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GROWTH OF INDIGENOUS BACTERIA *VIBRIO ALGINOLYTICUS* AND *DIETZIA* SP. ISOLATED FROM THE EAST COAST OF ALGERIA IN THE PRESENCE OF MONOAROMATIC HYDROCARBONS

East coast of Algeria suffers from several types of pollution, mainly hydrocarbons. The hydrocarbon-based bacteria of the littoral were investigated. 53 indigenous strains were isolated, identified according to morphological and biochemical nature. *Dietzia* sp. CNJ898 PLO4 and *Vibrio alginolyticus* PB-WC 11099 were selected for growth tests in the presence of monoaromatic hydrocarbons as a sole source of carbon and energy and total hydrocarbons at the end of growth. The results showed that both strains used monoaromatics to grow. The total hydrocarbons determined after growth showed that all monoaromatics were biodegraded with different rates. The maximum rate was obtained with *Dietzia* sp. in the presence of xylene with 4.33 mg C/dm^3 at the end of the process, followed by toluene with a level of 5.07 mg C/dm^3 and 5.60 mg C/dm^3 in the presence of *Vibrio alginolyticus*. The lowest degradation rate was obtained in the presence of benzene with *Dietzia* sp. with 15 mg C/dm^3 compared to the control, which was 27 mg C/dm^3 at the beginning of growth. The results obtained showed that both selected strains assimilated the monoaromatics tested and could be used for the bioremediation of the polluted littoral.

1. INTRODUCTION

Monoaromatic hydrocarbons constitute one of the major classes of aromatic compounds present in the environment with chloroaromatic hydrocarbons. The main natural production of monoaromatic compounds comes from the degradation of lignin [1]. In fact, considerable quantities of monoaromatic hydrocarbons encountered in the environment are mostly of petroleum or petrochemical origin and are the consequence of

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the much pollution caused by human activity [2]. Of these, benzene, toluene, ethylbenzene and xylene are referred to as BTEX. These four aromatic carbon compounds are mainly used in the petrochemical industry which transforms them to make other synthetic compounds. They are thus present in many products such as varnishes, solvents, plastics, leather, rubber, lacquers, waxes, and inks. Being naturally present in the oil, the principal sources of emission of BTEX are thus automobile exhausts, petroleum refining and evaporation of gasoline during storage, transportation and distribution [3].

Thus, xylene appears in the 30 most produced chemicals in the United States in terms of volume. With respect to benzene, its use as a solvent has greatly decreased because of its high toxicity. Because of their relatively high solubility and toxicity, BTEX pose a significant threat to human and animal health in contaminated environments. They are carcinogenic, neurotoxic and have been blacklisted by the Environmental Protection Agency (EPA) in the United Kingdom [4]. Chemical and physical methods to eliminate them are expensive and complex, leaving nonbiodegradable residues potentially toxic to the environment [5]. Bioremediation uses natural microorganisms to degrade contaminants and these biological means have proved effective and less costly in removing petroleum contaminants [6]. Petroleum hydrocarbons are organic pollutants that are subject to biodegradation by microorganisms [7]. Many microorganisms have been reported to use various petroleum hydrocarbons, including BTEX and PAHs as their only carbon and energy substrate [8].

However, aromatic compounds of low molecular weight such as benzene, toluene and xylene, which are among the toxic compounds present in petroleum, are also very easily degraded by marine microorganisms [9]. Isolation techniques have highlighted the ability of some strains to degrade BTEX alone. Many microorganisms such as *Ralstonia pickettii*, *Pseudomonas putida* strain F1, *Pseudomonas putida* strain mt-2 [10] were reported to degrade toluene, whereas *Rhodococcus opacus*, *Pseudomonas putida* strain ML2, *Alicycliphilus* as denitrificans [11] of benzene. Ethylbenzene, *m*-xylene and *o*-xylene could also be degraded by several pure strains [12].

The purpose of this work was to isolate and identify autochthonous hydrocarbonoclastics bacteria from marine environments chronically exposed to oil pollution. In vitro culture tests were carried out in the laboratory by monitoring the growth of the selected bacterial strains in the presence of different monoaromatic hydrocarbons: benzene C_6H_6, toluene $C_6H_5CH_3$ and xylene $C_6H_4(CH_3)_2$ of known concentrations as the only carbon source and energy.

2. EXPERIMENTAL

Study sites and sampling. Seawater samples were taken from two ports on the eastern Algerian coastline in the coastal towns of Annaba and El-Kala (Fig. 1). The city of Annaba has one of the main industrial ports of Algeria with a strong maritime activity

where it is exposed to chronic pollution of hydrocarbons. The port of El-Kala is a small port characterized by very strong fishing activity. Seawater samples were collected at the ports from oil and engine oil polluted stations. Two sites were selected: the port of Annaba with strong maritime activities with the following four sampling stations:

station 1: 36°54′15.21″N, 7°46′28.72″E,
station 2: 36°53′47.71″N, 7°45′40.39″E,
station 3: 36°53′45.52″N, 7°46′70.26″E,
station 4: 36°54′10.09″N, 7°46′39.68″E,

and the port of El-Kala with two stations:

station 1: 36°54′05.91″N, 8°25′15.07″E,
station 2: 36°53′56.62″N, 8°25′25.84.

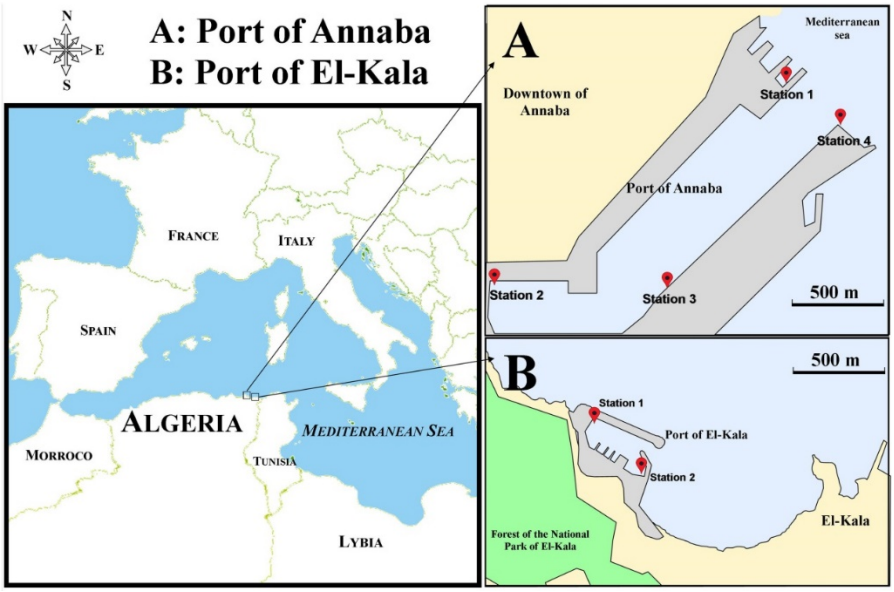


Fig. 1. Study sites and sampling stations

Physicochemical analyzes. Seawater samples from both ports, as well as Cap de Garde water (used in this study for control tests), were collected in 1 dm^3 capacity flasks and sent to the laboratory for physicochemical soil and water analysis in order to determine the total hydrocarbon content, total nitrogen and total phosphorus concentrations.

The total hydrocarbons rate was carried out to determine the seawater degree of pollution, and on the other hand, to assess monoaromatic hydrocarbons at the end of growth for each isolated strain. Hydrocarbons are detected following the five 5 steps: (i) extraction of hydrocarbons with pentane in a separatory funnel, (ii) polar substances were removed by filtering the sample with a filter characterized by a pore of 45 µm diameter, (iii) evaporation of the extraction solvent (pentane) in a heating block at 70 °C

for 30 min, (iv) oxidative decomposition of samples in a heating block for 2 h at 148 °C, and (v) determination of total hydrocarbons using a visible UV spectrophotometer at 436 nm. The rate is expressed in mg/dm^3.

Oxidative acid mineralization of all organic and inorganic nitrogen compounds was conducted in a heating block for 1 h at 100 °C. All organic and mineral nitrogen substances were oxidized to produce nitrates. The nitrates reacted with 2,6-dimethylphenol to form 4-nitro-2,6-dimethylphenol. Total nitrogen content was determined at 365 nm and expressed in mg/dm^3.

Orthophosphate ions reacted with ammonium molybdate to give phosphomolbydic acid, which was reduced to blue molybdenum. Oxidative mineralization at 100–120 °C was performed to release polyphosphates and organic phosphate. Total phosphorus content was measured at 690 nm and expressed in mg/dm^3. The analyses were performed within 24 h after collection by using a kit according to the Macherey–Nagel patent methods [13].

Bacteriological isolation and identification. The seawater samples from both sites collected in glass 250 cm^3 bottles previously sterilized were sent to the microbiology laboratory of the Department of Biochemistry (University of Badji Mokthar-Annaba) and kept at 4 °C before analyses. Sampling for bacteriological analyses was carried out in 2 periods of the year, the first period between March and July, the second between September and November. Isolation was achieved within 8 h of sampling. The isolation of the bacteria from the samples was carried out on the nutrient agar (GN), Mac-Conckey and Chapman medium. The dishes were incubated at 30 °C for 72 h. The identification was based on the study of morphological characters (Gram stain) as well as physiological traits such as mobility, catalase and oxidase according to the following tests. For mobility, a drop of fresh culture was observed at ×400 or ×600 magnification. Bacteria were considered mobile if different trajectories were observed, excluding Brownian motions. For the catalase test, a bacterial colony taken with a sterile Pasteur pipette was distributed in a drop of hydrogen peroxide. Appearing of bubbles indicates the decomposition of hydrogen peroxide under catalase action. For oxidase test, a bacterial colony fragment collected with a sterile Pasteur pipette was spread on an oxidase disk, moistened with sterile distilled water. Oxidase-positive species gave purple color immediately in 10 s. Molecular identification (PCR and sequencing of the 16s rRNA gene with primers 27f 1401r) was previously realized as described by Batisson et al. [14].

Bacterial selection and growth test. Isolated native bacteria were subjected to a preliminary agar growth test composed of synthetic seawater and light crude oil with N/P of 1 biostimulation. A sterile Erlenmeyer flask containing 99 cm^3 of sterile synthetic seawater (pH 8.18), enriched in nitrogen and phosphorus with an N/P ratio of 1, was prepared. Then 1 cm^3 of monoaromatic hydrocarbon (benzene, toluene and xylene) was added as the only source of carbon and energy with initial total hydrocarbon rate equal

to 27 mg C/dm^3 as chemical compound seeded with 1 cm^3 of bacterial suspension, then incubated at 30 °C in an agitator set at a speed of 150 min^{-1}. The control cultures of the selected bacterial strains were carried out without biostimulation. For this purpose, the spectrophotometric optical density (OD) at a wavelength of 600 nm was evaluated daily.

3. RESULTS AND DISCUSSION

3.1. PHYSICOCHEMICAL PARAMETERS

The results of seawater physicochemical analyzes show that the highest concentration of total hydrocarbons (THT) – 111 mg C/dm^3 – is recorded in the seawater of the port of Annaba. It is ten times higher than the Algerian standard [19], which recommends a total hydrocarbons limit value of 10 mg C/dm^3 in seawater. The concentration of THT at El-Kala fishing port is slightly higher than the standard – 11.20 mg C/dm^3, while the THT in the Cap de Garde seawater considered as a control station is 4.80 mg C/dm^3. These different concentrations result from the fact that the site of Annaba is a big seaport with commercial activity and that the port El-Kala is a small fishing port with simple infrastructure. It was noted that the concentrations of nitrogen and phosphorus remain low in the three sampling stations. The Algerian standard tolerates a maximum of 30 mg N/dm^3 for nitrogen and 10 mg P/dm^3 for total phosphorus [19]. The maximum concentration of nitrogen was recorded at Cap de Garde station with 5.4 mg N/dm^3, while the maximum concentration of phosphorus, 0.7 mg P/dm^3, was observed at the port of Annaba.

3.2. IDENTIFICATION AND SELECTION OF STRAINS

A total of 53 bacterial strains and consortia between Gram + and Gram – as well as some yeasts were isolated and have been subjected to a preliminary growth test by seeding on synthetic seawater agar [17] in the presence of light crude oil (1 cm^3) as the sole source of carbon and energy, with a biostimulation ratio N/P of 1, during an incubation period of 24 h to 15 days at 30 °C. Two indigenous seas bacterial strains (from Annaba and El-Kala) were selected (9.1 and 3.1) on the basis of the appearance of colonies after good growth on synthetic seawater medium and light crude oil (Table 1).

Table 1

Evolution of cultures of selected species on agar synthetic–hydrocarbon seawater

Duration of the test	24 h	48 h	72 h	96 h	7 days
Strain 9.1	–	–	+/–	+	+
Strain 3.1	–	+	+	+	+

The morphological, physiological and biochemical characters of both bacterial strains were determined (Table 2).

Table 2

Morphological, physiological and biochemical characteristics of selected bacteria

Bacterium	*Dietzia* sp.	*Vibrio alginolyticus*
Gram	+	–
Aggregation	isolated, grouped into clusters	isolated
Form of the bacterial cell	cocco bacilli	curved bacilli
Mobility	++++	– –
Oxydase	+	–
Catalase	+	+

3.3. MOLECULAR IDENTIFICATION AND GROWTH OF STRAINS

The molecular identification of both selected bacterial strains, after sequencing their DNAs, gave the following results: Strain 9.1 – *Vibrio alginolyticus* PB-WC 11099, strain 3.1 – *Dietzia* sp. CNJ898 PLO4[3]. The growth of isolated strains selected in the presence of different monoaromatic hydrocarbons (BTX) was analyzed.

Vibrio alginolyticus. BTEX-contaminated water and soil are mainly remedied through biodegradation by natural microbial populations [20]. Gram-negative bacteria are known for their biodegradation capabilities. Heldund and Staley [21] report detailed characterization of aromatic hydrocarbon degradation by the strain of *Vibrio cyclotriphicus* isolated from marine sediments contaminated with creosote and *Vibrio* sp. P-2P44T, that uses polycyclic aromatic hydrocarbons as a substrate such as naphthalene, 2-methylnaphthalene and phenanthrene. Moreover, *Vibrio* sp. KM1 can mineralize aerobically large volumes of chemical production of benzoic acids; this demonstrates that species of this genus can contribute to the removal of aromatic pollutants from estuarine environments [22].

The growth curves *of Vibrio alginolyticus* fed with monoaromatic hydrocarbons show that from the first hours (2 h) of cultures a quick adaptation with monoaromatic hydrocarbons in vitro was observed. The optical density (OD) evolved rapidly from 0.233 to 0.442 in the presence of benzene (Fig. 2a), from 0.161 to 0.868 in the presence of toluene (Fig. 2b) and from 0.27 to 0.431 in the presence of xylene (Fig. 2c). Next, the optical density continues to increase gradually but less rapidly, the OD has increased from 0.442 to the maximum value of 0.459 (Fig. 2a) and toluene from 0.868 to 0.895 and then to 0.962 (Fig. 2b) in contrast to the xylene with which the OD increased rapidly from 0.431 to 1.01 (Fig. 2c). The growth rate was fast with xylene (Fig. 2c) and the

[3]In the following text, these two strains, srain 9.1 *Vibrio alginolyticus* PB-WC 11099, and strain 3.1 *Dietzia* sp. CNJ898 PLO4 will be referred to as *Vibrio alginolyticus*, and *Dietzia* sp., respectively.

maximum OD displayed after 24 h was 1.01. In the presence of toluene, the OD increased from 0.161 to 0.962 after 48 h of culture. For benzene (Fig. 2a), the rate was slower and the maximum OD reached was 0.459 after 24 h of culture.

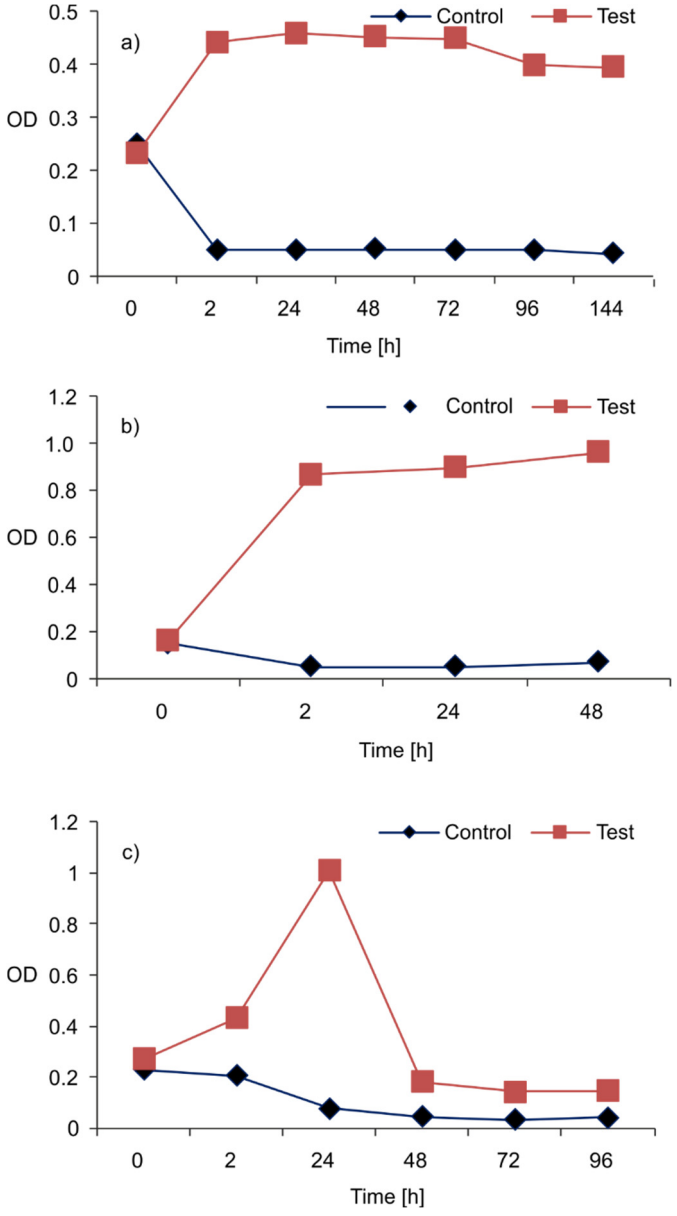


Fig. 2. Growth of *Vibrio alginolyticus* PB-WC 11099 in the presence of:
a) benzene, b) toluene, c) xylene as the sole source of carbon and energy

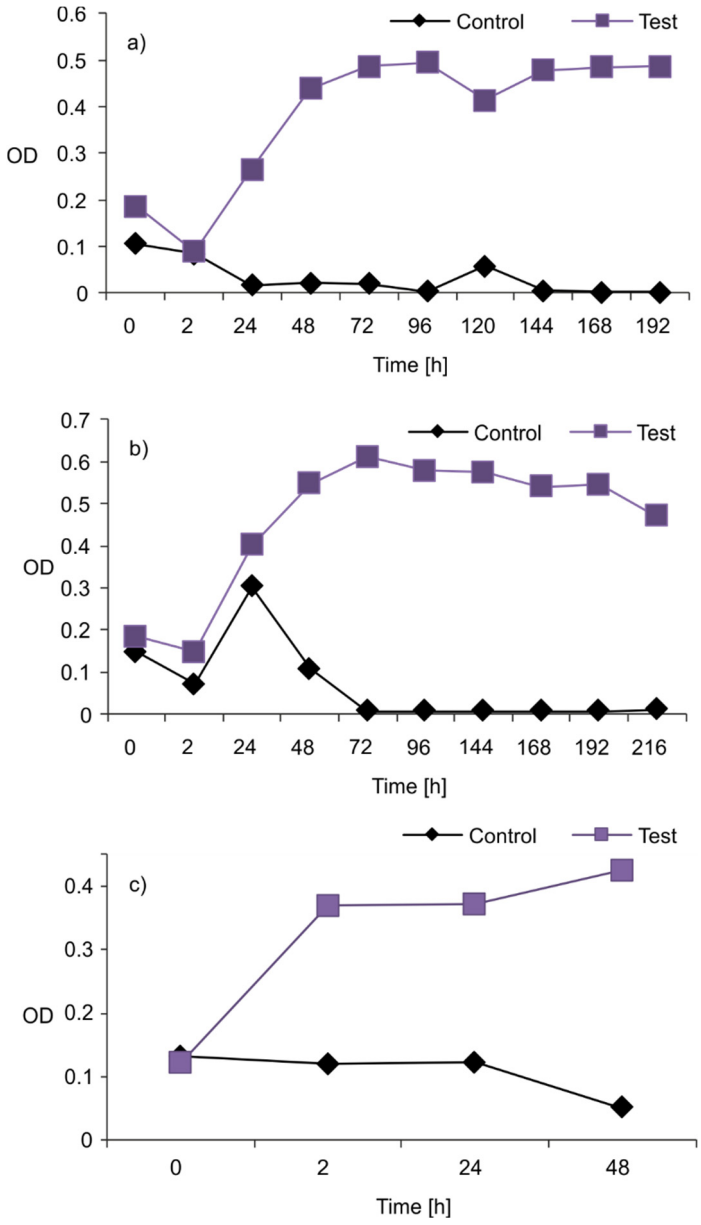


Fig. 3. Growth of *Dietzia* sp. CNJ898 PLO4 in the presence of:
a) benzene, b) toluene, c) xylene as the sole source of carbon and energy

Dietzia sp. Contrary to the growth curves of *Vibrio alginolyticus* with benzene and toluene, *Dietzia* sp. isolated from the seawater of El-Kala showed difficulties of adaptation at the beginning of culture with 2 carbon sources added to the in vitro media.

A decrease in the number of bacteria was noted, where the OD decreased from 0.187 to 0.089 when culturing *Dietzia* sp. with benzene (Fig. 3a) and from 0.184 to 0.147 with toluene (Fig. 3b). Exceeding this phase, the bacterium seems to be gradually adapting to the two carbon sources present, there is a resumption of growth and a gradual increase in OD or turbidity from 0.187 to 0.495 after 96 h with benzene (Fig. 3a) and from 0.184 to 0.612 with toluene (Fig. 3b). These results show that the indigenous El-Kala seawater has assimilated well and used the only source of carbon and energy present in the culture medium.

Dietzia sp. in the presence of xylene does not seem to have difficulties in adaptation. Thus, the OD increased from 0.121 to 0.425 after only 48 h of incubation (Fig. 3c). The growth rate of *Dietzia* sp. with xylene is faster than that of its growth with benzene and toluene. *Dietzia* sp. showed good growth with the three monoaromatic hydrocarbons tested. Previously, *Dietzia* sp. achieved good growth on agar-seawater synthetic-crude oil, with alkanes [13] and monoaromatic hydrocarbons and this has already been observed in studies of Xing Xang-Biao et al. [24] with *Dietzia* DQ 12-45-1b that uses a wide range of *n*-alkanes, aromatic compounds and crude oil as the sole source of carbon for growth. The total hydrocarbon levels achieved after growth of the selected indigenous bacteria *V. Alginolyticus* and *Dietzia* sp. are shown in Table 3.

Table 3

Concentration of hydrocarbons [mg C/dm^3] after growth of selected bacterial strains

Hydrocarbon	Initial concentration	*Vibrio alginolyticus*	*Dietzia* sp.
Benzene	27	6.13±0.13	15.00±1.00
Toluene		5.60±0.20	5.07±0.57
Xylene		5.23±0.37	4.33±0.73

All the monoaromatic hydrocarbons tested were biodegraded by the strains selected at different rates, the maximum rate of biodegradation being obtained with *Dietzia* sp. in the presence of xylene at 4.33 mg C/dm^3, i.e., a percentage of degradation was 16.03%, followed by degradation of toluene with a level of 5.07 mg C/dm^3 (18.77%). The latter was degraded with a rate of 5.60 mg C/dm^3 (20, 74%) in the presence of *Vibrio alginolyticus*. According to Dubinsky at al., not all bacterial species exhibit a similar rate of hydrocarbon degradation [25]. The biodegradation of the various compounds causes a variation in the carbon sources available in the medium as the biodegradation process progresses. It has been also noted that the lowest degradation rate was obtained in the presence of benzene with *Dietzia* sp. at a rate of 15 mg C/dm^3 (55,55%) compared to the initial concentration of 27 mg C/dm^3 at the beginning of growth.

4. CONCLUSION

Two selected strains have assimilated and consumed benzene, toluene and xylene added as sole source of carbon and energy in their growth medium in vitro. It can be

concluded that *Vibrio alginolyticus* PB-WC 11099 and *Dietzia* sp. CNJ898 PLO4 possess the capacity to use a wide range of hydrocarbons and can, therefore, be used for the bioremediation of oil-polluted sites in the east Algerian coast.

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