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Bacterial Isolates from Greek Sites and Their Efficacy in Degrading Petroleum

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Abstract: Polycyclic aromatic hydrocarbons (PAHs) are a major organic pollutant, not only because they do not self-degenerate but also because they accumulate in the food chain and give rise to serious repercussions in terms of biodiversity sustainability. Petroleum-degrading bacteria have long been used as a promising solution in the effort to biodegrade crude oil. In this study, new isolates from specific Greek environments displaying various levels of crude oil contamination, as well as isolates belonging to the ATHUBA collection, were thoroughly investigated for their capacity to degrade crude oil. Furthermore, the presence of *nah*H and *alk*J genes in the above bacterial isolates, as well as their ability to form agglomerates or release surfactants, was investigated. Two consortia were formed, and their ability to degrade crude oil was tested, achieving similar degrading capacities as those observed with the individual strains. A *Pseudomonas plecoglossicida* isolate demonstrated the highest percentage (76.7%) ability to degrade crude oil. The biodegradation rate of this isolate was further evaluated by measuring the alkanes/hopanes ratio over a period of ten days, exhibiting a higher degradation rate in short-chain (C11–C21) alkanes, whereas a decrease in the ratio was observed when the number of carbons in petroleum increased. This is the first detailed report on bacterial communities in oil-polluted areas of Greece that contain a variety of bacteria with the ability to degrade PAHs in contaminated sites and may provide a novel alternative to various bioremediation processes or be used as inocula in autochthonous bioaugmentation procedures for crude oil biodegradation.

Keywords: crude oil degradation; polycyclic aromatic hydrocarbons; alkanes/hopanes; bacterial degradation

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1. Introduction

In recent decades, the increase in petroleum consumption has given rise to a serious environmental issue [1,2]. Ecosystems are severely contaminated by crude oil and its derivatives. Polycyclic aromatic hydrocarbons (PAHs), among petroleum hydrocarbons, constitute a group of major organic pollutants, not only because of their high toxicity but also due to their tendency to accumulate in the food chain. Along with their epoxides, they can cause mutations and lead to serious carcinogenic consequences [3,4]. PAHs are mainly non-polar organic compounds formed by the incomplete combustion of various organic materials, such as tar, coal, oil and gas. Depending on the type of combustion employed, as well as the nature of the starting material, different PAHs are produced [5].

Among existing methods, biodegradation of oil is the most important process for the removal of oil spills and the subsequent restoration of the environment. It is an alternative and environmentally friendly process that is achieved through the metabolic activity of microorganisms. In the process of biodegradation, microorganisms use carbon contained in the various components of oil as a source of energy for their growth. The main categories of microorganisms that consume hydrocarbons are bacteria and fungi. There are more

than 200 species of bacteria and fungi that degrade oil, from its simplest compounds, such as methane, to the largest compounds containing more than 40 carbon atoms. In marine ecosystems, bacteria play a dominant role in the degradation of hydrocarbons [6]. The percentage of microbial degraders of hydrocarbons in relation to the overall microbial community varies depending on the ecosystem. One factor that can change this percentage is the continuous environmental exposure to hydrocarbons. In non-polluted ecosystems, consumers of hydrocarbons can occupy less than 1% of the total microbial community, whereas following an oil spill, this percentage can increase to 10%. In environments that suffer from chronic pollution, bacterial populations that degrade oil can occupy much larger percentages of the microbial community. At the beginning of the biodegradation process, lighter components of oil (simpler hydrocarbons) break down, whereas the decomposition of more complex hydrocarbons takes longer due to the adaptation time needed for bacteria to produce enzymes that can disintegrate these compounds. In general, the sequence of decomposition of the components, from the easiest to the most difficult to degrade, are n-alkanes, branched alkanes, low-molecular-weight aromatic compounds and cyclic alkanes [7].

Although there is evidence that bacteria can degrade PAHs anaerobically, the majority of bacteria prefer aerobic conditions via oxygenase-mediated catabolism. Dioxygenase hydroxylates an aromatic ring, forming a *cis*-dihydrodiol, which then turns into a diol intermediate by the action of a dehydrogenase [3]. These intermediates are then cleaved by dioxygenases, leading to the formation of catechols, which are further converted to tricarboxylic acid (TCA) cycle intermediates [8]. The genes involved in naphthalene-degrading pathways have been extensively studied. *nah*H is among the 11 genes involved in the pathway of naphthalene catabolism and encodes for a catechol 2,3-dioxygenase. As far as aliphatic degradation is concerned, one of the most common genes involved in the process is *alk*J, which encodes a key aliphatic alcohol dehydrogenase [9] capable of oxidizing the primary alcohol to the aldehyde, possibly providing a better-defined substrate and product specificity. On the basis of its amino acid sequence, the enzyme has been classified as a member of the glucose-methanol-choline (GMC) oxidoreductase family [10]. It has been reported [11–13] that endogenous microorganisms expressing alkane catabolic genes show a high ability to degrade oil in contaminated soils or sediments, even under extreme environmental conditions in the Arctic or Antarctic [14,15]. Detection of specific genes is crucial, not only for the estimation of the potential of a bacterial strain to be used in bioremediation processes but also as a biomarker for the characterization of the contamination level of a specific area [12].

Microbial consortia composed of multiple strains exhibit improved oil degradation efficiency [9]. Different bacterial species may provide a variety of enzymes used during oil bioremediation and follow diverse metabolic pathways in order to degrade PAHs [16]. It has been shown that when a successful bacterial consortium is constructed, the synergistic effect significantly improves oil degradation ability. However, the criteria imposed for successful selection of each member of the consortium are still under consideration [17].

One modern approach to treat oil spills is autochthonous bioaugmentation. In this procedure, microorganisms isolated from the specific polluted environments are used as inocula for decontamination. These are called autochthonous (Greek word for endogenous) rather than allochthonous (derived from "allo", meaning a different environment from where microorganisms were isolated). Allochthonous (foreign) microorganisms cannot easily compete with autochthonous microorganisms. Consortia comprised of autochthonous isolated strains are much more likely to lead to a successful outcome than those containing foreign microorganisms [18], as various factors, such as the microbial diversity of the population, as well as the chemical structure and concentration of pollutants, have a considerable effect on the progress of the bioremediation process.

In this study, we attempted to elucidate the role of autochthonous isolates with respect to their ability to degrade oil independently or in a consortium. To this end, we isolated bacterial strains from four sites in Greece displaying varying levels of oil contamination.

Among them, various high-efficiency oil-degrading bacterial strains were isolated and classified according to their 16S rRNA sequences. Their biochemical profiles, as well as the occurrence of *alk*J and *nah*H genes, were examined. In one specific strain that displayed high biodegradation capacity, the rate of oil degradation in liquid cultures was measured by gas chromatography-mass spectrometry (GC-MS). Based on the strains' characteristics, two consortia were constructed, and their biodegradation capacity was examined in relation to that of each individual strain.

2. Materials and Methods

2.1. Soil Sampling and Media

All samples were collected in October 2017. Each of the four contaminated sampling sites displayed varying stages of oil contamination: Sampling Sites 1 and 2 (Glyfada Beach and Attika, Greece, 37°856,173′ N, 23°745,566′ E and 37°856,334′ N, 23°745,433′ E respectively) seemed to be affected by oil pollution due to an oil spill, whereas Sampling Site 3 (Glyfada Beach, Attika, Greece 37°856,303′ N, 23°745,221′ E) was unaffected. Sampling site 4 is a paleoenvironmental setting of *Keri Lake* on Zakynthos Island (Zakynthos Island, Greece 37°685,225′ N, 20°835,265′ E). The site is not commercially exploitable and has remained unchanged for a recorded historical period of at least 2000 years [19]. Approximately 100 g of sand/pebbles was collected from a depth of 20 cm using a sterile 8 cm diameter plastic core. The total sampling area was 10 m^2, and five samples (consisting of soil, sand and gravel) were collected from each sampling site. The soil samples were spaced 2 to 5 m apart from each other.

Each sample was placed separately in a new Ziploc plastic bag, transported to the laboratory, stored at 4 °C and analyzed within 3 days. A total of 80 g of each individual sample from each sampling site was then mixed to provide a representative sample (400 g) for each sampling site. All representative samples were divided into two halves. One half was transferred to the National Center for Scientific Research "Demokritos" (NCSR) for hydrocarbon analysis, and the other half was further processed in the NKUA lab. In all cases, pH, temperature, salinity and dissolved oxygen were estimated before sample collection using an aquameter provided by Aquaread.

Media used for isolation and cultivation of the isolates were a minimal salt medium with agar (MSM) [20] and arginine glycerol salts agar (AGS) [21]. With the aim isolating oil hydrocarbon degraders, the following modifications to the media composition were performed: in MSM agar and AGS agar, the carbon source was replaced by crude oil 5% (*w/v*). The new media were named MSMCO and AGSCO, respectively. The oil used in all the experiments was donated by Motor Oil Hellas, and it was added to each medium after sterilization. Its physicochemical properties are as follows: 0.89 kg/L density at 15 °C, 5.4 psi vapor pressure at 37.8 °C and −25 °C flow point. As to its chemical composition, the oil contained 3.35% (*w/v*) sulfur, 36 ppm vanadium, 14 ppm nickel, 0.06% (*v/v*) water and 7% (*w/v*) asphalt.

Following isolation, batch cultures were set up to test for the capacity of microorganisms to grow in naphthalene and oil. To this end, MSMCO and AGSCO liquid media of were prepared, with a crude oil 5% carbon source. In addition, the same media were prepared, but instead of oil, they contained naphthalene at a final concentration of 10 mM. The new media were named nMSM and nAGS, respectively. For biochemical identification and DNA isolation, nutrient broth [22] was used in all isolated strains.

2.2. Isolation of Oil Hydrocarbon Degraders

Amounts of 100 g of each representative sample were placed in sterile Erlenmeyer flasks, mixed with 1000 mL sterile Ringer's solution (0.25 strength,) and shaken on an orbital shaker at maximum speed (220 rpm) for 2 h. Mixtures were allowed to settle before preparing serial dilutions (up to 10^{-8}) of the supernatant fluids and plating on AGSCO and MSMCO media in triplicate. After incubation at 30 °C for 72 h, each morphologically distinct bacterial colony was picked up and purified by further cultivation on MSM and

AGS medium plates. All isolates were preserved in a 30% (*w/v*) glycerol solution as a protective agent at −80 °C.

2.3. Phenotypical Characterization of Bacterial Isolates

For preliminary biochemical identification, all isolates, as well as the previously deposited ATHUBA bacterial strains (Athens University Bacteria and Archaea Culture Collection), were inoculated on NA or AGS plates and incubated at 30 °C for 24–48 h. Gram staining was performed using a Bactident aminopeptidase kit (Merck, Darmstadt, Germany). A slide method was used to estimate catalase activity. Colonies were transferred to a dry glass slide. A drop of 3% (*v/v*) H_2O_2 was placed on the glass slide, where cells had already been transferred. The evolution of oxygen bubbles was observed and recorded. A cytochrome oxidase test was established according to the technical annex of a Microbact biochemical identification kit (Thermofisher scientific, Waltham, MA, USA). For examination of endospore formation, one colony of each strain was suspended in a final volume of 1 mL sterile Ringer's solution. The suspensions were placed in an 80 °C water bath for 10 m to deactivate any vegetative formations. After the treatment, 25 μL of the suspension was transferred onto NA or AGS plates. Observation of the colonies after 24 h of incubation at 30 °C indicated endospore formation.

2.4. Isolation of Genomic DNA and Detection of the 16S rDNA Gene, Catechol 2,3-Dioxygenase Enzyme (nahH) Gene and Aliphatic Alcohol Dehydrogenase Enzyme (alkJ) Gene

Each strain was cultured in NA broth for 48 h at 200 rpm and 30 °C. A volume of 1 mL of the culture was centrifuged for 10 m at 12,000 rpm. Biomass was resuspended in 500 μL of lysis solution (25 mM Tris-HCl, 25 mM EDTA, 0,3 M sucrose, 10 mg/mL lysozyme) and incubated for 2 h at 37 °C. Thereafter, 125 μL of SDS 10% (*w/v*) was added, and the mixture was incubated for 30 m at 65 °C. After the incubation, the mixture was centrifuged for 10 m at 13,000 rpm. The supernatant was transferred into a new tube, and 216 μL of potassium acetate solution (5M) was added and incubated in an ice bath for 45 m. After centrifugation for 10 m at 13,000 rpm, the supernatant was transferred into new tubes, and an equal amount of isopropanol was added for DNA precipitation. After 15 m, the tubes were centrifuged for 20 m at 14,000 rpm. Precipitate was washed twice by ethanol 99.5% (*v/v*). The pellet was dried out and resuspended in sterilized distilled water. The DNA concentration was determined by a spectrophotometer and preserved at −80 °C. Before each PCR reaction, the concentration of DNA was adjusted to 40 ng/μL.

The strains were characterized according to 16S rRNA sequence. The primers used for the amplification were 27F 5′-AGAGTTTGATCCTGGCTCAG-3′ [23] and 1492R 5′-GGTTACCTTGTTACGACTT-3′ [24]. The PCR mixture contained 4 μL 10× standard Taq reaction buffer (10 mM Tris—HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 0.3 μL Taq DNA polymerase (1.25 units), 0.4 μL dNTPs (10 mM/dNTP), 2,0 μL primer 27F (0.05–0.5 mM), 2.0 μL primer 1492R (0.05–0.5 mM), 1.5 μL DNA (5 pg–0.5 μg) and 9.8 μL H_2O. The conditions for PCR reaction were as follows: denaturation of target DNA at 95 °C for 3 m followed by 35 cycles at 95 °C for 30 sec, primer annealing at 55 °C for 30 sec, primer extension at 72 °C for 2 m and a final extension at 72 °C for 10 m. Amplified PCR products were separated by gel electrophoresis on 1.2% (*w/v*) agarose gel and purified using a NucleoSpin extract PCR kit (MACHEREY-NAGEL, Duren, Germany). The 16S rDNA fragment (>900 bp) was fully sequenced in both directions (VBC-Biotech Service GmbH, Eurofins, Vienna). Similarity comparisons to the GenBank database were performed by BLAST (www.ncbi.nih.gov, accessed on 14 March 2021). Sequences retrieved from this study have GenBank codes MW719562 (ATHUBA687, *Bacillus wiedmannii*), MW719566 (ATHUBA689 *Streptomyces* sp.), MW719589 (ATHUBA 691, *Bacillus flexus*), MW719612 (ATHUBA697, *Isoptericola chiayiensis*), MW719875 (ATHUBA700, *Halomonas aquamarina*), MW719877 (ATHUBA706, *Streptomyces flavoviridis*), MW719879 (ATHUBA707, *Pseudomonas plecoglossicida*), MW720656 (ATHUBA708, *Paenibacillus abyssi*), MW720667 (ATHUBA716, *Kocuria arsenatis*), MW720699 (ATHUBA717, *Brevundimonas vesiculari*), MW729331 (ATHUBA718, *Pseudomonas flavescens*),

MW729323 (ATHUBA725, *Nitratireductor aquamarina*) and MW729318 (ATHUBA727, *Pseudomonas plecoglossicida*)

The *nah*H and *alk*J genes were also amplified, with sequences of approximately 476 bp and 352 bp, respectively. All the primers and conditions used in this study are described in [25]. The PCR mixture for both genes contained 2.5 μL 10× Kappa Taq reaction buffer (10 mM Tris—HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 0.1μL Taq DNA polymerase (1.25 units), 1.25 μL dNTPs (4 mM/dNTP), 0.5 μL primer *alk*JF-*nah*HF (0.05–0.5 mM), 0.5 μL primer *alkJ*R-*nah*HR (0.05–0.5 mM), 2.0 μL DNA (5 pg–0.5 μg), 1.0 μL DMSO (100%) and 17.1 μL H_2O. The PCR program is described in [25]. The amplified sequences were electrophorized in a 1.5% (*w/v*) agarose gel and purified as described in the previous paragraph.

2.5. Estimation of Biodegradation Activity in Batch Cultures

Liquid cultures were prepared for the study of the biodegradation by isolated bacteria. To this end, 100 mL conical flasks containing 50 mL medium were used. Each bacterium, depending on the medium used for isolation, was inoculated either into MSMCO/nMSM medium or AGSCO/nAGS medium. The flasks were incubated at 30 °C, for 14 days at 220 rpm. The inoculum was 10^9 cells/mL derived from cultures grown for 24 h. The same procedure was used for the study of carbohydrate degradation by bacterial consortia containing as many as three bacterial strains in each conical flask. For this study, in which two strains were used, the inoculum concentration was 0.5×10^9 cells/mL each, and when three strains were used, the inoculum concentration was 0.34×10^9 cells/mL. As a control, a series of flasks treated under the same conditions without inoculation was used. All cultures were performed in triplicate. Growth was measured by absorbance at OD_{600nm}. Samples for biomass estimation were collected aseptically and carefully so that no spoilage by oil occurred. Concerning the sample collected on the 14th day, the biomass production was also estimated by dry weight determination. Each sample was collected and mixed with an equal volume of hexane (agent dissolving the oil) and, after vigorous stirring, were centrifuged (9000 rpm, 10 m, 4 °C), washed twice with distilled water and dried by lyophilization for 24 h.

2.6. Analysis of Petroleum Hydrocarbons in Crude Oil and Liquid Naphthalene Cultures

PAHs were determined by the NCSR laboratory "DEMOKRITOS" with an in-house developed and validated method based on [26]. Soil samples (100 g) were extracted with 50 mL methanol overnight under stirring. A volume of 20 mL of methanolic extract was transferred in 500 mL ultra-pure water, then purified and preconcentrated by solid phase extraction (SPE) using ENVI-C18 cartridges (500 mg, 6 mL). The final extract was analyzed by reverse-phase liquid chromatography with fluorescence detection (HPLC-FLD). The following substances were detected: naphthalene, anthracene, fluoranthene, benzo (b) fluoranthene, benzo (k) fluoranthene, benzo (a) pyrene, benzo (g,h,i) perylene and indeno (1,2,3-cd) pyrene. The instrument used was an HPLC/DAD/FLD system AGILENT 1100 SERIES equipped with a Vydac 201 TP 54 column, with a sample volume of 50 μL. The column oven temperature was set at 25 °C, and the flow rate was set at 1 mL min^{-1}. Mobile phase A was acetonitrile, and mobile phase B was acetonitrile/water 50:50. Gradient elution ranged from 50% A to 65% A at 43 min and up to 100% A at 1 min. For the determination of cyclopentapyrene, DAD was set at 222 nm. For the other PAHs, FLD was programmed as follows: excitation program: 0 min at 270 nm, 9 min at 290 nm and 40 min at 270 nm; emission program: channel A: 0 min at 400 nm, channel B: 420 nm, channel C: 470 nm, channel D: 0 min at 370 nm and 9 min 510 nm. Retention times (RT) and channels where each PAH was measured are listed in Table 1a in [26].

Table 1. Physicochemical parameters of *Keri Lake* and Glyfada Beach.

Physicochemical Parameter	Keri Lake	Glyfada Beach
pH	8.5	8.0
Temperature	19.0 °C	22.0 °C
Salinity	6.9 ppt *	37.8 ppt *
Dissolved Oxygen	10.1 mg/L	8.5 mg/L

* (parts per trillion, ppt).

Hydrocarbon concentrations of the batch cultures of the strain *P. plecoglossicida* (ATHUBA 707), which showed the highest degradable capacity, were determined by gas chromatography-mass spectrometry (GC-MS) at the NCSR laboratory "DEMOKRITOS", as described in [25]. Cultures were extracted using an equal volume of n-hexane. The n-hexane soluble fraction was analyzed by gas a chromatography-mass spectrometer (GC-MS hp6890) equipped with a Saturn 4D ion trap detector with a split or unsplit injector and a fused silica column (DB5) (30 m × 0.25 mm i.d.), using He as a carrier gas. The initial oven temperature was 60 °C, and the temperature was increased to 290 °C at a rate of 15 °C min^{-1}. The injector was set at 290 °C in unsplit mode, and then changed to the split mode of 1/10 to the column. The MS had a mass range of 30–400 atomic mass units at a scan rate of 0.5 s $scan^{-1}$. The acquisition time was set to 30 min. The oil analysis standard contained saturated alkanes in the range of C11 through C35, as well as phytane and pristane, which are common components of crude oil, and hydrocarbon isoprenoids of phytol; phytane is the isoprenoid alkane (C20) formed when phytol loses its hydroxyl group, whereas pristane is derived from phytol when one carbon atom is lost (C19) [25].

3. Results

3.1. Physicochemical Parameters of the Sampling Sites

Each of the four contaminated sampling sites displayed different stages of oil contamination: Sampling Sites 1 and 2 seemed to be affected by oil pollution due to oil spillage, whereas Sampling Site 3 was unaffected. Sampling site 4 is a paleoenvironmental setting of *Keri Lake* on Zakynthos Island. The physicochemical parameters of each sampling site are presented in Table 1. According to observations, the only parameter that displays a significant difference is the salinity of the two areas, with that of *Keri Lake* being five times higher than that of Glyfada Beach.

3.2. Determination of Polycyclic Aromatic Hydrocarbons (PAHs) at the Sampling Sites

The concentration of aromatic hydrocarbons was examined at the four sampling sites, and the results are presented in Table 2. Sampling Sites 1, 2 and 3 correspond to Glyfada Beach Sampling Sites A, B and C, respectively. These three sampling sites correspond to three areas displaying varying levels of environmental pollution. At Sampling Site 3, pollution is not detectable, according to the measurements taken. Sampling Sites 1 and 2 are affected by oil pollution. Sampling Site 4, representing samples from *Keri Lake*, displayed the highest values of PAHs, probably due to the long-term exposure of the lake to various PAH pollutants.

3.3. Bacterial Strains Displaying Petroleum-Degrading Ability

For the isolates, we performed a preliminary biochemical identification (paragraph 2.3); thereafter they, were identified by *16S r*DNA gene sequence analysis. A proportion of 53% of the isolated strains was classified as Gram (+), whereas 47% were Gram (-). The majority of the Gram (+) bacteria belonged to the genus *Streptomyces* and *Bacillus*, whereas the dominant genera of Gram (-) isolates was *Pseudomonas*. The majority of the strains were oxidase- and catalase-positive. Most of the Gram (+) strains formed endospores.

In area A, the strains that displayed the highest ability to degrade were three Gram (+) bacteria and one Gram (-), all displaying a positive reaction to catalase and oxidase tests. One of the Gram (+) strains had the ability to produce endospores. In area B, two of

the four most active strains were Gram (+), and all exhibited positive oxidase and catalase activity. At *Keri Lake* (Sampling Site 4), three strains displayed the highest degrading ability, all displaying positive oxidase and catalase activity, whereas two of them were Gram (-). According to observations, none of the bacterial strains isolated from Sampling Site 3 were capable of degrading petroleum hydrocarbons. Concerning the ATHUBA bacterial strains we examined, only two displayed the ability to degrade hydrocarbons. Both were Gram (-) and catalase- and oxidase-positive.

Table 2. Measurement of PAHs of the three sampling sites of Glyfada Beach and Keri Lake sampling site. Measuring unit: μg/kg. Limit of detection (LOD): 0.83 μg/kg for NAPH and FLUO and 0.08 μg/kg for the remaining PAHs. Limit of quantification (LOQ): 2.5 μg/kg for NAPH and FLUO and 0.25 μg/kg for the remaining PAHs.

Sampling Site	Naphthalene (NAPH)	Anthracene (ANTH)	Fluoranthene (FLUO)	Benzo(b) Fluoranthene (B[b]F)	Benzo(k) Fluoranthene (B[k]F)	Benzo(a) Pyrene (B[a]P)	Benzo(ghi) Perylene (B[ghi]P)	Indeno (1,2,3-cd) Pyrene (IP)
Sampling Site 1 Glyfada Beach A	8.63	0.55	7.01	46	7.36	22.4	7.16	5.69
Sampling Site 2 Glyfada Beach B	3.3	32.2	79.7	122	17	60	19.2	7.27
Sampling Site 3 Glyfada Beach C	NT *	NT *	NT *	<LOQ **	NT *	NT *	NT *	NT *
Sampling Site 4 Keri Lake D	9	35.7	75.5	131	17	62	17.8	7.51

* NT: not traceable. ** LOQ: limit of quantification.

3.4. Presence of Petroleum-Hydrocarbon-Degrading Genes alkJ and nahH in the Specific Bacterial Strains

The abundance of the two genes at all the sampling sites is shown in Table 3. The *alk*J gene was traced in 52.6% of the isolated strains and in 40% of the ATHUBA Collection strains. The highest percentage of this gene's presence was observed in *Keri Lake* strains, followed by area B, where microorganisms were exposed at the location of a petroleum oil slick. Finally, the gene was also present at a lower concentration in strains isolated from Area C. The *nah*H gene was traced in 60.5% of the isolated strains and in 40% of the ATHUBA Collection strains. The frequency pattern of the *alk*J gene differed from that of the *nah*H gene among the sampling areas. According to observations, the presence of the *nah*H gene was higher than that of the *alk*J gene, as it was present in 23 of the 38 isolated strains and in 4 of 10 ATHUBA Collection strains. The presence of the *alk*J gene was observed in 20 isolated bacterial strains and in 4 ATHUBA Collection strains. Areas A and B, as well as Keri Lake areas that were exposed to oil pollution, displayed the highest presence of the specific genes. With respect to the simultaneous presence of both genes in one bacterial strain, this frequency was rather low, except in isolates retrieved from Sampling Site B, where 5 of 11 bacterial strains contained both genes. Data on the presence of the two genes in each strain are presented in detail in Supplementary Table S1.

3.5. Growth of Isolated Bacterial Strains in Crude Oil or Naphthalene Batch Cultures

Every isolated bacterial strain was grown in liquid cultures containing crude oil or naphthalene as the sole carbon source. Results are presented in Table 4. As shown in Supplementary Table S1, at Sampling Site 3 (Glyfada Beach C), none of the bacterial strains had any biodegradability. With respect to bacterial population growth, generally, the strain that had the highest biodegradation ability also displayed the highest dry biomass production in both carbon sources examined.

Table 3. Frequency of *alk*J and *nah*H genes in bacterial strains isolated from specific sites.

Sampling Site	Total Isolated Bacterial Strains	Strains with Ability to Degrade Crude Oil	Strains with *alk*J Gene	Strains with *nah*H Gene	Strains with Both Genes Present
Sampling Site 1 Glyfada Beach A	19	16 (84.21%)	7 (46.7%)	9 (60.0%)	2(12.5%)
Sampling Site 2 Glyfada Beach B	15	11 (73.33%)	7 (63.6%)	7 (63.6%)	5 (45.5%)
Sampling Site 3 Glyfada Beach C	31	4 (12.9%)	1 (25.0%)	3 (75.0%)	1 (25.0%)
Sampling Site 4 Keri Lake D	8	7 (87.5%)	5 (71.4%)	4 (54.1%)	2 (28.6%)
ATHUBA Culture Collection	-	10	4 (40.0%)	4 (40.0%)	2 (20.0%)

Table 4. Biodegradability percentage of crude oil and biomass production using crude oil or naphthalene as the unique carbon source. The isolates showing the highest percentages are highlighted and were selected for further investigation.

Sampling Site	Strain Name and Classification	Biodegradability Percentage of Crude Oil (%)	Biomass (g/L) of Crude Oil	Biomass (g/L) of Naphthalene
Sampling Site 1	*Streptomyces flavoviridis,* ATHUBA 682	25.80 ± 0.69	0,11 ± 0,03	0.10 ± 0.02
	Streptomyces aridus, ATHUBA 683	27.80 ± 1.01	0.44 ± 0.05	0.21 ± 0.01
	Staphylococcus sp., ATHUBA 684	30.40 ± 0.99	0.38 ± 0.04	0.27 ± 0.02
	Isoptericola chiayiensis, ATHUBA 685	23.40 ± 0.65	0.19 ± 0.02	0.11 ± 0.01
	Isoptericola chiayiensis, ATHUBA 686	38.70 ± 1.21	0.34 ± 0.03	0.28 ± 0.01
	***Bacillus wiedmannii* sp., ATHUBA 687**	**57.70 ± 0.36**	**0.61 ± 0.04**	**0.41 ± 0.02**
	Staphylococcus warneri, ATHUBA 688	32.10 ± 0.51	0.19 ± 0.01	0.17 ± 0.01
	***Streptomyces* sp., ATHUBA 689**	**66.10 ± 0.56**	**0.49 ± 0.04**	**0.42 ± 0.02**
	Paenibacillus polymyxa, ATHUBA 690	40.00 ± 2.30	0.12 ± 0.01	0.09 ± 0.01
	***Bacillus flexus,* ATHUBA 691**	**60.80 ± 1.14**	**0.67± 0.05**	**0.47 ± 0.02**
	Isoptericola chiayiensis, ATHUBA 692	33.00 ± 0.39	0.13 ± 0.01	0.06 ± 0.01
	Bacillus sp., ATHUBA 693	35.50 ± 0.69	0.11 ± 0.02	0.05 ± 0.01
	Isoptericola sp., ATHUBA 694	45.50 ± 1.00	0.22 ± 0.02	0.10 ± 0.01
	Bacillus sp., ATHUBA 695	40.20 ± 1.27	0.16 ± 0.02	0.12 ± 0.01
	Brevundimonas sp., ATHUBA 696	38.70 ± 1.91	0.21 ± 0.01	0.13 ± 0,01
	***Isoptericola chiayiensis,* ATHUBA 697**	**52.00 ± 1.36**	**0.66 ± 0.04**	**0.25 ± 0.01**
Sampling Site 2	*Streptomyces* sp.,ATHUBA 698	45.60 ± 0.98	0.64 ± 0.04	0.41 ± 0.02
	Halomonas lionensis, ATHUBA 699	27.00 ± 0.87	0.14 ± 0.01	0.14 ± 0.01
	***Halomonas quamarina,* ATHUBA 700**	**50.00 ± 1.03**	**0.41 ± 0.03**	**0.39 ± 0.02**
	Isoptericola chiayiensis, ATHUBA 701	37.50 ± 1.63	0.27 ± 0.01	0.38 ± 0.02
	Isoptericola halotolerans, ATHUBA 702	14.50 ± 0.62	0.19 ± 0.02	0.21 ± 0.01
	Isoptericola rhizophila, ATHUBA 703	43.20 ± 0.28	0.10 ± 0.01	0.12 ± 0.01
	Isoptericola chiayiensis, ATHUBA 704	28.80 ± 1.35	0.15 ± 0.01	0.12 ± 0.01
	Bacillus sp., ATHUBA 705	21.00 ± 1.28	0.37 ± 0.02	0.30 ± 0.02
	***Streptomyces flavoviridis,* ATHUBA 706**	**51.10 ± 1.87**	**0.49 ± 0.02**	**0.41 ± 0.02**
	***Pseudomonas plecoglossicida,* ATHUBA 707**	**76.70 ± 1.23**	**0.73 ± 0.04**	**0.66 ± 0.04**
	***Paenibacillus abyssi,* ATHUBA 708**	**69.30 ± 0.99**	**0.53 ± 0.03**	**0.48 ± 0.03**

Table 4. *Cont.*

Sampling Site	Strain Name and Classification	Biodegradability Percentage of Crude Oil (%)	Biomass (g/L) of Crude Oil	Biomass (g/L) of Naphthalene
Sampling Site 3	*Bacillus cereus*, ATHUBA 709	37.80 ± 0.68	0.10 ± 0.01	0.05 ± 0.01
	Bacillus sp., ATHUBA 710	21.10 ± 2.01	0.15 ± 0.01	0.09 ± 0.01
	Halomonas sp., ATHUBA 711	10.80 ± 1.27	0.15 ± 0.01	0.12 ± 0.01
	Bacillus sp., ATHUBA 712	10.00 ± 0.36	0.19 ± 0.02	0.13 ± 0.01
Sampling Site 4	*Pseudomonas wadenswilerensis*, ATHUBA 713	46.70 ± 0.87	0.47 ± 0.04	0.48 ± 0.03
	Pseudomonas sp., ATHUBA 714	29.10 ± 0.61	0.23 ± 0.01	0.31 ± 0.02
	Halomonas sp., ATHUBA 715	27.80 ± 0.53	0.25 ± 0.01	0.19 ± 0.01
	***Kocuria arsenatis*, ATHUBA 716**	**61.30 ± 1.41**	**0.88 ± 0.05**	**0.75 ± 0.5**
	***Brevundimonas vesiculari*, ATHUBA 717**	**59.20 ± 1.18**	**0.71 ± 0.05**	**0.79 ± 0.06**
	***Pseudomonas flavescens*, ATHUBA 718**	**52.10 ± 1.39**	**0.68 ± 0.04**	**0.56 ± 0.04**
	Bacillus sp., ATHUBA 719	38.70 ± 0.97	0.27 ± 0.01	0.31 ± 0.2
ATHUBA Culture Collection	*Streptomyces griseus*, ATHUBA 720	25.20 ± 0.67	0.32 ± 0.02	0.13 ± 0.01
	Streptomyces chromofuscus, ATHUBA 721	18.20 ±0.57	0.41 ± 0.03	0.32 ± 0.4
	Streptomyces lividans, ATHUBA 722	21.90 ± 0.61	0.47 ± 0.02	0.45 ± 0.04
	Streptomyces rochei, ATHUBA 723	24.00 ± 0.32	0.60 ± 0.04	0.35 ± 0.02
	Streptomyces griseus, ATHUBA 724	25.20 ± 0.32	0.83 ± 0.06	0.66 ± 0.05
	***Nitratireductor aquamarina*, ATHUBA 725**	**50.80 ± 1.25**	**0.27 ± 0.01**	**0.15 ± 0.01**
	Thalassospira sp., ATHUBA 726	47.00 ± 1.10	0.50 ± 0.04	0.47 ± 0.03
	***Pseudomonas plecoglossicida*, ATHUBA 727**	**60.10 ± 1.38**	**0.73 ± 0.05**	**0.27 ± 0.01**
	Nitratireductor sp., ATHUBA 728	41.50 ± 0.99	0.28 ± 0.01	0.28 ± 0.02
	Pseudomonas sp., ATHUBA 729	40.00 ± 0.87	0.80 ± 0.06	0.45 ± 0.03

Investigation of the average biodegradation ability of all bacterial strains per area revealed that those of *Keri Lake* displayed the highest biodegradation ability, followed by areas B and A. The biodegradation ability of the ATHUBA Culture Collection strains is also worth mentioning. The lowest biodegradability percentage was observed at Sampling Site 3 (Glyfada Beach C).

Investigation of the average dry biomass of bacterial strains per sampling area revealed that dry biomass in a liquid culture using crude oil as the unique carbon source had a slightly higher average dry biomass than that of a culture using naphthalene as the carbon source. The bacterial strains of the region with the highest values of biodegradability were those of *Keri Lake*, followed by the strains of the ATHUBA Culture Collection and regions A and B. Finally, the average dry biomass of region C displayed the lowest values of all sampling sites examined. A total of 13 bacterial strains were selected for further investigation according to their ability to biodegrade crude oil and their biomass, as shown in Table 4.

As shown in Table 4, the highest biodegradation percentage was observed in the strains *Pseudomonas plecoglossicida*, ATHUBA 707 (76.7%), and *Paenibacillus abyssi*, ATHUBA 708 (69.3%), both isolated from Sampling Site 2. The strain *Kocuria arsenatis*, ATHUBA 716, displayed the highest biomass production of all strains grown under the same conditions. The strain *Nitratireductor* sp., ATHUBA 725, although showing a very low biomass production, still displayed a considerable biodegradation ability.

3.6. Patterns of Biodegradability of Specific Bacterial Strains

Bacterial strains with a degradation rate of more than 40% showed obvious differences in biodegradation pattern when they were incubated in liquid cultures, with crude oil as the carbon source (liquid AGSCO). The bacterial cultures were grown for 14 days in the

media as described in Section 2.4. Based on the achieved biodegradation process, they were grouped into those that produced oil agglomerates that possibly contain high-molecular-weight alkanes and those that released surfactants (Figure 1).

Figure 1. Liquid bacterial cultures containing crude oil as a carbon source. (**A**) Control liquid broth with no microorganism added. (**B**) Liquid bacterial culture in which agglomerates were observed after 14 d of incubation. (**C**) Liquid bacterial culture in which surfactants were released after 14d of incubation.

The following isolated strains produced agglomerates: *Bacillus* sp., ATHUBA 687; *Streptomyces* sp., ATHUBA 698; *Pseudomonas wadenswilerensis*, ATHUBA 713; *Pseudomonas* sp., ATHUBA 718; and *Nitratireductor* sp., ATHUBA 728.

The following isolated strains released surfactants when grown on crude oil: *Streptomyces* sp., ATHUBA 689; *Paenibacillus polymyxa*, ATHUBA 690; *Bacillus flexus*, ATHUBA 691; *Isoptericola* sp., ATHUBA 694; *Bacillus* sp., ATHUBA 695; *Isoptericola chiayiensis*, ATHUBA 697; *Halomonas aquamarina*, ATHUBA 700; *Isoptericola halotolerans*, ATHUBA 702; *Streptomyces flavoviridis*, ATHUBA 706; *Pseudomonas plecoglossicida*, ATHUBA 707; *Paenibacillus abyssi*, ATHUBA 708; *Kocuria arsenatis*, ATHUBA 71; *Brevudimonas sp.*, ATHUBA 717; *Nitratireductor sp.*, ATHUBA 725; *Thalassospira sp.*, ATHUBA 726; *Pseudomonas sp.*, ATHUBA 727; and *Nitratireductor sp.*, ATHUBA 728.

According to observation, the release of surfactants occurred much more frequently than the production of agglomerates. The strains with the highest percentage of biodegradation (ATHUBA 707 and ATHUBA 708) both released surfactants, whereas ATHUBA 687 and ATHUBA 718, which produced agglomerates, were among those displaying high biodegradability percentages.

3.7. Development of Consortia for Crude Oil Degradation

The successful combination of various bacteria in a consortium that can better biodegrade crude oil has been demonstrated in various bioremediation treatments [18]. To this end, we formed two distinct consortia. The strains were selected for each consortium, not only for their ability to degrade crude oil but also for their ability to form agglomerates or to produce surfactants that would facilitate crude oil biodegradation. Each consortium had one representative strain from Sampling Sites 1, 2 (A and B) and 4 (Keri Lake). Within this scope, the first consortium was comprised of *Streptomyces sp.*, ATHUBA 689 (Area A, Biodeg %); *Pseudomonas plecoglossicida*, *ATHUBA 707* (Area B, Biodeg %); and *Pseudomonas wadenswilerensis*, ATHUBA 713 (Area C, Biodeg %). The second consortium included the following strains: *Bacillus flexus*, ATHUBA 691 (Area A, Biodeg %); *Paenibacillus abyssi*, ATHUBA 708 (Area B, Biodeg %); and *Kocuria arsenatis*, ATHUBA 716 (Area C, Biodeg %). According to the results, the highest degradation yield of total hydrocarbons was recorded by Consortium 1 (63.10%), followed by Consortium 2, (58.30%) after 14 days of incubation in 5% crude oil. Although both biodegradation percentages were high, they did not surpass the percentages displayed when specific microorganisms were used. The dry biomass of the Consortium 2 (1.31 g/L) was slightly higher than that of Consortium 1 (1.22 g/L), whereas both consortia resulted in the production of surfactants, and at Consortium 1, small agglomerates were formed. This result was expected, as ATHUBA 713, which participates in Consortium 1, produced visible agglomerates when grown in 5% crude oil liquid cultures.

3.8. Alkanes/Hopanes Ratio during Crude Oil Degradation by Pseudomonas plecoglossicida

The strain ATHUBA 707, which, according to its identification through sequence analysis of the 16S rDNA gene, exhibits a phylogenetic similarity to *Pseudomonas plecoglossicida*, was the strain with the highest rate of biodegradation. Owing to its action, this strain was selected to investigate the ratio of alkanes/hopanes concentration by gas chromatography-mass spectrometry (GC-MS) (Figure 2), both in short alkanes (C11-C21) and in long alkanes (C22-C35). In the first two days of incubation in a liquid AGSCO medium, a decrease in the ratio of alkanes/hopanes was observed, which indicates a higher degradation efficiency for alkanes than hopanes. By the 10th day of incubation, this decrease was significant. Furthermore, the strain showed significant degradation in both short alkanes (C11-C21) and long alkanes (C22-C35). Furthermore, short alkanes were present in higher concentrations and appeared to be degraded at a higher rate than longer alkanes appearing in lower concentrations.

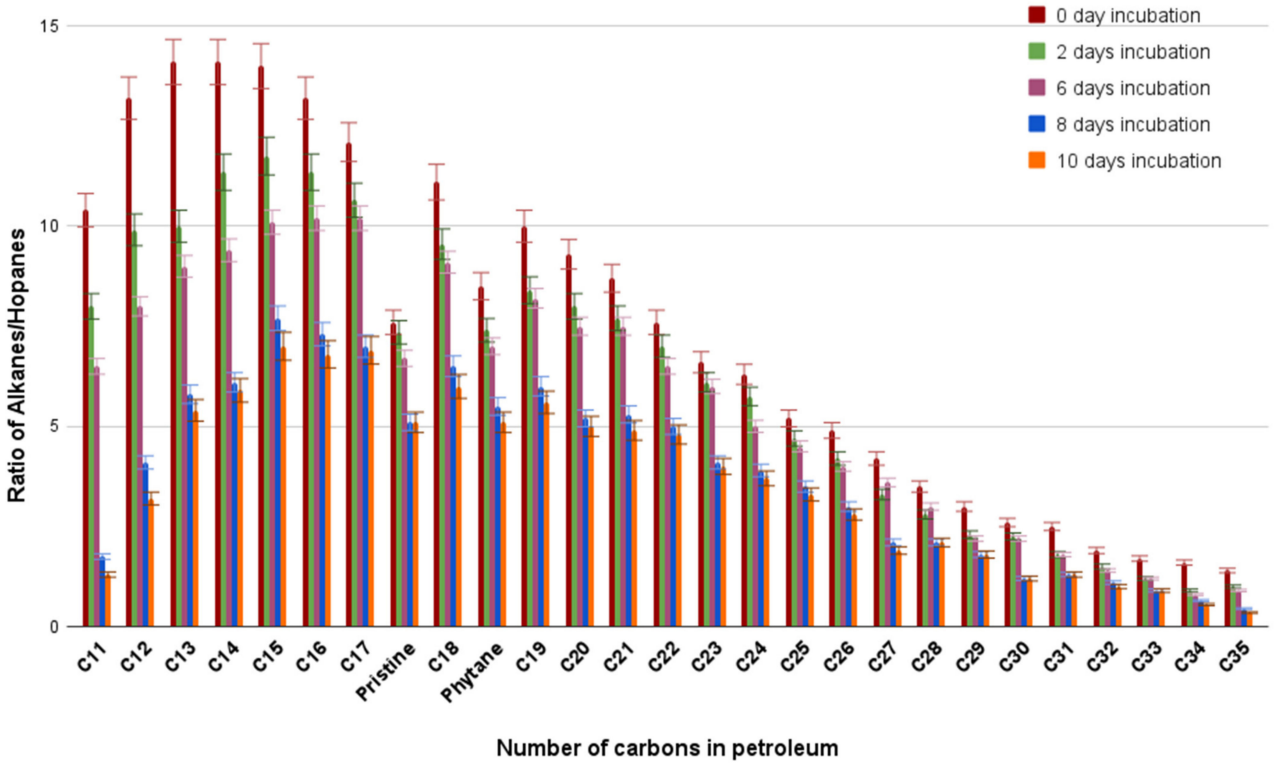

Figure 2. Ratio of alkanes/hopanes for hydrocarbons of various lengths when degraded by *Pseudomonas plecoglossicida* strain ATHUBA 707 after incubation in liquid culture with single-carbon-source 5% (*w/v*) oil after 0, 2, 6, 8 and 10 days at 250 rpm and a temperature of 25 °C. Pristane and phytane are common components of crude oil and are hydrocarbon phytol isoprenoids; phytane is the isoprenoid alkane (C20) formed when phytol loses its hydroxyl group, whereas pristane is derived from phytol when one carbon atom is lost (C19).

4. Discussion

4.1. Physicochemical Parameters of Sampling Sites and Microbial Diversity

We analyzed the physicochemical parameters of the contaminated areas, as well as the occurrence of various PAHs in the collected samples. In the present work, various bacterial isolates were collected from four specific Greek sampling sites, representing varying degrees of oil contamination. The lower microbial diversity observed at Keri Lake may be related to its higher salinity. Liu et al. [11] observed that higher salinity and pH were limiting factors for the biodegradation of polycyclic hydrocarbons in soils of the Dagang Oilfield (China). The more extreme the environmental conditions of a specific site, the lower the microbial biodiversity. Recently, it was reported that salinity was the most

important environmental factor that controlled the microbial community in five lakes and six rivers in the semi-arid Inner Mongolia Plateau area [27]. The authors concluded that salinity resulted in dramatic declines in species diversity. In accordance with these findings, increased salinity usually imposes a strong selection stress, which, in combination with high growth rates, may lead to a bacterial community comprised of autochthonous species capable of surviving in oil-contaminated niches.

4.2. Occurrence of alkJ and nahH Genes in Bacteria Populations Is Correlated with the Level of Contamination

In all isolates, as well as in other bacterial strains belonging to the ATHUBA bacterial collection (collection of the Laboratory of Microbiology, Section of Botany, Department of Biology, School of Science, National and Kapodistrian University of Athens), the *nah*H and *alk*J genes were detected by PCR using specific primers [28–30].

The three sampling sites of the Glyfada Beach area displayed varying degrees of oil contamination, with the Sampling Site 1 being the most polluted area (high levels of PAHs, as shown in Table 2) and Sampling Site 3 showing the lowest PAH concentration. *Keri Lake* is environmentally burdened by its long-term exposure to pollutants. This could explain the fact that *Keri Lake* not only displayed the highest percentage of total isolates with the ability to degrade crude oil (seven of eight; 87.5%) but was also the only area where isolates harbored both examined genes. Sampling Sites 1 and 2 followed, displaying large numbers of isolates capable of degrading petroleum, as well as high frequencies of *nah*H and *alk*J genes presence. Petroleum-degrading-related genes have been used as an assessment index for the pollution of specific areas [11]. Because naphthalene dioxygenases (*nah*s) display a wider range of target substrates compared to alkane monooxygenases (*alk*s), it is possible that they appear more frequently in oil-contaminated soils. This could explain the higher percentage concentration of *nah*H genes compared to *alk*J genes in the various sampling sites.

4.3. Bacterial Population Diversity in Contaminated Sites

The sustainability of various environmental areas is often maintained due to microbes that can degrade petroleum and are frequently isolated from areas that are heavily polluted. As stated by Liu et al. [11], many endogenous oil-degrading microorganisms have been identified to date, such as *Pseudomonas* strain GPo1 (C5–C12, n-alkanes) [31], *Acinetobacter* sp. strain DSM17874 (C10–C40, n-alkanes) [32], *Rhodococcus* sp. strain Q15 (C12–C32, n-alkanes) [33], *Pseudomonas putida* G7 (naphthalene) [34], *Burkholderia* sp. strain JS15 (BTEX) [35] and *Pseudomonas aeruginosa* JI104 (toluene) [36]. In our current study, the isolates belong mainly to *Streptomyces* sp., *Isoptericola* sp., *Halomonas* sp. and *Bacillus* sp., whereas the isolate that displayed the highest biodegradability was isolated from Sampling Site 2 and belonged to *Pseudomonas plecoglossicida* species, with both genes (*nah*H and *alk*J) present in its genome. Recently Bakaeva et al. [37] used a *P. plecoglossicida* strain that displayed high ability to degrade petroleum hydrocarbons (around 70%) in soil contaminated with petroleum experiments in order to examine the possible phytoremediation ability of the specific strain and whether it is capable of inducing plant growth. The results were satisfactory, as treatment with *Pseudomonas* strain oil-degrading bacteria seemed to promote plant growth that had been inhibited as a result of petroleum stress.

4.4. Cometabolism of Hydrocarbons by Microbial Community and Consortia Development

Taking into consideration the patterns of biodegradability and the ability of each strain to degrade crude oil, two consortia were formed and used for biodegradation studies in liquid cultures. The consortium that included the specific isolate yielded a total hydrocarbon degradation of 63.10% and did not exceed that of the specific isolate when used alone (76.7%). It is possible that the antagonistic relationships between the consortium bacteria did not permit a higher degradation percentage, although, as stated previously, extensive crude oil biodegradation is achieved when each bacterium degrades a fraction

of the compounds present in the complex crude oil [9,38,39]. It is generally believed that microbial consortia composed of multiple strains exhibit better oil degradation efficiency. The diversity of the strains used in a consortium implies a variety of enzymes used during oil bioremediation, as well as the use of various catabolic pathways of PAH degradation [16]. Oil degradation ability can be significantly improved with an appropriate selection of microorganisms. Although scientists agree that the selection of autochthonous strains can achieve better results, many aspects of the microorganisms' selection criteria need to be considered in order to obtain a successful microbial consortium with improved degrading capacity [17].

4.5. Selection of a Specific Strain and Its Potential Use in Autochthonous Bioaugmentation

A *P. plecoglossicida* isolate displayed the highest biodegradability. It was isolated from Sampling Site 2, and both genes (*nah*H and *alk*J) were present in its genome. This specific strain had the ability to release surfactants when grown on crude oil. As previously stated [37,40–42], *Pseudomonas* strains can be used for the biodegradation of petroleum in soils and thus enhance phytoremediation processes. Furthermore, the ratio of alkanes/hopanes during *P. plecoglossicida* crude oil degradation indicates a high degrading ability of the specific autochthonous isolate. The biodegradation procedure can lead to a final product that may provide a possible substrate for further bioremediation by other microorganisms able to degrade hydrocarbons of lower molecular weight, or it can be used in phytoremediation procedures as an initial inoculum that strengthens the ability of plants to remediate highly contaminated soils. This specific strain, which achieves a biodegradation percentage of slightly less than 80%, may be further used alone or in combination with other microorganisms to effectively degrade oil in contaminated sites.

5. Conclusions

The aim of the above work was the isolation and identification of biotechnologically important strains from Greek environments displaying various degrees of oil contamination. As a result of the above work, some important conclusions were drawn. First of all, salinity led to a decline in bacterial biodiversity at the examined sites. Secondly, the *alk*J and *nah*H genes can be used as biomarkers of oil contamination. Their presence is frequent in isolates from heavily oil burdened areas, with *nah*H appearing more frequently than *alk*J. Furthermore, there seems to be a linear correlation between the degrading ability of the strains and exposure to high oil concentration. In this study, various autochthonous Greek isolates displayed high biodegradation capacity. One strain achieved 77% biodegradation capacity and was capable of quickly catabolizing high concentrations of short and long alkanes. In the constructed consortium, the biodegradation capacity did not exceed that of the individual selected strain.

Because selected strains isolated from Greek environments have repeatedly been used in various biotechnological procedures (antibiotics production [21,43], waste detoxification and management [25,44], biomass production etc.), it can be readily assumed that Greek isolates may pave the way for new approaches with respect to adequately effective biodegradation procedures for crude oil.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su14159562/s1, Table S1: The results from the comparison of the 16rRNA sequence with the existing databases analysis where the microorganism closely related to each strain tested and the data concerning the presence of the two genes, *alk*J and *nah*H, in each strain.

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