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Crude Oil Degradation by a Novel Strain *Pseudomonas aeruginosa* AQNU-1 Isolated from an Oil-Contaminated Lake Wetland

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Abstract: In this study, a novel crude oil degrading bacterium was isolated from an oil-contaminated freshwater lake using crude oil as the sole carbon source. The strain was named *Pseudomonas aeruginosa* AQNU-1 based on the analyses of its morphological characteristics and 16S rRNA gene sequencing. Gas chromatography-mass spectrometry (GC-MS) was carried out to investigate the degradation of crude oil fractions under dynamic (37 °C, 180 r/min) and static (37 °C, 0 r/min) cultivation over three months of continuous enrichment in the laboratory. It was found that strain AQNU-1 exhibited stronger biodegradation efficiency for *n*-alkanes of C13–C35 under dynamic cultivation with degradation ratios of 87–100% compared to ratios of 74–100% under static cultivation. Furthermore, this strain could fully utilize alkylcyclohexane (M/Z 82), alkylbenzene (M/Z 92) and alkyltoluene (M/Z 106) in crude oil under both conditions. It also had better biodegradability of partial aromatic compounds in the crude oil, showing differences within compound families of aromatic hydrocarbons. Further, the potential degradation ability of this isolated strain decreased with increasing molecular weight, with the dynamic condition performing better in general. These results suggest that the isolated strain has great potential to assimilate indigestible crude-oil contaminants under different hydrological conditions, providing a valuable microbiological resource for in situ remediation of natural wetlands.

Keywords: lake wetland; crude-oil contamination; *Pseudomonas*; dynamic and static cultivation; aromatic compound family

Citation: Liu, H.; Yang, G.; Jia, H.; Sun, B. Crude Oil Degradation by a Novel Strain *Pseudomonas aeruginosa* AQNU-1 Isolated from an Oil-Contaminated Lake Wetland. *Processes* **2022**, *10*, 307. https://doi.org/10.3390/pr10020307

Academic Editor: Andrea Petrella

Received: 24 December 2021
Accepted: 1 February 2022
Published: 4 February 2022

1. Introduction

Crude oil is composed of diverse organic fractions, thus it can be used as energy and also as a raw material to synthesize products for our daily life and agricultural fertilizers. Due to human activities, such as refining, processing, storage and transportation, petroleum chemicals have become a major pollutant in the aquatic environment [1]. In general, the presence of petroleum hydrocarbons can reduce and shift the microbial diversity in various ecosystems. Notably, it also successfully stimulates the enrichment of hydrocarbon-degrading bacteria [2,3]. It is well known that natural wetlands are able to provide many ecosystem services, such as species storage, climate regulation and ecological protection. However, the issue of hydrocarbon contamination in natural wetlands cannot be ignored. As summarized by Zhang et al. [4], the Sundarban Mangrove Wetland in India (634 ng/g), the Liaohe Estuarine Wetland (1001.9 ng/g) and Yellow River Delta (150.9 ng/g) in China, and the Seine River Basin in France (450 ng/g), as well as other wetlands, suffer from severe polycyclic aromatic hydrocarbon (PAH) contamination. In addition, the Σ16PAH concentration in wetland sediment that we studied ranged from 450–3640 ng/g. Thus, more attention should be paid to wetlands contaminated by petroleum hydrocarbons to ensure their ecological balance and sustainability.

As an alternative technology to the traditional methods of dumping, landfilling and incineration, microbial remediation is considered as environmentally benign, highly efficient and widely acceptable for removing crude oil contaminants in various environments [5,6]. Moreover, some microorganisms utilize *n*-alkanes, PAHs, resins, asphaltenes and other hydrocarbons as carbon sources in oil-polluted soils, displaying tolerability under different concentrations of these chemicals. For example, Jia et al. [7] showed that *Rhodococcus* sp. T1 is able to deplete 90.81% of *n*-hexadecane (2% v/v) and 42.79% of pyrene (200 mg/L) within 5 days, while Varjani and Upasani [8] revealed that 60.63% of crude oil (C8-C36) was assimilated by *Pseudomonas aeruginosa* NCIM 5514. In addition, Kshirsagar et al. [9] isolated six asphaltene-degrading bacterial strains from oil-contaminated sites, with the bacterium *Ochrobactrum intermedium* removing 30% of the asphaltene within 15 days. Until now, most studies have focused on the biodegradation of a few representative components in crude oil, while reports on the degradation of most components by highly efficient strains are not common. Generally, the hydrocarbon components in crude oil-contaminated wetlands are extremely complex; however, isolating and the screening of highly efficient hydrocarbon-degrading bacteria may provide a potential resource for oil-contaminated ecological bioremediation.

Microorganisms exhibit diverse functional mechanisms related to crude oil degradation, such as possessing multiple catabolic genes, which enhance access to target petroleum hydrocarbons and mineralizes hydrocarbon substrates. There are many genes responsible for the transformation and metabolism of petroleum hydrocarbons, such as *alkB*, *LadA*, *pcaG*, *nahAc*, *nahH* and *alkJ*, encoding alkane monooxygenase, ring-hydroxylating dioxygenase, naphthalene dioxygenase, catechol 2,3-dioxygenase and aliphatic alcohol dehydrogenase, respectively [10–14]. Furthermore, some microbial biosurfactants can effectively reduce the surface and interfacial tension between the aqueous and organic phases, which is especially important for improving the low solubility and bioavailability of hydrophobic hydrocarbon-related contaminants [15]. Special functional secretions are produced by a variety of microorganisms, such as the genera *Pseudomonas*, *Bacillus*, *Acinetobacter* and *Meyerozyma* [16–18]. Furthermore, many enzymes show broad substrate specificity during the petroleum hydrocarbon degradation process. For example, catechol 2,3-dioxygenase, dehydrogenase and peroxidase can mediate the degradation of three-ring PAHs, such as phenanthrene, anthracene and fluorene [19]. Additionally, ring-hydroxylating dioxygenases, dihydrodiol dehydrogenases and ring-cleaving dioxygenases can cooperate to efficiently degrade fluoranthene [11]. In general, various alkane hydroxylases present different degradation abilities, which are associated with the chain length of alkanes. Medium-chain *n*-alkanes can usually be degraded by most of the AlkB or AlkB-like family, as well as by cytochrome P450, while LadA and AlmA prefer to assimilate long-chain *n*-alkanes larger than C20 [20]. Meanwhile, the degree of petroleum hydrocarbon degradation is associated with multiple factors related to the reaction system, such as nutrients, salts, pH, oxygen content, temperature, and substrate concentration. Adding appropriate nutrients can enhance the microbial degradation process of petroleum hydrocarbons. Sun et al. [21] demonstrated that sufficient nitrogen and phosphorus content could ensure the survival and degradation activity of the engineered strain *Acinetobacter* sp. HC8-3S-9 during bioaugmentation of crude oil. Additionally, high salinity can significantly inhibit biodegradation. The hydrophobicity of the cell surface is reduced under high salinity, restricting the productivity of bio-emulsifiers and the degradation of insoluble long-chain *n*-alkanes [22]. Furthermore, molecular oxygen is involved in substrate oxidation mediated by oxidase during the aerobic degradation process, aiding in ring cleavage and serving as a terminal electron acceptor [23]. In natural lake wetlands, oxygen can be a limiting factor for hydrocarbon biodegradation due to the low solubility. Meanwhile, there are various factors that affect oxygen availability, such as the disturbance of wave and water flow, the physical state of the crude oil and the amount of available substrates [24].

Presently, studies addressing lake wetlands exposed to long-term oil pollution are still scarce. Screening endogenous and efficient crude-oil degrading bacteria from oil-

contaminated lake wetlands should be helpful for in situ ecological restoration of lake contamination. In this study, a novel strain *P. aeruginosa* AQNU-1, isolated with crude oil as the sole carbon source, was profiled by studying its morphological characteristics, 16S rRNA gene sequence, carbon metabolic characteristics through Biolog-EcoPlate™ microplate and crude oil degradation by GC-MS. The objectives of this study were to: (1) screen highly efficient hydrocarbon-degrading bacteria from an oil-contaminated lake wetland; (2) assess the hydrocarbon-degrading potential and metabolic characteristics of the isolated bacteria; and (3) discover the degradation differences under dynamic and static culture processes in order to explore the bioremediation capability of the functional strain for oil-contaminated lake wetlands.

2. Materials and Methods

2.1. Water Sampling

For screening highly efficient hydrocarbon-degrading bacteria, water was sampled from a long-term oil-contaminated lake wetland (30°32′04″ N, 117°02′50″ E) close to a large number of petrochemical industries in Anqing City. This sampling area is located in the middle and lower reaches of the Yangtze River. Statistically, a large amount of industrial wastewater from the petrochemical industries (over 8 million tons per year) is emitted into the lake wetlands. The oil content of the partial wetland water reaches 0.18 mg/L, while the total nitrogen, ammonium nitrogen and total phosphorus contents are 3.5 mg/L, 0.8 mg/L and 0.02 mg/L, respectively [25].

2.2. Isolation and Selection of Crude Oil-Degrading Bacteria

First, 5% wetland freshwater was inoculated in 250 mL Erlenmeyer flasks containing 100 mL of mineral salts medium (MSM). The medium (pH 7.2) contained 5.0 g L^{-1} of NaCl, 5.0 g L^{-1} of KH_2PO_4, 2.0 g L^{-1} of $NaNO_3$, 1.0 g L^{-1} of $(NH_4)_2SO_4$, 1.0 g L^{-1} of $KH_2PO_4 \cdot 3H_2O$, 0.25 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 0.02 g L^{-1} of $FeCl_2 \cdot 4H_2O$, 0.02 g L^{-1} of $CaCl_2$ and 1.0 g L^{-1} of crude oil, and was sterilized at 121 °C for 30 min. All flasks were sealed with 0.4 μm sealing film, covered with craft paper to reduce evaporation, and then enriched for 1 month at 37 °C and 180 rpm on a rotary shaker. The culture was inoculated three times consecutively by transferring 5 mL of inoculum to 100 mL of fresh MSM. Finally, a portion of the enriched medium was plated onto the aforementioned MSM containing 2.0% of agarose, and cultured in a thermostatic culture box for 7 days to obtain various pure colonies [26]. At present, all isolated strains are preserved in the microbiology laboratory at our university.

2.3. Analysis of Morphological Observation

The morphological characteristics of *P. aeruginosa* AQNU-1 were profiled by Gram staining and scanning electron microscopy (SEM). Gram staining was carried out after culturing for 18 h in beef extract-peptone liquid medium (3.0 g L^{-1} of beef extract, 10.0 g L^{-1} of peptone, 5.0 g L^{-1} of NaCl; pH 7.2), and the results were photographed using a Leica DM500 microscope (Leica Microsystems, Wetzlar, Germany). Gram-positive bacteria have a violet color, while Gram-negative bacteria are pink. For SEM analysis, bacterial colonies from a beef-extract solid medium were fixed with 2.5% glutaraldehyde overnight at 37 °C. Cell pellets were then collected and rinsed thrice with 0.1 M phosphate buffered solution (PBS, pH 7.0) for 15 min. The cell sample was post-fixed for 1–2 h in 1% osmium tetroxide, after which the osmium tetroxide was carefully removed. The sample was then dehydrated twice using an ethanol gradient (30%, 50%, 70%, 80%, 90% and 95%; 20 min per concentration). Next, the specimen was treated for 30 min with ethanol-isoamyl acetate (1:1 v/v), and further processed for 1 h with isoamyl acetate. Finally, the specimen was dried overnight in a fume hood, mounted on aluminum stubs, sputtered-coated with gold and then visualized using an electronic microscope.

2.4. 16S rRNA Gene Sequencing

Total DNA of *P. aeruginosa* AQNU-1 was extracted using the SDS method [27]. The 16S rRNA sequence was PCR-amplified using the universal primer of 27f (5′-AGAGTTTGA TCCTGGCTCAG-3′) and the reverse primer of 1492r (5′-TACGGCTACCTTACGACTT-3′) [26]. The amplification reaction was performed in a total volume of 25 μL containing 15.5 μL of sterile deionized water, 2.0 μL of Mg^{2+} (25 mM), 2.0 μL of dNTPs (2.5 mM), 1.0 μL of each forward and reverse primer (25 μM), 0.5 μL of DNA template, 0.5 μL of *Taq*DNA polymerase (5 u μL^{-1}) and 2.5 μL 10 × buffer solution (20 mM). Amplification was performed using the GeneAmp® PCR System 9700. The temperature profile of the PCR was as follows: 95 °C for 5 min (predenaturation); 35 cycles at 94 °C for 30 s (denaturation), 53 °C for 1 min (annealing) and 72 °C for 2 min (extension); and finally 72 °C for 10 min (extension). Amplicons were checked by 1.0% agarose gel electrophoresis, purified with the Tiangel Midi Purification Kit (Sangon Biotech Co., Ltd., Shanghai, China) and then sequenced (Sangon Biotech Co., Ltd., Beijing, China). The phylogenetic tree was constructed using the neighbor-joining method with the MEGA 5.0 software.

2.5. Carbon Source Utilization

First, 1.5 mL of cultivation (beef extract-peptone liquid medium, 18 h) was placed in a 2.0 mL centrifuge tube, centrifuged at 10,000 r/min for 20 min, and then the supernatant was discarded. Next, 1 mL of sterilized NaCl solution (0.85%) was added and shaken for 5 min, and then the mixture was centrifuged at 10,000 r/min for 20 min. This process was repeated twice to remove carbon sources in the seed liquid. Another 1 mL of NaCl solution was then added and shaken for 5 min, and the mixture was centrifuged at 2000 r/min for 1 min. The supernatant was diluted with 20 mL of sterilized NaCl solution to an optical density at 590 nm (OD_{590}) of 0.13 ± 0.02. Finally, 150 μL of the diluted solution was inoculated in each well of a Biology Ecoplate™ and incubated at 20 °C for 5 days. All processes required aseptic manipulation. The OD_{590} and OD_{750} were measured directly every 24 h for 5 days, using a Multi-mode Reader (Thermo Fisher Multiskan FC, Waltham, MA, USA). To minimize the risk of contamination, all solutions were prepared with sterile water.

2.6. Growth Assay with Different Mediums

Growth assays of strain AQNU-1 were conducted using beef extract-peptone liquid medium and liquid MSM. Seed liquid of the tested strain was incubated at 37 °C and 180 r/min for 18 h in beef extract-peptone liquid medium. Then, 5 mL of seed liquid was collected and washed twice with 7.5 mL of sterilized NaCl solution. Each washed inoculation was added to 100 mL of sterilized beef extract-peptone liquid medium or MSM containing 5.0 g crude oil. All experiments were carried out under aseptic conditions. Cultivation flasks were sealed with 0.4 μm sealing film and covered with craft paper to reduce the evaporation. The cultivation in the beef extract-peptone liquid medium was conducted at 37 °C and 180 r/min for 96 h, while MSM was chosen for dynamic (180 r/min) and static (0 r/min) cultivations at 37 °C for 148 h. The growth in different mediums was assessed by measuring the turbidity at 600 nm.

2.7. Degradation Properties

In order to explore the potential degrading capability of *P. aeruginosa* AQNU-1 in the oil-contaminated lake wetland, degradation experiments were designed under dynamic and static culture processes. The approach was divided into two parts: dynamic cultivation (37 °C, 180 r/min) and static cultivation (37 °C, 0 r/min). Briefly, 5 mL of activated seed liquid was inoculated into 100 mL of MSM medium with 1.0% crude oil as the sole carbon source. Each cultivation was performed in triple using 250 mL Erlenmeyer flasks. All flasks were sealed with 0.4 μm sealing film and tightly covered with craft paper. The dynamic cultivations were incubated on a rotary shaker at 37 °C and 180 rpm, while the static cultivations were conducted in a constant temperature incubator at 37 °C. After the continuous enrichment culture finished, the residual oil of the last batch was extracted with dichloromethane,

and then dehydrated and cleaned using anhydrous Na_2SO_4 columns and Al_2O_3 columns. Hydrocarbon fractions were determined by GC-MS using a 7890A gas chromatograph coupled to a 5975C MS spectrometer (HP-5MS, 30 m × 0.25 mm i.d., 0.25 μm film). The internal conservative molecular marker 17 α(H),21β(H)-hopane (M/Z 191) was selected as an internal standard to calculate the degradation rate of *n*-alkanes and aromatic compound families. Determination of *n*-alkanes was based on the special fragment ion of M/Z 85, while detection of aromatic compound families was conducted according to corresponding molecular ions: naphthalenes, N-N4 (M/Z 128, 142, 156, 170, 184); fluorenes, F-F3 (M/Z 166, 180, 194, 208); phenanthrenes, P-P3 (M/Z 178, 192, 206, 220); dibenzothiophenes, D-D3 (M/Z 184, 198, 212, 226); fluoranthenes and pyrenes, Py-Py3 (M/Z 202, 216, 230, 244); and chrysenes, C-C3 (M/Z 228, 242, 256, 270) [25].

3. Results

3.1. Isolation and Identification of a Crude Oil-Degrading Strain

A highly efficient hydrocarbon-degrading strain was successfully isolated from a lake wetland. This strain grows rapidly on beef extract-peptone solid medium. Colonies are yellow-brown and have spreading irregular edges (Figure 1a). In addition, this strain produces light pink pigment in the liquid MSM (data not shown). The strain is Gram-negative and has a short-rod shape with a size of about 0.4 μm × 1.0 μm according to SEM analysis (Figure 1b,c). The 16S rRNA gene sequence contains 1389 bp, with a G+C content of 54.2%. In addition, the 16S rRNA gene fragment shows a stable genetic clade with reported strains of *Pseudomonas*, with nearly 100% similarity to *P. aeruginosa* PAO1CT according to the neighbor-joining method (Figure 2). Phylogenetic analysis revealed that this strain belongs to the genus *Pseudomonas*. Thus, this strain was named *P. aeruginosa* AQNU-1 in our study.

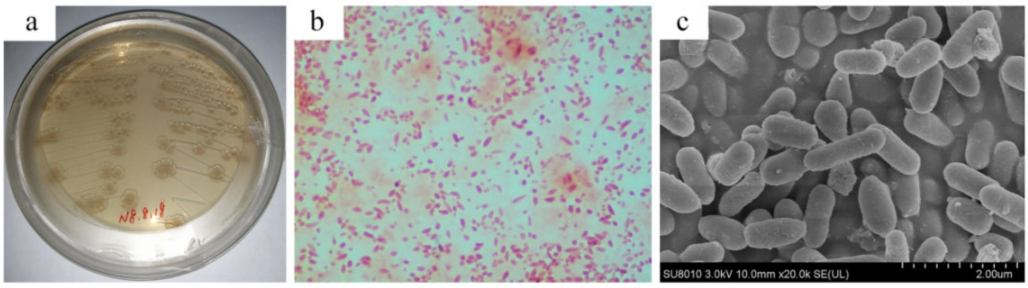


Figure 1. Morphological observations of *P. aeruginosa* AQNU-1. (**a**) Bacterial colonies on the beef extract-peptone solid medium, (**b**) Gram staining of strain AQNU-1, and (**c**) SEM photograph of strain AQNU-1 (10.0 mm × 20.0 k).

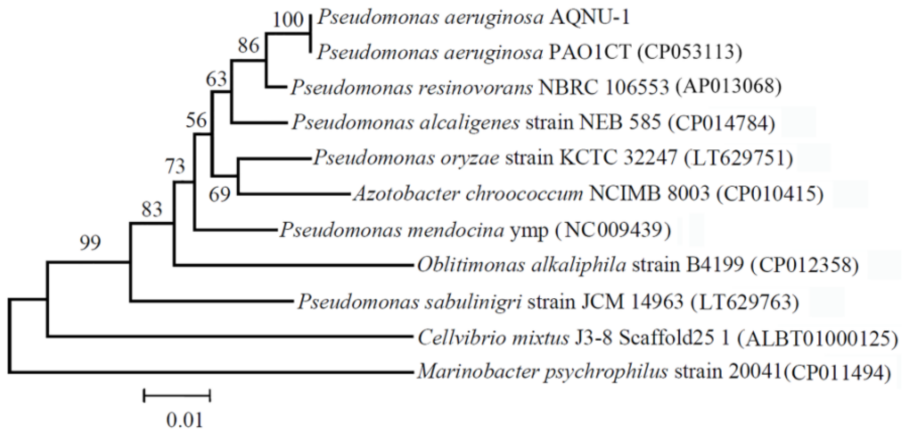


Figure 2. Phylogenetic relationship between *P. aeruginosa* AQNU-1 and related species using software Mega 5.0 with the neighbor-joining method based on 16S rRNA sequences.

3.2. Growth Characterization

The growth curves of *P. aeruginosa* AQNU-1 in two different mediums are shown in Figure 3. In beef extract-peptone liquid medium, strain AQNU-1 showed a complete growth curve over 96 h including the delay, exponential, plateau and decline phases, whereas the decline phase did not emerge during 148 h in the MSM. In addition, this strain quickly reached the plateau phase after 12 h, and new fast growth happened during the stable period, which could be related to the metabolism of a microbial-mediated substrate. At the same time, the cell turbidity reached OD_{600} 1.03. Furthermore, strain AQNU-1 still maintained a higher cell turbidity (>0.39 OD_{600}) during a relatively long decline phase. However, this strain possessed a longer delay period of 52 h in the MSM with 1.0% crude oil under dynamic cultivation, indicating that high hydrophobicity and toxicity of petroleum hydrocarbons may limit the microbial growth. After adapting to the harsh environment, strain AQNU-1 started a longer exponential phase of 48 h, and the cell turbidity reached OD_{600} 0.90. Furthermore, this strain started another fast growth period after a short decrease in growth, with the turbidity reaching OD_{600} 1.44 over 48 h. After the one-month cultivation, the turbidity of strain AQNU-1 was OD_{600} 1.5~1.7 in our experiments. Furthermore, the delay period was longer (up to 80 h) for the static culture, while the turbidity still reached OD_{600} 1.3~1.4 over one month. It was found that once strain AQNU-1 adapted to the crude oil, it could utilize crude oil and metabolic intermediates as a carbon source for rapid growth. The growth curves demonstrate that strain AQNU-1 has good potential for oil-contaminated remediation.

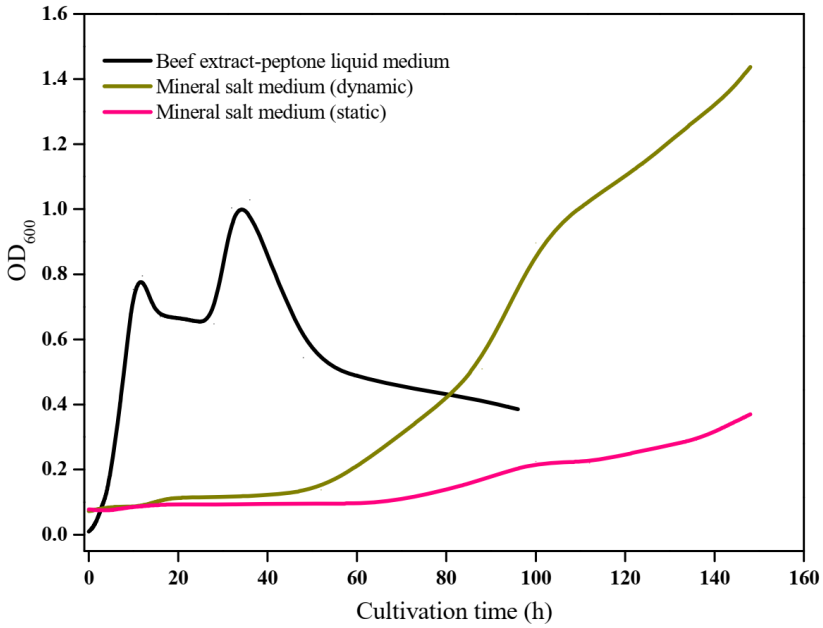


Figure 3. Growth curves of *P. aeruginosa* AQNU-1 on beef extract-peptone and crude oil as the carbon and energy source at 37 °C. Beef extract-peptone liquid medium (180 r/min for 96 h), mineral salt medium (180 r/min and 0 r/min for 148 h, respectively).

3.3. Carbon Source Utilization

The Biolog EcoPlate™ is a useful approach for studying the physiological profiles of microorganisms based on the carbon substrate utilization. The principle is that electrons produced by microbial respiration change the color of the redox dye within each micropore, revealing microbial growth. The carbon source utilization of strain AQNU-1 was determined using the absorbance at OD_{590} and OD_{750} over 5 days. As shown in Table 1, the carbon substrates utilized by strain AQNU-1 were mainly in six categories, namely, carbohydrates, amino acids, carboxylic acids, polymer, phenolic acids and amines, and included thirteen different carbon substrates (D-xylose, D-mannitol, N-acetyl-D-glucosamine,

L-asparagine, L-arginine, L-serine, γ-hydroxybutyric acid, itaconic acid, pyruvic acid methyl ester, Tween 40, Tween 80, 4-hydroxybenzoic acid and putrescine). The optical density for each substrate over 5 days is profiled in Figure 4. In this study, the OD_{590} was a better indicator of carbon source metabolism. Additionally, strain AQNU-1 showed an extensive carbon source utilization ability, especially in relation to polymers, and carboxylic or phenolic acids. These compounds are highly consistent with hydrocarbons in crude oil or their metabolites. The substrate utilization properties indirectly demonstrate that strain AQNU-1 has good crude oil degradation potential.

Table 1. Carbon source utilization of *P. aeruginosa* AQNU-1 using a Biolog EcoPlate™ analysis.

	Categories	Carbon Sources
1	Carbohydrates	D-xylose
		D-mannitol
		N-acetyl-D-glucosamine
2	Amino acids	L-asparagine
		L-arginine
		L-serine
3	Carboxylic acids	γ-hydroxybutyric acid
		Itaconic acid
		Pyruvic acid methyl ester
4	Polymer	Tween 40
		Tween 80
5	Phenolic acids	4-hydroxybenzoic acid
6	Amine	Putrescine

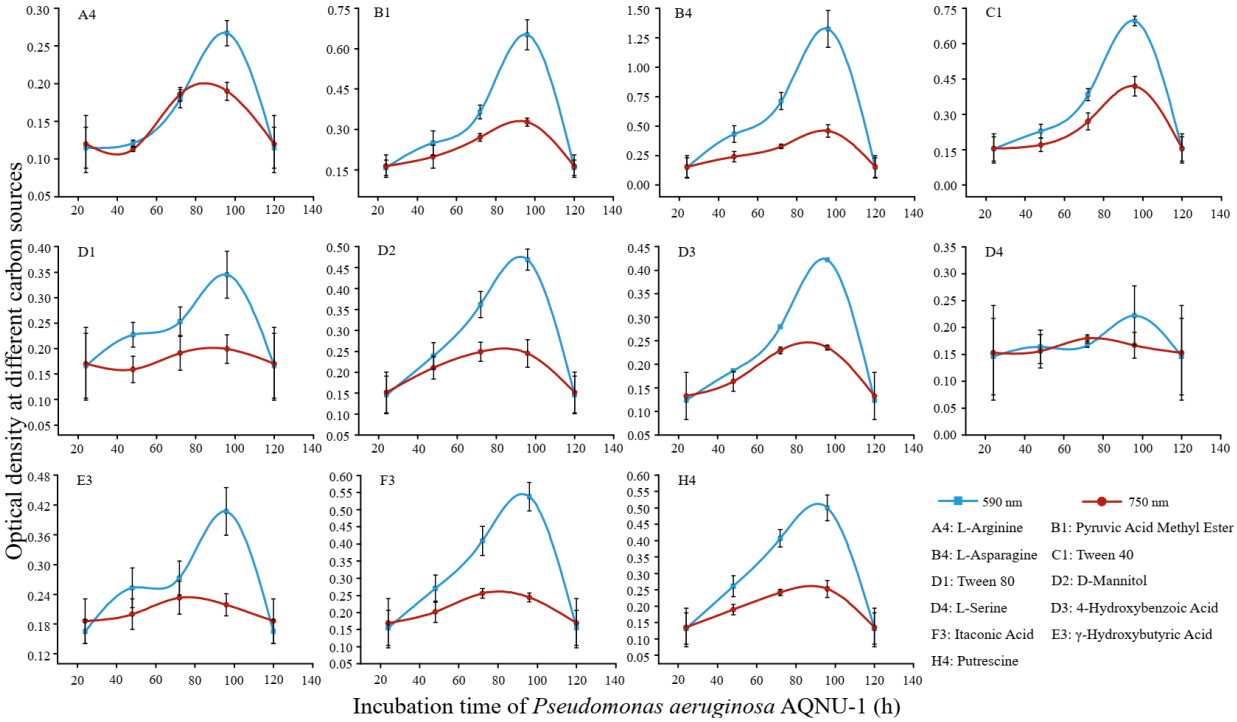


Figure 4. Optical density properties of different carbon sources used by *P. aeruginosa* AQNU-1 during a 120 h incubation at 20 °C.

3.4. Crude Oil Degradation under Dynamic and Static Cultivation

During the strain AQNU-1 cultivation process, crude oil heavily dispersed into the aqueous phase, especially under the dynamic condition. The crude oil degradation properties in different experimental groups were profiled using GC-MS. Crude oil was significantly depleted under both the dynamic and static cultivation due to the microbial metabolic

process, and most of them were completely consumed based on the ion chromatogram analysis. Moreover, hopane (M/Z 191), as an indigenous molecular marker, did not change in all experimental groups, and thus was used as the internal standard to assess crude oil degradation. In our study, the crude oil had abundant *n*-alkanes (nC_{13}-nC_{35}, M/Z 85). Interestingly, for both the dynamic and static cultivation, all *n*-alkanes were greatly reduced, indicating that strain AQNU-1 has high potential for *n*-alkane degradation (Figure 5). Additionally, nC_{13}-nC_{18} were completely depleted by strain AQNU-1 in all of the different groups, indicating that low molecular weight *n*-alkanes are more susceptible to degradation (Table 2). For higher molecular weight *n*-alkanes (nC_{19}-nC_{35}), the dynamic degradation efficiency was better than the static condition. The degradation ratios were 87–100% for dynamic cultivation and 74–98% for static cultivation. In particular, for *n*C34-*n*C35, the degradation efficiency appeared to decrease with a percentage of 74–87% in both conditions. According to our observation, the degradation effects of strain AQNU-1 showed a downward trend with increasing molecular weight, which is consistent with current reports related to hydrocarbon degradation. Additionally, the isolated strain still had extensive degradation capacity under dynamic cultivation. At the same time, linear alkylcyclohexanes (M/Z 82), alkylbenzenes (M/Z 92) and alkyltoluenes (M/Z 106) were severely degraded by strain AQNU-1 in the different groups. However, the endogenous isoprenoid alkanes, pristane (Pr) and phytane (Ph), in the crude oil were hard to reduce with the strain (Figure 6), thereby demonstrating the limited degradation capacity of branched alkanes. In general, strain AQNU-1 shows a high degradation potential for *n*-alkanes and alkyl-compounds under both dynamic and static cultivation conditions.

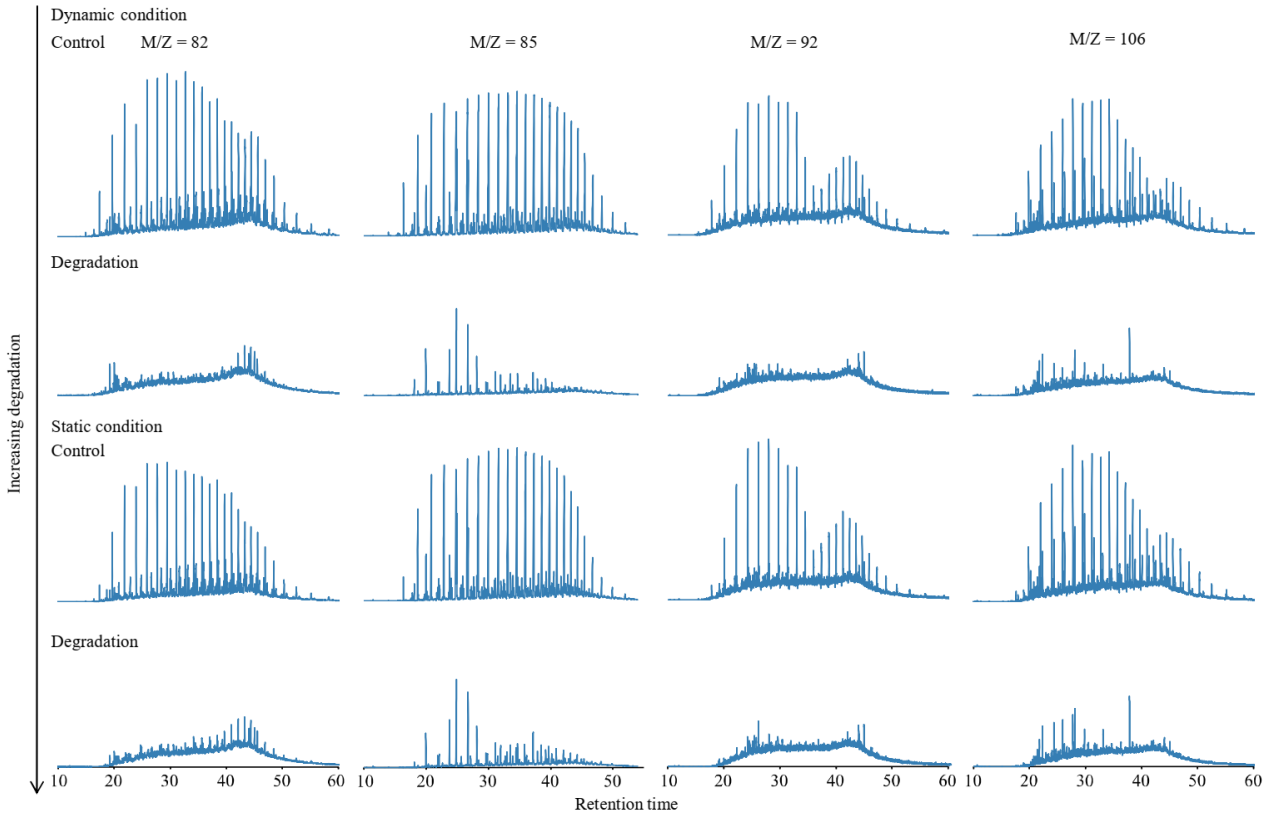
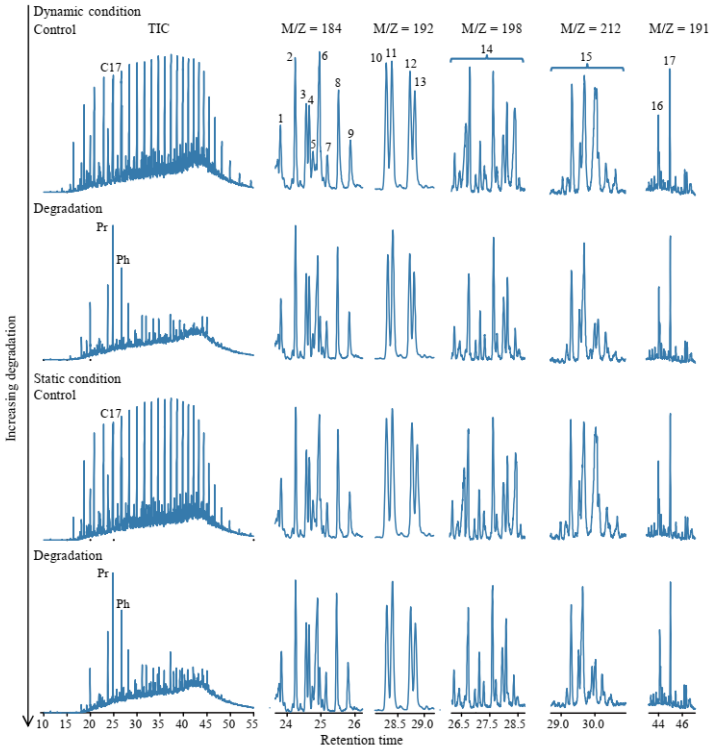


Figure 5. Ion chromatograms of linear alkylcyclohexanes (M/Z 82), *n*-alkanes (M/Z 85), alkylbenzenes (M/Z 92), and alkyltoluenes (M/Z 106) in crude oil samples with different degradation degrees by *P. aeruginosa* AQNU-1 under the dynamic and static cultivation (180 r/min and 0 r/min at 37 °C, respectively).

Table 2. Degradation ratio of *n*-alkanes and aromatic compound families by *P. aeruginosa* AQNU-1 under dynamic and static cultivation conditions.

Compound (*n*-Alkane)	Degradation Ratio (%)		Compound (*n*-Alkane)	Degradation Ratio (%)		Compound (PAHs)	Degradation Ratio (%)	
	Dynamic	Static		Dynamic	Static		Dynamic	Static
n-C13	100	100	*n*-C25	92	89	M/Z = 138	37	65
n-C14	100	100	*n*-C26	96	93	M/Z = 142	72	67
n-C15	100	100	*n*-C27	99	96	M/Z = 152	15	33
n-C16	100	100	*n*-C28	100	95	M/Z = 154	56	49
n-C17	100	100	*n*-C29	100	95	M/Z = 154 *	100	100
n-C18	100	100	*n*-C30	100	94	M/Z = 168	72	61
n-C19	98	98	*n*-C31	100	92	M/Z = 184	29	21
n-C20	96	96	*n*-C32	100	95	M/Z = 192	26	24
n-C21	100	98	*n*-C33	100	93	M/Z = 198	16	10
n-C22	100	97	*n*-C34	87	74	M/Z = 212	25	27
n-C23	93	94	*n*-C35	87	75			
n-C24	98	96						

Degradable aromatic compounds detected were as follows: Decalin (M/Z 138); C1-naphthalenes (M/Z 142); Acenaphthylene (M/Z 152); Acenaphthene and Biphenyl * (M/Z 154); Dibenzofuran (M/Z 168); C4-naphthalenes (M/Z 184); C1-phenanthrenes (M/Z 192); Dibenzothiophenes, C1-C2 (M/Z 198, 212).



Peak	Diagnostic ion (M/Z)	Compound	Peak	Diagnostic ion (M/Z)	Compound
Pr	85	pristane	9	184	1,2,5,6-/1,2,3,5-TMN
Ph	85	phytane	10	192	3-MP
1	184	1,3,5,7-TMN	11	192	2-MP
2	184	1,3,6,7-TMN	12	192	4-/9-MP
3	184	1,2,4,6-/1,2,4,7-/1,4,6,7-TMN	13	192	1-MP
4	184	1,2,5,7-TMN	14	198	C1-dibenzothiophenes
5	184	2,3,6,7-TMN	15	212	C2-dibenzothiophenes
6	184	1,2,6,7-TMN	16	191	17α(H),21β(H)-30-norhopane
7	184	1,2,3,7-TMN	17	191	17α(H),21β(H)-hopane
8	184	1,2,3,6-TMN			

Figure 6. GC-MS ion current (TIC) profiles and ion chromatograms of C4-naphthalenes (M/Z 184), C1-phenanthrenes (M/Z 192), C1-dibenzothiophenes (M/Z 198), C2-dibenzothiophenes (M/Z 212) and terpanes (M/Z 191) for the same samples. Each profile is presented on a scale relative to the largest peak. The identification of the different isomers is mentioned below.

Meanwhile, isolated strain AQNU-1 possessed degradation potential for aromatic compounds to a certain extent in different cultivations (M/Z 138, 142, 152, 154, 168, 184, 192, 198 and 212) (Figure 6, Table 2). The degradation of alkylated PAH analogues and aromatic compound families was profiled by the absence or increase in ion chromatogram peaks, as well as an increase or decrease in their intensity. According to the determination of aromatic compounds in the residual crude oil, the degradation ratios of PAHs were 15–100% under dynamic cultivation, while they were 10–100% under the static condition. Interestingly, biphenyl (M/Z 154) showed a high degradation efficiency of 100% among the different groups, and in regard to the biodegradation efficiency under static cultivation, 2-ring or 3-ring PAHs (such as naphthalene, biphenyl, acenaphthene and dibenzofuran) were easier to fully degrade than high molecular weight 3-ring PAHs (such as phenanthrene, dibenzothiophene and their alkylated analogues). The degradation trend of aromatic hydrocarbons was similar to that of alkanes, and the degradation potential decreased with the increasing number of aromatic rings and alkyl substituents. These results are consistent with previous reports [28,29]. Furthermore, there were apparent differences in degradation among aromatic compound families within the same M/Z index (Figure 6), and the PAH compound families also had a similar degradation trend under the two cultivations. For example, 1,2,6,7-TMN (tetramethylnaphalenes) was easier to degrade compared with other TMN isomers, while 3-MP (methylphenanthrenes) was more easily consumed than other MP isomers. When investigating the degradation of crude oil in an extreme reservoir, Jiménez et al. [30] showed that 1- and 3-MP are more easily utilized by the microbial communities. In our study, it was found that higher molecular weight C1-dibenzothiophenes (M/Z 198) and C2-dibenzothiophenes (M/Z 212) were also degraded according to the obvious change in specific ion peaks. However, Cheng et al. [29] pointed out that 4-methyldibenzothiophene was most readily biodegraded, while 1-methyldibenzothiophene showed greater resistance to microbial utilization. Generally, strain AQNU-1 has good degradation potential for different PAHs and aromatic compound families under both dynamic and static cultivation conditions, and the biodegradability decreases with an increasing number of aromatic rings and alkyl groups.

4. Discussion

Wetlands are considered the most biologically diverse ecosystem on earth, and they perform diverse ecological functions and provide socio-economic value, such as water purification, sediment stabilization, carbon sequestration and food supplementation [25]. However, as a fragile ecosystem, global wetlands have been lost or degraded rapidly in the past decades. Thus, there is a pressing need to protect this delicate ecosystem so as to maintain ecological balance and sustainability. In actuality, crude oil contamination, which commonly occurs due to natural spillage and anthropogenic activity, is a significant driver of wetland degradation. Moreover, contamination is particularly common in coastal wetlands; thus, it is gaining increasing attention [31]. Nevertheless, studies related to long-term crude oil contamination in freshwater wetlands are still very scarce. Additionally, how to execute ecological restoration and functional reconstruction of contaminated wetlands is an important research topic. The existence of indigenous functional microorganisms provides a potential tool to solve this scientific problem. Microorganisms not only serve as a sensitive indicator for versatile contaminants, but also drive the biogeochemical cycling of various elements [32,33]. In particular, hydrocarbon-degrading microorganisms in wetlands could be screened and acclimatized for use in hydrocarbon contamination remediation. Wu et al. [34] used high-throughput DNA sequencing to show that urban wetlands possess a strong potential for PAH degradation due to the wide distribution of dioxygenase and dehydrogenase genes. Nkem et al. [35] isolated two strains, namely, *Cellulosimicrobium cellulans* DSM 43879 and *Acinetobacter baumannii* ATCC 19606, from Rhu Sepuluh beach that exhibited great potential for degradation of diesel-oil alkanes with a degradation efficiency of 10–95.4% and 0.2–95.9%, respectively. Additionally, Sakshi et al. [19] revealed that *Kocuria flava* and *Rhodococcus pyridinivorans*, isolated from oil-contaminated soil, could

fully utilize 3-ring PAHs, such as phenanthrene, anthracene and fluorine. In our study, a novel strain of *P. aeruginosa* AQNU-1 was successfully isolated from a crude-oil polluted freshwater wetland, and this strain was clearly able to utilize crude oil as the sole carbon source. Furthermore, the isolated strain exhibited good potential for the degradation of major crude oil components, including *n*-alkanes, alkylcyclohexane, alkylbenzene and alkyltoluene, as well as various PAHs and aromatic compound families in crude oil under both dynamic and static cultivation conditions. Crude-oil contaminated wetlands are an important source of hydrocarbon-degrading bacteria, and screening indigenous bacteria with high degradation efficiency and the ability to degrade a wide range of crude oil components could provide a valuable microbial resource for wetland restoration.

To better understand the substrate utilization of strain AQNU-1, a Biolog EcoPlate™ with 31 carbon sources was chosen in our study. According to our observations, the isolated strain has a wide metabolic substrate range within six categories, namely, carbohydrates, amino acids, carboxylic acids, polymer, phenolic acids and amines. More interestingly, half of the carbon sources that can be utilized by strain AQNU-1 are hydrocarbon analogues (Tween 40 and Tween 80) or their metabolic intermediates (γ-hydroxybutyric acid, itaconic acid, pyruvic acid methyl ester and 4-hydroxybenzoic acid). Furthermore, the degradation rate for these specific substrates is relatively rapid according to the substrate utilization curves (Figure 4). This finding suggests that the isolated strain possesses good degradation potential for different crude oil components. As reported by Reddy et al. [36], Tween 40 and Tween 80 not only enhance fluorene degradation by *Paenibacillus* sp. PRNK-6, but also serve as carbon substrates. An alkane-degrading strain *Alkanindiges illinoisensis*, isolated from oil-field soil, exhibited a narrow carbon source range, as only Tween 40 and Tween 80 support significant growth [37]. Carboxylic or phenolic acids are usually metabolic intermediates of hydrocarbon biodegradation. For instance, Muralidharan et al. [38] showed that halotolerant bacterial strains mediate the oxidative cleavage of the aromatic ring of Benzo[a]pyrene to form intermediary metabolites, including carboxylic acids. In addition, Janniche et al. [39] demonstrated that a groundwater microbial community, with similar substrate utilizations revealed by Biology EcoPlate™, has the ability to mineralize petrochemical products such as atrazine and isoproturon. The carbon-source utilization properties provided a valuable perspective in our screening of highly efficient hydrocarbon-degrading microorganisms.

According to the observations, strain AQNU-1 has an efficient wide hydrocarbon degradation range under dynamic cultivation. However, the degradation capacity decreases with increases in the molecular weight of the hydrocarbons under dynamic cultivation. This degradation trend is consistent with previous reports. The results suggest that the microbial degradation efficiency of larger molecular weight hydrocarbons not only depends on the inherent potential of microorganisms involved in functional genes, but is also closely associated with external habitats. Under aerobic conditions, various functional genes involved in transport and catalysis determine the diversity and differences in the degradation pathways. For instance, some bacteria prefer to utilize alkanes, while others can metabolize alicyclic or aromatic compounds. Sabirova et al. [40] demonstrated that *Alcanivorax borkumensis* encodes an outer membrane lipoprotein Blc to uptake small alkanes based on the alkane-induced gene *blc*. Furthermore, when studying the alkane hydroxylase AlkB structure of *Pseudomonas putida* GPo1 and *Alcanivorax borkumensis* AP1, van Beilen et al. [41] found that the chain-length of the utilized alkanes that the enzyme AlkB catalyzes is closely related to the specific amino acid composition in the transmembrane helix, and that these strains can degrade alkanes longer than *n*C13 if tryptophan in the enzyme domain is replaced by smaller serine or cysteine. At the same time, long-chain alkanes (>*n*C12) can be degraded by flavin-binding monooxygenase AlmA and LadA of the luciferase protein families [14,42]. For alkyl-substituted cycloaliphatic compounds, oxidative decomposition may occur at side-chain alkanes or alicyclic rings through a more complicated enzymatic system. As revealed by Abbasian et al. [43], cyclohexane degradation by *Acinetobacter* sp. depends on five enzymes co-encoded by two operons, namely, the chnBER and chnADC ORFs. Generally, linear alkanes can be degraded more easily than

branched-chain alkanes and cycloalkanes, while branched alkanes are more preferentially degraded than cycloalkanes [44]. These differences are closely associated with inherent functional genes carried out by the microorganisms. Thus, the structure and function of genes involved in hydrocarbon degradation by strain AQNU-1 will be a focus of our subsequent experiments.

At the same time, strain AQNU-1 can efficiently utilize various PAHs and aromatic compound families under both dynamic and static cultivation conditions. Generally, the biodegradation of aromatic compounds exhibits a decreasing trend with an increasing number of aromatic rings and alkyl substitutions. Many factors affect the degradation process of aromatic compounds, such as the bioavailability, toxicity and presence of degradation-related functional genes. PAH bioavailability is an important factor affecting the degrading process. Bezza and Chirwa [45] reported that biosurfactants are able to alleviate the low bioavailability caused by high hydrophobicity and lipophilicity of high molecular weight PAHs (5–6 rings), thus helping to accelerate the degradation process. Further, substituents such as methyl, hydroxyl and carboxyl groups, can usually alter the stability of aromatic compounds in favor of an electrophilic reaction, thereby improving bioavailability. For strain AQNU-1, the differences in degradation within aromatic compound families should be closely related with their specific structures. Cheng et al. [29] reported that the susceptibility of PAH isomers is associated with the positions and stereochemical structures of alkyl substituents. Moreover, aromatic compounds with a similar molecular structure still have different fates. Our study reveals that aromatic sulfur derivatives, such as dibenzothiphenes, are more resistant to degradation compared to dibenzofurans, suggesting that these compounds may have greater toxicity than aromatic oxygen derivatives. In addition, Pasumarthi et al. [46] showed that *P. aeruginosa* and *Escherichia fergusonii* exhibit differences in degradation among the 4-ring aromatic hydrocarbons pyrene and chrysene, suggesting that increased hydrophobicity is caused by the greater size and angularity of the aromatic rings. In theory, the aromatic compound degradation process depends on the inherent functional genes and their encoded enzymes. As revealed by Ferraro et al. [47], Rieske oxygenase is the key enzyme that aerobically degrades aromatic compounds; a characteristic that is closely related to the specificity, stereoselectivity and regioselectivity of the metabolites. Additionally, Khara et al. [48] showed that the α- and β-subunit structures of Rieske oxygenase can affect the substrate specificity of the enzyme, and reported that the specificity relies on the properties of the catalytic subunit, specifically, the volume, size and shape. In addition, many factors influence enzymatic reactions, including the structure of the catalytic center, entry and exit channels of substrates, and the physicochemical properties of size, geometry and hydrophobicity of the ligands [49]. Furthermore, the unique loop structure linking the α- and β- subunits in Rieske oxygenase plays a significant role in maintaining its structural stability and catalytic activity, and is important for NADPH binding and screening substrates [50]. In essence, the functional variability of aromatic oxygenases should stem from their encoding genes, which ultimately leads to significant differences in the biodegradation of aromatic compounds.

In fact, the hydrocarbon degradation capability not only relies on the inherent potential related to functional genes, but is also connected to the external habitats of microorganisms. As revealed by Liu et al. [51], the low biodegradation rate of hydrocarbons with a larger molecular weight is related to their poor water-solubility. Additionally, Cerniglia [52] reported that incomplete biodegradation of diesel hydrocarbons is intrinsic to the limited amount of dissolved oxygen in the reaction system. Cai et al. [53] also revealed that the aerobic oxidation of alkanes and aromatics to organic acids is a significant process for the microbial degradation of petroleum, according to the determination of metabolic products such as fatty acid, naphthenic acid and aromatic carboxylic acid from aerobic culture. Furthermore, Hamzah et al. [54] noted that the rapid growth of a microbial population during crude oil degradation will result in lower dissolved oxygen in the aquatic system, thereby decreasing the hydrocarbon degradation rate. In our experiment, the isolated strain AQNU-1 was selected for its ability to assimilate crude oil under both dynamic and static

cultivation conditions. The results reveal that the degradation efficiency under the dynamic condition is better than that under the static condition, especially for *n*-alkanes with a larger molecular weight. This phenomenon demonstrates the effect of dissolved oxygen on the degradation of petroleum hydrocarbons to a certain extent. The dynamic degradation process is not only conducive to full contact between cells and hydrocarbons, but it also effectively strengthens oxygen delivery between the two gas and liquid phases. According to our research, the isolated strain possesses good environmental adaptability, despite living in harsh conditions with recalcitrant substrates and a lack of oxygen. Isolating functional microorganisms from a hydrocarbon-polluted wetland is very important for its in situ bioremediation. Moreover, exploring the degradation potential under dynamic and static conditions can provide basic information about the microbial adaptability under various flow regimes, such as the static state, slow-flow state and turbulent movement of wetland waters.

5. Conclusions

A crude oil-degrading strain, *P. aeruginosa* AQNU-1, was isolated from a long-term hydrocarbon-polluted lake wetland, and it presented a high degradation efficiency for major crude oil components. Specifically, *n*-alkane, alkylcyclohexane, alkylbenzene and alkyltoluene were fully depleted. Additionally, partial aromatic hydrocarbons were assimilated to a certain extent, and aromatic compound families exhibited differences in degradation. Although the degradation capacity decreases with increasing hydrocarbon molecular weight, strain AQNU-1 still possesses a wide hydrocarbon-substrate range under both dynamic and static cultivation conditions. Based on the experimental design, which considered both dynamic and static cultivation conditions, the isolated strain demonstrated good environmental adaptability for the different hydrologic conditions of the wetlands, despite living in harsh conditions with recalcitrant substrates and a lack of oxygen. Thus, isolating functional microorganisms from hydrocarbon-polluted wetlands can provide useful microbial resources for in situ bioremediation.

Author Contributions: Conceptualization, methodology, supervision and writing, H.L.; experimental execution, data collection and editing, G.Y.; visualization, H.J.; experimental execution, B.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported in part by grants from the National Natural Science Foundation of China (41603083), Key Project Supported by the Natural Science Foundation of the Higher Education Institutions of Anhui Province (KJ2020A0501), the Program for Innovative Research Team at Anqing Normal University (Pollution Ecology in the Wetland), and the Funding Project for Cultivating Top Talents in Universities of Anhui Provincial Education Department (gxgnfx2021129). We gratefully acknowledge all the support provided.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available from the corresponding author upon request, which are not publicly available due to privacy.

Conflicts of Interest: The authors declare no conflict of interest.

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