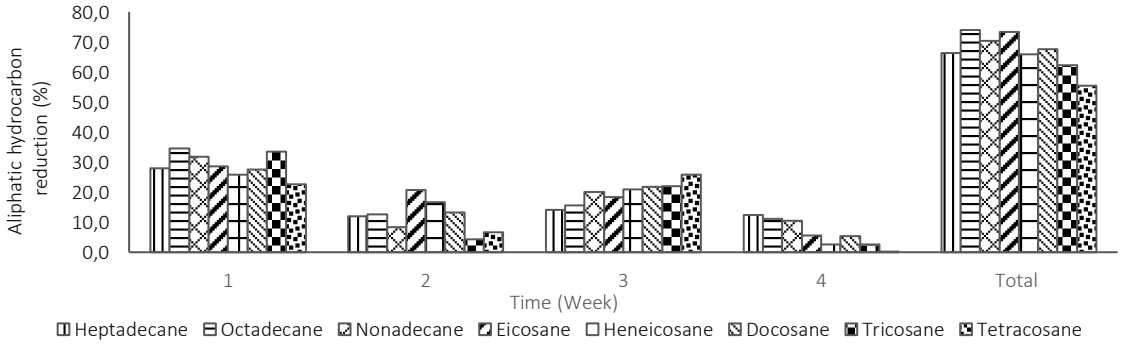
The reduction percentage of the individual hydrocarbons was calculated and is shown in Figure 3 (a-c) to identify the biodegradation capability of crude oil by single and consortia cultures of *C. tropicalis* RETL-Cr1 and *P. aeruginosa* BAS-Cr1. Eight types of hydrocarbon aliphatic components were chosen in this study: heptadecane ($C_{17}H_{36}$), octadecane ($C_{18}H_{38}$), nonadecane ($C_{19}H_{40}$), eicosane ($C_{20}H_{42}$), heneicosane ($C_{21}H_{44}$), docosane ($C_{22}H_{46}$), tricosane ($C_{23}H_{48}$), and tetracosane ($C_{24}H_{50}$) was chosen in this study and identified based on the standard hydrocarbon profile. The results show that the selected aliphatic hydrocarbon reduced by a single culture of *C. tropicalis* RETL-Cr1 was octadecane (75.77%) > eicosane (75.60%) > docosane (71.99%) > nonadecane (69.73) > heneicosane (69.38%) > tricosane (67.35%) > tetracosane (65.64) > heptade (58%). For *P. aeruginosa* BAS-Cr1, the reduction sequence was as follows: eicosane (42.33) > docosane (38.65) > octadecane (37.18) > tricosane (37.39) > heneicosane (36.31) > nanodecane (35.27) > tetracosane (34.43) > heptadecane (32.76). Results for consortia cultures showed that the percentage reduction was octadecane (73.93%) > eicosane (73.23%) > nonadecane (70.43) > docosane (67.64%) > heptadecane (66.36%) > heneicosane (65.94%) > tricosane (62.28%) > tetracosane (55.39%). According to GC-MS analysis, all the peak heights and areas for long-chain alkanes (C12) can be clearly observed after incubation for 1 month. As suggested by Musat (2005), this may be due to incomplete mineralization in the present study. However, the biodegradation of n-alkanes in crude oil was fast compared to previous research. It was unexpected that the total percentage of individual hydrocarbon degraded by single cultures of *C. tropicalis* RETL-Cr1 was relatively similar to the consortia cultures. The single culture of *P. aeruginosa* BAS-Cr1 exhibited poor performance in terms of the reduction percentage based on individual hydrocarbons. Since the results of reduction percentage shown by the consortia cultures were highly similar to the single cultures of *C. tropicalis* RETL-Cr1 rather than the single culture of *P. aeruginosa* BAS-Cr1, it can be said that *C. tropicalis* RETL-Cr1 has great potential to become an effective petroleum hydrocarbon degrader in the marine environment.



(a)



(b)



(c)

Figure 3 Percentage degradation of individual hydrocarbons present in crude oil by single cultures of (**a**) *C. tropicalis* RETL-Cr1 (**b**) *P. aeruginosa* BAS-Cr1, and (**c**) consortia cultures

The striking emulsification of crude oil in conical flaks containing single and consortia cultures is shown in Figure 4, which complements the evidence of enhanced biodegradation provided by the gas chromatogram. According to Saad et al. (2019), the emulsification of crude oil is evidence that biodegradation has occured. Moreover, the experiments were carried out at the microcosm level in controlled laboratory conditions; therefore, the availability of sunlight for the natural weathering of crude oil was nonsignificant. Hence, the preliminary work was based on biodegradation activity.

(a) (b) (c)

Figure 4 The emulsification activity in single cultures of (a) *C. tropicalis* RETL-Cr1 (b) *P. aeruginosa* BAS-Cr1 and (c) consortia cultures after 14 days of biodegradation study

3.4. Biodegradation Ratio of Crude Oil

A confirmatory experiment was conducted to verify the isoprenoids pristane (Pr) and phytane (Ph), which are commonly used as biomarkers for evaluating the biodegradation of crude oil. The n-C_{17}/Pr, n-C_{18}/Ph, and Pr/Ph values for the residual crude oil after biodegradation by the single and consortia cultures of *C. tropicalis* RETL-Cr1 and *P. aeruginosa* BAS-Cr1 were calculated based on the data obtained from the GC-MS analysis. Table 4 shows data on initial n-C_{17}/Pr and n-C_{18}/Ph ratios, which were 3.06 and 4.34, respectively, before the biodegradation process started. Relying on the n-C_{17}/pristane and n-C_{18}/phytane ratio indexes, consortia cultures of *C. tropicalis* RETL-Cr1 and *P. aeruginosa* BAS-Cr1 achieved considerable biodegradation after 28 days of incubation. The ratios of n-C_{17}/Pr and n-C_{18}/Ph decreased from 3.06 to 1.04 and from 4.34 to 1.13, respectively, while the single cultures of *C. tropicalis* RETL-Cr1 decreased to 1.20 and 1.13, respectively. *P. aeruginosa* BAS-Cr1 showed less biodegradation performance, with values of 2.05 and 2.73, respectively. By comparing the results obtained for each week of incubation, it was clearly exhibited that the n-C_{17}/Pr ratio was always lower than the n-C_{18}/Ph ratio for all single and consortia treatments. These results are consistent with Diaz et al. (2000), who suggested that there was preferential biodegradation of phytane in a preliminary study. Hence, the selected microbes tested in the present study were comparatively better and more potent hydrocarbon degraders. The greatest biodegradation was achieved by the consortia, as they had the lowest ratios of biomarker chemistry in both n-C_{17}/Pr and n-C_{18}/Ph. The ratios of consortia in n-C_{17}/Pr and n-C_{18}/Ph after 28 days of treatment were 1.11 and 1.15, respectively. These data highlighted that the smaller the ratio, the higher the degradation of the hydrocarbon fractions (Makeen et al., 2013). This indicated that the consortia had excellent potential for biodegradation in the marine environment compared to the single cultures of *C. tropicalis* RETL-Cr1 and *P. aeruginosa* BAS-Cr1. It was observed that the peak area ratios of n-C_{17}/Pr and n-C_{18}/Ph were higher than 1.0. According to Okoro and Amund (2009), weight ratios of n-C_{17}/Pr and n-C_{18}/Ph higher than 1 indicated that the hydrocarbon present in the simulated oil-contaminated seawater was not biodegraded. However, this disagreement can be explained through a study conducted by Diaz et al. (2000), which showed a constantly higher ratio of n-C_{17}/Pr and n-C_{18}/Ph in natural seawater than in synthetic media. Thus, it can be concluded that the hydrocarbon present in the preliminary study underwent biodegradation.

Table 4 Comparison of the n-C_{17}/pristane and n-C_{18}/phytane peak area ratios from gas chromatograms of the saturated hydrocarbons.

Experimental Condition	Time (d)	n-C_{17}/Pr	n-C_{18}/Ph	Pr/Ph
C. tropicalis RETL-Cr1	7	2.56	2.50	4.75
	14	1.85	1.71	4.75
	21	1.39	1.41	4.75
	28	1.20	1.13	4.74
P. aeruginosa BAS-Cr1	7	2.51	3.39	4.75
	14	2.11	2.83	4.75
	21	2.09	2.77	4.75
	28	2.05	2.73	4.75
Consortia cultures	7	2.20	2.84	4.75
	14	1.83	2.29	4.75
	21	1.41	1.62	4.75
	28	**1.04**	**1.13**	**4.69**

4. Conclusions

Based on this study, it can be concluded that the reduction of hydrocarbons by both single and consortia cultures varies. Consortia cultures displayed high degradation of hydrocarbon, which was 2-fold higher as compared to the single culture of *C. tropicalis* RETL-Cr1 and of *P. aeruginosa* BAS-Cr1, respectively. This has been proven with chromatogram profiles, where the crude oil element has been degraded and undergoes emulsification activity. The results concluded that consortia cultures have great potential for microbial-enhanced oil recovery in real field sites, especially in polluted marine water. To advance biodegradation studies, future research should focus on treatment with high concentrations of crude oil and carry out biocompatibility tests of two different species to identify the presence of toxins or any potentially harmful effects among the consortia cultures.

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