Evaluation of the Biodegradation of Petroleum Contaminants by *Pseudomonas Aeruginosa* in the Caspian Sea Coastline Waters

Samira Mehrdad [1], Keivan Saeb[*2], Lobat Taghavi [1], Masood Ghane [3]

[1.] Department of Natural Resources and Environment, Science & Research Branch, Islamic Azad University. Tehran, Iran.
[2.] Department of Environment, Tonekabon Branch, Islamic Azad University. Tonekabon, Iran.
[3]. Department of Microbiology, Tonekabon Branch, Islamic Azad University. Tonekabon, Iran

Article Info

Article Type:
Original Research

Article History:
Received: 05.05.2019
Accepted: 01.07.2019

**** Corresponding Author:***
Keivan Saeb
Department of Environment, Tonekabon Branch, Islamic Azad University. Tonekabon, Iran.
E-mail: keivansaeb@gmail.com

A B S T R A C T

Background:

Water plays an essential role in supporting life on earth and sea worldwide, requiringclean, safe, high quality and sustainable resources. Nowadays, many water resources have been contaminatedwith toxic compounds originating from petroleumdue to economical and industrial developments. There is anurgent need to clean up the waters with environmentally friendlyand inexpensive methods. This study investigated the biodegradation of total petroleum hydrocarbons (TPH) using *Pseudomonas aeruginosa (P. aeruginosa)* in southern Caspian Sea coastline.

Methods:

Coastline sediment samples were collected, with *P. aeruginosa* being the predominant strain. The bacteria were cultured in triplicates in the presence of 0.5, 1, 2 and 4% of gasoline andunder specific experimental conditions of varying temperature, pH, salinity, shaker speed, and incubation periods. The data representing the gasoline biodegradation in the samples were statistically analyzed.

Results:

At optimized experimental conditions for temperature, pH, salinity, incubation period, and shaker speed, maximum biodegradation of TPH was achieved by culturing *P. aeruginosa* strains with the sea water samples containing varying concentrations of gasoline.

Conclusion:

The gram-negative bacteria, *P. aeruginosa,* almost completely biodegraded TPH contaminants from the samples' culture media over 28 days of incubation. We conclude that the use of *P. aeruginosa* is an efficient method for the biodegradation of Caspian coastal waters contaminated with TPH.

Keywords:

Caspian Sea Waters, Gas Chromatography, Gasoline Biodegradation, Petroleum Hydrocarbons, *Pseudomonas Aeruginosa*.

How to cite this paper

Mehrdad S, Saeb K, Taghavi L, Ghane M. Evaluation of the Biodegradation of Petroleum Contaminants by *Pseudomonas Aeruginosa* in the Caspian Sea Coastline Waters.Iran J Toxicol.2019; 13 (3):21-26

INTRODUCTION

The unique and essential role of the body of waters in the world in the development of human society is well justified. This requires clean, healthy, safe, high quality and sustainable water resources and leads to environmental biodiversity (1). Otherwise, water will lose its benefits to humans and environment (1). Coastal waters are one of the most valuable universal water resources with great impact on the economy, aquatic life, and human nutrition and recreation.

Nowadays, water resources have been adversely affected by the diverse societal, economical and industrial developments, primarily due to increasing pollutions and contaminations worldwide (2). The release of contaminants, such as petroleum and its byproducts into the environment is one of the main causes of global pollution (3). The main source of oil contamination into the sea is due to natural seepage (47%) and other causes such as oil consumption (38%), transportation (12%) and production (3%) (4). A good example is the adverse changes in the pH level of sea waters, that have been forecasted to increase progressively, ranging from about 0.40 to 0.77 units by the year 2100 (5). Various human interventions are believed to be the main factor contributing to the environmental contaminations with toxic and hazardous compounds (6). Along with the development of petrochemical and transportation industries, the

leakage of total petroleum hydrocarbons (TPH) have commonly and progressively contaminated human lives on land and in aquatic environment alike (7). These petrochemical pollutants are known to be soluble, volatile and biodegradable compounds (8).

The contaminated sites are currently cleaned up using chemical and engineering methods, such as crystallization and thermal stability processes, or a combination of these methods; however, they are expensive and energy consuming (9). Alternatively, there is a growing interest in the application of biodegradation, using bacteria, fungi and plantsthat are environmentally friendly, self-sustaining and of low-costs (10-12). Microorganisms are other practical choices for the biodegradation of petrochemical pollutions in sea waters (13-15). Recent studies have shown that isolated strains of *Pseudomonas aeruginosa* (*P. aeruginosa*) and *P. fluoresces* utilize petroleum hydrocarbons as a carbon source, with the former strain having the highest ability to decompose hydrocarbons (16,17).

Considering the findings presented above, we focused on the potential role of the indigenous micro-organisms found along the southern coastline of the Caspian Sea in the biodegradation of TPH, as an environmentally friendly, self-sustaining and low-costs approach. Following the initial discovery that *P. aeruginosa* was a major adaptable micro-organism, populating the Caspian Sea coastline, we investigated its capacity to biodegrade TPH under well defined *in vitro* conditions. Our findings suggest that *P. aeruginosa* is a good candidate to biodegrade TPH, providing an environmentally friendly method to decontaminate the southern Caspian Sea coastal waters efficiently and at low costs.

MATERIALS AND MEHTHODS

Sampling and Culture: Following a careful literature search on the microorganisms prevalent in the Iranian aquatic environment, three sediment samples were collected from the Caspian sea coastline at Noshahr from a depth of 0-30cm, and were kept in sterile plastic bottles (50ml), marked with date and the collection site information. In this study, all tests under all categories were performed in triplicate.

Isolation & Identification of Bacteria: To isolate the bacteria from the sediment samples, 1ml of the sediment solution was diluted in 50ml tryptic soya broth medium and incubated at 36°C for 24 hours. Serial dilutions of 1ml aliquot of the bacteria culture solution were spread on several soy-agar plates and incubated. To identify *P. aeruginosa*, several biochemical reactions were performed, such as: triple sugar iron agar (TSI), oxidation fermentation (OF), methyl red/voges-proskauer (MR-VP), and citrate tests (18).

Bacteria Storage: Following the confirmation of the bacteria identity, approximately four colonies were mixed in test tubes containing 1.5ml soy broth media and 20% glycerol, and incubated at 37°C for two hours. Upon turbidity of the culture media in test tubes, indicating adequate bacterial growth, they were kept in a fridge for four hours (4°C) followed by storing them in a freezer at -70°C. To establish that the frozen bacterial batches were functional, some tubes were defrosted randomly and cultured on MHB media, on a weekly, monthly, bimonthly or every 3 months basis.

DNA Extraction & Molecular Identification: The *P. aeruginosa* DNA was extracted and verified by 1% agarose gel electrophoresis according to an established method (19) and stored in test tubes in a freezer at -20°C. To amplify the bacterial 16sr-RNA gene, two specific primers (Table 1) were obtained from Tak Co. (Copenhagen, Denmark) and utilized for the subsequent polymerase chain reaction (PCR). To prepare the reaction mixture for each sample, 10µL master mix, 3µl sterile distilled water, 1µl forward primer, 1µl reverse primer, and 5µl of the DNA sample were added to 0.2ml test tubes. These tubes were subjected to the PCR in a thermocycler and processed according to specific procedure for the amplification of 16sr-RNA gene. The product was subjected to 1% agarose gel electrophoresis and the 1400bp band was extracted, using the fermentase (K0513) procedure. The PCR product was sequenced by Gen Fanavaran Ltd., Tehran, Iran (20).

Table 1. The primer specifications used for polymerase chain reaction.

Primer	Primer Sequence	Segment Length
27-F	5'-AGAGTTTGATCCTGGCTCAG-3'	1247
1492-R	5'-GGTTACCTTGTTACGACTT-3'	

Bacterial Growth Kinetics: To determine the growth curve of *P. aeruginosa*, batches of the bacteria were cultured in nutrient broth (NB) and brain heart infusion (BHI) media and the optic density measured at 600 nm on a spectrophotometer. Bacterial colonies were cultured on a solid medium, transferred to a test-tube containing the NB medium, and incubated at 38°C for 24 hours.

Gasoline Biodegradation by Bacteria: We added 0.5, 1, 2 and 4% of gasoline to test tubes containing 1ml of the bacterial culture media, and incubated them on a shaker at 12, 20, 25 and 28°C. The gasoline concentration in each test tube was determined on a gas chromatography unit at time zero, and 14 or 28 days after incubation under each of the experimental conditions as listed below:

- **Temperature:** 12, 20, 25 or 28°C.
- **pH:** 6.5, 7, 7.5, 8 or 8.5.
- **Salinity:** 12.2, 12.3, 12.4, 12.5 or 12.6 mg/L.
- **Shaker Speed:** 140, 150 or 160 round per minute (rpm).
- **Incubation Time:** Immediately (0), 14 or 28 days.

RESULTS

Bacteria Identification: Following the laboratory growth of bacteria samples found in the Caspian Sea coastline waters at Noshahr and their staining, the predominant species were identified and separated as

pink colonies. Other sporadic bacterial species identified from the water samples were blue-greenish colonies. Upon performing various biochemical analyses on the colonies, it was determined that the pink colonies belonged to gram-negative *P. aeruginosa*. The results of DNA sequencing and genetic homology of thesespecies, as examined by an established method (19) further supported the true identity of *P.aeruginosa*. This was achieved by gene 16sr-RNA amplification, PCR analysis of two primers and gel electrophoresis of the 1400bp genetic product. The kinetic growth curve of *P. aeruginosa* is illustrated in Figure 1.

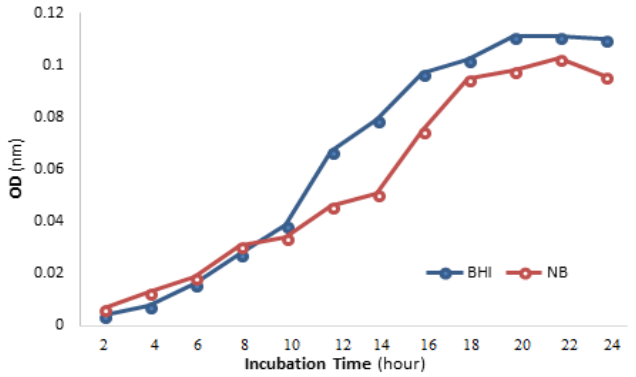

Figure 1. The growth kinetics of *P. aeruginosa*inthe culture media. *Culture media*: **BHI** = Brain heart infusion; **NB** = Nutrient broth.

Effect of Temperature: The effect of various temperature (12, 20, 25 and 28 °C) on the ability of *P. aeruginosa* to digest and dissipate TPH from the culture media over 28 days of incubation, as measured by gas chromatography, demonstrated that the TPH concentration (part per million = ppm) reached its minimum level at 28°C. The next best incubation temperature was found at 25°C (Fig. 2).

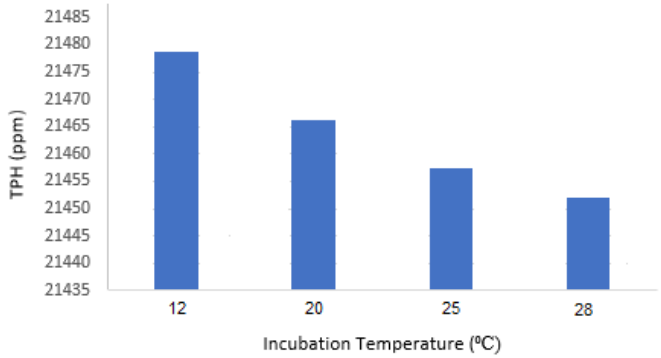

Figure 2. Effect of temperature (12-28°C) on the TPH concentration (ppm) in theculture mediaafter 28 days of incubation with *P. aeruginosa.*

Effect of pH: The effect of various pH levels (6.5, 7, 7.5, 8, and 8.5) on the on the ability of the bacteria to digest TPH from the culture media over 28 days of incubation, as measured by gas chromatography, showed that at pH 7 the TPH concentration reached its minimum. The next best experimental results were obtained at pH 6.5 (Fig. 3).

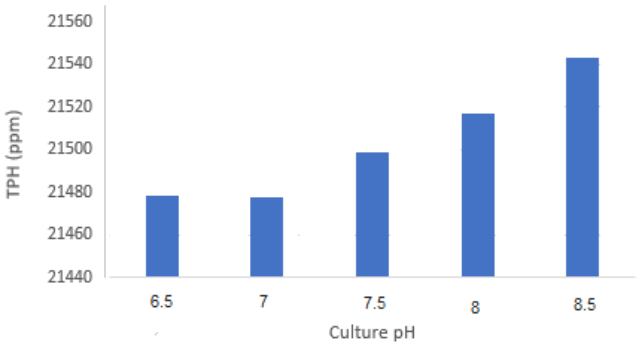

Figure 3. Effect of pH (6.5-8.5) on the TPH concentration (ppm) in the culture media after 28 days of incubation with *P. aeruginosa.*

Effect of Salinity: The effect of various salt concentrations (12.2, 12.3, 12.4, 12.5 and 12.6) in the culture media on the ability of the bacteria to digest TPH over 28 days of incubation showed that the lowest TPH level was obtained at the salinity level of 12.2 mg/L (Fig. 4).

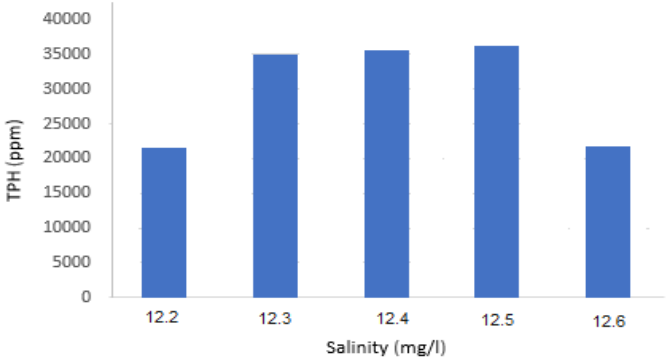

Figure 4. Effect of salinity (12.2-12.6 mg/L) on the TPH concentration (ppm) in culture media after 28 days of incubation with *P. aeruginosa.*

Effect of Shaker Speed: As shown in Figure 5, the effect of the shaker speed as measured at 140, 150 and 160 rpm, demonstrated that incubation with the shaker at 140 rpm over 28 days of incubation, resulted in the lowest TPH concentration in the culture media, with the next best level being 160 rpm.

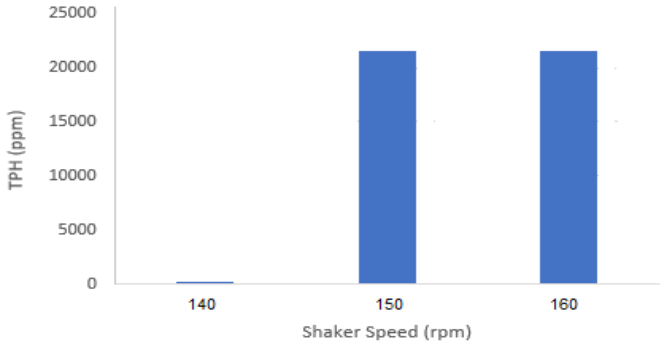

Figure 5. Effect of shaker speed (rpm) on the TPH concentration (ppm) in the culture media after 28 days of incubation with *P. aeruginosa.*

Effect of Incubation Time: The effect of the duration of incubation (0, 14 & 28 days) on the ability of the bacteria to digest TPH from the media revealed that gasoline concentration reached its minimum level on day 28, with the next best results seen on day 14 (Fig. 6).

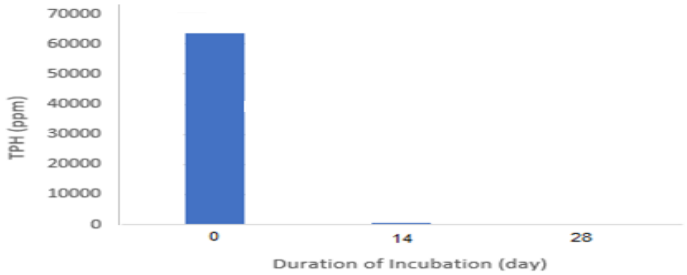

Figure 6. Effect of duration of incubation (0, 14 or 28) with *P. aeruginosa* on the TPH concentration (ppm) in the culture media.

Statistical Analyses: Table 2 represents the Pearson correlations among measured variables. The results of two-way ANOVA revealed a positive and significant relationship ($P<0.05$) between salinity vs pH; duration of incubation vs temperature; duration vs pH; duration vs salinity; TPH concentration vs pH; and TPH concentration vs duration of experiment (Table 3).

Table 2. Pearson's correlation results among measured variables.

Variable	Conc.	Temp	pH	Salinity	Shaker speed	Day	TPH
Conc.	1	-	-	-	-	-	-
Temp.	192	1	-	-	-	-	-
pH	301	551	1	-	-	-	-
Salinity	554	658	*0.001	1	-	-	-
Shaker Speed	77	662	71	77	1	-	-
Day	702	*0.005	*0.003	*0.003	0.65	1	-
TPH	601	872	*0.006	501	0.34	*0.000	1

* Correlation was significant at p< 0.05 level; - = Not applicable.
Conc. = Concentration; **Temp.** = Temperature

Table 3. Two-way analysis of variance.

Variable	Squared Sum (Type 3)	Deg. ofFreedom	Mean²	F	P Value
Conc.	1.298E9	3	4.327E8	17.595	.000
Temperature	4867.669	3	1622.556	.000	1.000
pH	37081.916	4	9270.479	.000	1.000
Salinity	2.847E9	4	7.116E8	28.940	.000
Shaker Speed	32433.412	2	16216.706	.001	.999
Day	1.408E9	2	7.040E8	28.629	.000
Conc. x Temp.	73829.918	9	8203.324	.000	1.000
Conc. x pH	118190.744	12	9849.229	.000	1.000
Conc. x Salinity	1.855E9	12	1.546E8	6.286	.000
Conc. x Shaker	324825.146	6	54137.524	.002	1,000
Conc. x Day	6.901E10	6	1.150E10	467.739	.000
Temp. x pH	.000	0	-	-	-
Temp. x Salinity	.000	0	-	-	-
Temp. x Shaker	.000	0	-	-	-
Temp. x Day	16412.599	6	2735.433	.000	1.000
pH x Salinity	.000	0	-	-	-
pH x Shaker	.000	0	-	-	-
pH x Day	84013.930	8	10501.741	.000	1.000
Salinity x Shaker	.000	0	-	-	-
Salinity x Day	1.582E9	8	1.977E8	8.041	.000
Shaker x Day	108896.136	4	27224.034	.001	1.000
Error	1.918E9	78	2.459E7	-	-
Total	3.678E11	168	-	-	-
Corrected Total	2.666E11	167	-	-	-

Key: **Conc.** = Concentration; **Temp.** = Temperature; **Shaker** = Shaker speed; **-** = Not applicable

DISCUSSION

The abundant use of gasoline in Iran has polluted many environmental habitats (21,22). This study was conducted to investigate the microbial potential of local waters ofthe Caspian Sea coastline at Noshahr for the removal of the petroleum contaminants. Our early findings revealed that the coastline waters were heavily populated with *P. aeruginosa*, which supported their adaptability to the aquatic environment. This was a fortunate discovery in favor of applying this resource as a natural solution to a long standing environmental problem, i.e., coastline water contamination with TPH. Microorganisms, such as bacteria, algae, fungi and yeasts have been shown to have the capacity to oxidize hydrocarbons in varying degrees (9,12,16,17,23). Consistent with the results of these studies, our findings confirmed the utility of gram-negative bacteria, such as *P. aeruginosa* in the biodegradation of TPH.

Temperature and pH Effects: *P. aeruginosa* had the best performance for biodegrading TPH contaminants at 28°C and pH 7 under the experimental conditions. This finding is a fortunate biological feature of *P. aeruginosa* that supports the oxidative process of TPH biodegradation and suitsthe climate temperature at the Caspian coastal waters at Noshahr most of the time annually. At these conditions aquatic bacteria normally adapt themselves to the environment (17, 24).

Salinity and Shaker Speed Effects: Our finding that the salinity level at 12.2 mg/L promoted the best ability for *P. aeruginosa* to digest TPH (Fig. 4) is consistent with that reported by a previous study for sea and ocean waters (25). The effect of optimal shaker speed (140 rpm) on the best activity of *P. aeruginosa* has not been reported by previous studies and, therefore, it is being reported for the first time by this study (Fig. 5). The advantage of this method is that itmakes the TPH particles accessible to the bacteria evenly throughout the test tubes for biodegradation.

Duration of Incubation: This study found that the TPH concentration reached the minimum level after 28 days of incubation (Fig. 6). The relatively long duration of incubation needed to obtain a significant degradation of TPH contaminants in this study was shorter than those reported by a previous study (17) for other aerobic bacteria to biodegrade 92-96% of crude pollutants in sea and ocean waters over 35-45 days. The experimental conditions employed in this study, including the optimal temperature, salinity and use of a shaker during incubation, might have influenced the optimal outcomes over a significantly shorter duration.

CONCLUSIONS

Based on the methodology designed by this study, gram-negative *P. aeruginosa* bacteria were able to almost completely biodegrade TPH contaminants from the experimental culture media over 28 days of incubation.

Optimal experimental conditions, such as temperature, pH, salinity, incubation period, and the use of a shaker (140 rpm) during the incubation periods, are essential to achieve the maximum biodegradation of TPH contaminants in culture by *P. aeruginosa.*

The use of *P. aeruginosa* and other bacteria to biodegrade TPH is potentially an efficient method of biodegrading contaminated coastal waters at low costs. Application of these experimental conditions to the actual aquatic environment awaits further research.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the support of the Department of Environmental Science and Research, Islamic Azad University, Tehran, Iran. Funding for this study was provided by the primary author. This manuscript was extracted from a doctoral thesis project conducted by the primary author, Ms. Samira Mehrdad, at the Department of Environmental Science and Research, Islamic Azad University, Tehran, Iran.

CONFLICT OF INTEREST

There was not conflict of interests of any kind in conducting this study.

REFERENCES

1. Saeedi SN, Chaldawi R, Mousavi Jerf SK. The Control of Impact of Marine Industries on Marine Environment for Sustainable Development. Journal of Human and Environment. 2012;10(2):1-12.

2. Aguilera PA, Castro H, Rescia H, Schmitz MF. Methodological Development of an Index of Coastal Water Quality: Application in a Tourist Area. Environmental Management. *2001*;27(2):295-301.

3. Silva, R.D.C.F. Almeida, D.G. Rufino, R.D. Luna, J.M. Santos, V.A. Sarubbo, L.A. Applications of biosurfactants in the petroleum industry and the remediation of oil spills. Int. J. Mol. Sci. 2014;15(7):12523–12542.

4. Prince RC, Lessard RR, Clark JR. Bioremediation of Marine Oil Spills. Oil & Gas Science and Technology – Rev. IFP. 2003;58(4):463-468.

5. Krause E, Wichels A, Giménez L, Lunau M, Schilhabel MB, Gerdts G. Small changes in pH have direct effects on marine bacterial community composition: A microcosm approach. PLOS One. 2012;7(10), e47035. Doi.org/10.1371/journal.pone.0047035.

6. Binark N, Güven KC, Gezgin T, Ünlü S. Oil Pollution of Marine Algae. Bull Environ Contam Toxicol. 2000;64:866-872.

7. Elmendorf DL, Haith CE, Douglas GS, Prince RC. Relative rates of biodegradation of substitiuted polycyclic Aromatic hydrocarbon. In: Hinchee, RE, lesson A, Semprini L, Ooong SK. (Eds). Bioremediation of chlorinated and polycyclic Aromatic Hydrocarbon Compounds. Lewis, Boca Raton, FL. 1994;88-202.

8. Seyed Alikhani S, Shorafa M, Asgharzadeh A. Efficiency of Bacillus and *Pseudomonas* Bacteria in Bioremediation of a Soil Contaminated with Hydrocarbons. Journal of Water and Soil Science. 2011;3(21):16-19.

9. Ebrahimi M, Fallah A, Sarikhani MR. (2014). Isolation and Identification of Oil Degrading Bacteria from Oil-Polluted Soils and Assessment of Their Growth in the Presence of Gas Oil, Journal of Water and Soil Science. 2014;23(1):109-121.

10. Talaie Khozani A, Jafarzadeh Haghighifard N, Talaie Khozani M, Beheshti M. The Determination of Biokinetic Coefficients of Crude Oil Biodegradation using Pseudomonas Aeruginosa Bacteria. Iranian Journal of Health and Environment. 2010;3(2):111-122.

11. Besharati H. Microbial Remediation of Petroleum Contaminated Soils and Rhizosphere Role in Microorganisms Efficiency. Iranian Journal of Soil Research. 2013;28(3):

12. Rossel-Mele A. Oil pollution in soils and sediments from the Northern Peruvian Amazon. Science of the Total Environment. 2018 Jan.1:610–611:1010-1019. Doi:10.1016/j.scitotenv.2017.07.208.

13. Singh, K. and Chandra, S. Treatment of petroleum Hydrocarbon polluted environment through bioremediation: A review. Pakistan journal of biological sciences. 2014;17(1):1-8.

14. Das, N. Chandran, P. Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview. SAGE-Hindawi Access to Research Biotechnology Research International, pp 13, 2011.

15. Zaki, M.S. Authman, M. N. and Abbas, H.H. Bioremediation of Petroleum Contaminants in Aquatic Environments (Review Article). Life Science Journal; 12(5): 127-139, 2015.

16. Benchouk A, Chibani A. Petroleum hydrocarbons biodegradation by Pseudomonas strains isolated from

hydrocarbon-contaminated soil. Journal of Fundamental and Applied Sciences. 2017:9(2):713-726.

17. Efeovbokhan VE, Hymore FK, Ayoola AA, Adeeyo OA. Comparison of Aerobic and Anaerobic Bioremediation of Polluted Water Samples. American International Journal of Contemporary Research. 2014;4(4):120-126.

18. Davies JA, Anderson GK, Beveridge TJ, Clark HC. Chemical mechanism of the Gram stain and synthesis of a new electron-opaque marker for electron microscopy which replaces the iodine mordant of the stain. J Bacteriol. 1983;156(2):837-845.

19. Sambrook J, Russell DW. Purification of Nucleic Acids by Extraction with Phenol: Chloroform. Cold Spring HarbProtoc; 2006. Doi:10.1101/pdb.prot4455.

20. Fakhrzadegan I, Hassanshahian M, Askari M. Isolation and Identification of Crude Oil Degrading Bacteria from Mangrove Forests of Khamir and Minab Ports Located at North of Persian Gulf. Journal of Aquatic Ecology. 2016;5(3):70-79.

21. Bour M, YousefiKebria D. Soil Pollutants and Methods for its Purification. At proceeding, 1st National Conference on Civil Engineering and Sustainable Development. Rasht, Iran, 2011.

22. Haghani, Safiyeh, et al. "An Early 'Little Ice Age': Brackish Water Invasion along the South Coast of the Caspian Sea (Sediment of Langarud Wetland) and Its Wider Impacts on the Environment and People. The Holocene.2016;26(1):3-16. Doi: 10.1177/0959683615596835

23. Safari M, Ahmadi S, Soltani N. Investigation of the ability of Schizothrixvaginata ISC 108 to bioremediation of crude oil. Journal of Health and Environment. 2014;7(3):363-374.

24. Cornelis P, Dingemans J. Pseudomonas aeruginosa adapts its iron uptake strategies in function of the type of infections. Front Cell Infect Microbiol. 2013;14(3):75. Doi:10.3389/fcimb.2013.00075

25. Dobaradaran S, Nabizadeh R, Mahvi AH, *et al*. Kinetic and degradation efficiency of trichloroethylene (TCE) via photochemical process from contaminated water. African Journal of Biotechnology. 2012;11(8):2006-2012. Doi.org/10.5897/AJB11.3276.