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ISOLATION AND IDENTIFICATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) DEGRADING BACTERIA FROM ARAK PETROCHEMICAL WASTEWATER

Zahra Fathi, Golamhossein Ebrahimipour*

Address(es): Zahra Fathi,
Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, Tehran, Iran.

*Corresponding author: : zahrafathi.microbiology94@gmail.com

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ABSTRACT

Oil pollutions reduce the potential of soils for optimal use in agriculture and crop production. Polycyclic aromatic hydrocarbons are a large group of oil contaminants with carcinogenic, mutagenic, teratogenic effects. Phenanthrene, fluorine, anthracene and pyrene are tri and four-cyclic aromatic hydrocarbons that are found in high concentrations in polycyclic aromatic hydrocarbon-contaminated sediments, surface soils and waste sites. Today, Bioremediation is one of the most effective and affordable methods in degradation of these compounds from the contaminated environment. In this study, the ability of two native bacterial species isolated from Arak petrochemical wastewater (*Pseudomonas aeruginosa* and *Serratia marcescens*) were examined to degradation of phenanthrene, anthracene, fluorene and pyrene. The degradation potential of these compounds by mixed bacterial cultures was evaluated with protein assay and gas chromatography in the mineral salt medium containing 250 mg/l anthracene and fluorene, 200 mg/l phenanthrene and 150 mg/l pyrene as the sole carbon and energy source during 10 days' incubation. Biochemical tests and 16s rDNA gene sequence analysis revealed that isolated bacteria are similar to *Pseudomonas aeruginosa* and *Serratia marcescens* with 99% similarity. The bacterial mixtures have good growth in PAHs compounds and could degrade more than of 98% of fluorine, 94% of anthracene, 97% of phenanthrene, 45% of pyrene and showed high potential to biodegradation of PAHs compounds in contaminated areas.

Keywords: Polycyclic aromatic hydrocarbons, Bioremediation, Phenanthrene, Anthracene, Fluorene, Pyrene

INTRODUCTION

PAHs are a large group of organic compounds with two or more fused aromatic rings in linear, angular, or cluster arrangements which product by human activities such as composting fossil fuels, petrochemical predicting, coal refining process, vehicle exhaust, forests fires (**Coral et al., 2005**). These compounds have a relatively low solubility in water but are highly lipophilic and very stable in the environment (**Johnsen et al., 2005**). In addition, these pollutions have many hazardous effects for all organisms and humans, for example, carcinogenic, mutagenic, teratogenic effects, genotoxicity was observed after long-term PAHs exposure and confirmed by toxicological experiments (**Janošek et al., 2007**). PAHs can enter the body through breathing contaminated air, eating food or drinks water that is contaminated with PAHs and also skin touches PAH contaminated soil or products like heavy oils, coal tar, roofing tar or creosote (**Samanta et al., 2002**). The PAHs can also affect agricultural soils and severely reduce crop production in this section. These compounds are listed as priority pollutants by the US Environmental Protection Agency (**Heitkamp and Cerniglia 1988**).

Although PAHs can exist in over 100 different combinations, the most common are treated as a group of 15, they are: acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(ghi)perylene, benzo(k)fluoranthene, chrysene, dibenzo(a,h)anthracene, fluoranthene, Fluorine, indeno(1,2,3cd)pyrene, phenanthrene, pyrene (**Kasai et al., 2003**). Fluorine, anthracene and phenanthrene are tri-cyclic aromatic hydrocarbons that are found in high concentrations in polycyclic aromatic hydrocarbon (PAH)-contaminated sediments, surface soils and waste sites (**Johnsen et al., 2005**). The main source of this compounds is a petrochemical industry that enters these compounds to the surrounding environment by petrochemical wastewater. These compounds are resistant to decomposition and these are very stable in environments and be considered as a serious threat to humans and the environment (**ErfanManesh and Afyouni, 2008**). Pyrene is another polycyclic aromatic hydrocarbon (PAH) consisting of four fused benzene rings, resulting in a flat aromatic system. Pyrene molecular weight and its stability at environment are greater than the other PAHs compounds.

Natural processes that decomposition PAHs from environments, including evaporation, photo-oxidation, and microbial degradation. Photo-oxidation by singlet oxygen, HO- radical, and other oxidants appears to be the most dominant process for the breakdown of PAHs and other organics in water (**Zafrriou, 1977**). Evaporation and photo-oxidation remove low molecular weight components in the aromatic compounds (**Bagi, 2013**). PAHs are subject to biodegradation by microorganisms present in the soil, sewage, and water. Due to the absence of optimal conditions and lack of effective bacteria in the environment, natural processes that remove PAHs compounds from contaminated environments are relatively slow.

For quick removing of PAHs compounds from the contaminated environment can use physical, natural and biological process. Using bioreactors are also effective but doing this requires that contaminated soil from the area be transferred to the reactor and also be expensive and cause the loss of beneficial microorganisms in the soil (**Rock, 1997**).

The microorganisms isolated from hydrocarbon contaminated environments have been found more benefit in bioremediation contaminated soils (**Bayoumi, 2009; Vinas et al., 2005**). There are a very diversity of microbial communities inhabiting the PAH-contaminated soil. These communities are a response to different biostimulation or bioaugmentation strategies that are able to adapt and exploit such habitats (**Vinas et al., 2005; Larkin et al., 2005**). Bioremediation is dependent on the environmental conditions, type and number of the microorganisms and the chemical compound which they biodegraded/biotransformed into less complex metabolites and mineralized into inorganic minerals, H_2O, CO_2 (aerobic) or CH_4 (anaerobic). Bioremediation rate is depending on pH, temperature, oxygen, microbial population, the degree of acclimation, accessibility of nutrients, the chemical structure of the compound, cellular transport properties, and chemical partitioning in growth medium (**Coulon et al., 2007; Zaidi and Imam, 1999**). Among the different ways to create a healthy environment that contaminated with PAHs, bioremediation is of particular importance due to high compatibility with the environment. In bioremediation, PAHs that are toxic and hazardous compounds transform to non-toxic compounds by microorganisms. Physical factors such as pH, temperature, and salinity have been shown to play a major role in controlling microbial growth and activity during PAHs biodegradation (**Coulon et al., 2007**). Bioremediation

which is based on microbial transformation and degradation is one of the most promising methods applied in the field of environmental biotechnology for cleanup of contaminated environments (**Atlas, 1981; Wilson and Jones, 1993**). Until now, various bacteria have been isolated that are capable of degrading individual PAHs (**Yaghmaei and Safekordi, 2001**). There are several mechanisms or combinations by microbial communities that can adapt to the presence of PAHs in their environment. There can be an increase in population size of those organisms that tolerate or even degrade the compound by induction of appropriate genes. The potential for decomposition of PAHs in soil has been a research area of great interest in recent years and many efforts have been made to isolate bacteria that could degrade PAHs. These studies have focused on the isolation of PAH-degrading bacteria experiments, with special emphasis on the characterization of bacteria and degradation potential of PAHs (**Anderson et al., 1994**).
This study aimed to isolate and identify effective bacteria in degradation PAHs compounds such as fluorine, anthracene, phenanthrene, pyrene in Arak petrochemical wastewater and determine the capacity and optimum conditions of this bacteria(s) for degradation of these compounds.

MATERIAL AND METHODS

Sampling

Samples were collected from Arak petrochemical wastewater and stored at 4 °C until use.

Materials and growth conditions

Mineral salts medium (MSM) including phenanthrene as carbon and energy source were used for the enrichment and isolation of phenanthrene-degrading strain. This medium contains 1.0 g of NH4Cl, 5.0 g KH2PO4, 0.1 g MgSO4×7H2O, 5 mg of Fe(SO4)2 and 1.0 mL of trace elements solution. The trace element solution contained per liter (Tian et al., 2002): 23 mg MnCl2×2H2O, 30 mg MnCl4×H2O, 31 mg H3BO3, 36 mg CoCl2×6H2O, 10 mg CuCl2×2H2O, 20 mg NiCl2×6H2O, 50 mg ZnCl2, and 30 mg Na2MoO4×2H2O. First, with the aim of enriching, 10 ml wastewater poured into 90 ml MSM containing 250 mg fluorine, 250 mg anthracene, 200 mg phenanthrene and 150 mg pyrene (individually) in 250 ml flask. The flasks shook in dark condition at 30 °C, pH 7 with the orbital shaking of 140 rpm for 10 days. For further enrichment, during the transport process, 1 ml of the previous culture medium was added to the new culture medium. Re-adding was performed at least 4 times and in 10-day intervals.

Isolation and identification

At the end of the enrichment process, solid MSM used for bacterial strains isolation in the consortium. After closing the plate's environment, in order to create a homogeneous environment, 1% solution of PAHs (fluorene, anthracene, phenanthrene and pyrene) with acetone sprayed on the surface of each plate and used as a special solid medium in bacteria isolation. Using the technique of spraying, bacteria were inoculated into the medium. Plates were incubated 2 to 5 days at 35 °C. After 5 days' colonies of bacteria were observed with a clear halo around. Creating clear halo reflects degradation of PAHs compounds (phenanthrene, pyrene, anthracene and fluorine). Then, bacterial colonies that have best grow in the medium were picked off from the plates and purified by repetitive streaking into nutrient agar plates for purification. Purified strains were then identified by biochemical tests and molecular identification of the strains performed by 16S rDNA gene sequences analysis.

Extraction and analysis of Phenanthrene

The concentration of phenanthrene was determined according to Wu et al (**Kelly and Cerniglia, 1991**). 50 ml ethyl acetate added to the culture medium and was shaken for 15 min. After forming two phases, phenanthrene-containing non-polar organic solvent phase collected, aqueous phase re-extracted by another aliquot of 50 ml ethyl acetate. Finally, two extracts were combined together, dried by anhydrous Na2SO4 and the volume was adjusted to 100 ml. Extract samples analyzed by GC-FID. The GC-FID was equipped with an HP-5MS fused silica capillary column (60m × 0.25 mm ID × 0.25 lm thickness, Agilent Technologies, USA) with the injector and detector temperature of 280 °C and 300 °C, respectively. Nitrogen was used as the carrier gas. The oven temperature program was set as follows: The oven temperature program was 80 °C (for 2 min) to 120°C at a rate of 10 °C/min and from 120 °C to 300 °C at a rate of 4 °C/minute and held at 300 °C for 15 min. The identification and quantification of chemicals were conducted based on matching their retention times of standards (**Churchill et al., 1999**).

Optimization factors in the degradation of PAHs

The influence of the environmental factors, pH, temperature, and carbon/nitrogen (molar) ratio on phenanthrene degradation and growth of the isolate was determined. For optimization used one factor at one time methods. To determine the effect of temperature, log-phase cells were cultivated in 5 ml MSM containing 200 mg/l phenanthrene, in which the temperature was adjusted at 25 °C to 45 °C (by 5 °C intervals) with shaking at 140 rpm. After determination of optimal temperature, in order to determine the effect of pH, the pH of the MSM was adjusted 6 to 10, and the temperature adjusted at 35 °C with shaking at 140 rpm. Finally, to determine the effect of C/N ratio, various concentrations of NH4CL (0.5 to 2.5 g/L) were used and other conditions were as 35 °C, pH 8 and shaking at 140 rpm. Experiments were performed in triplicate. At two-day intervals, 1 ml of each medium was taken for measurements of bacterial growth using with protein assay (Bradford) and obtained average values were used in the drawing diagrams.

Evaluate degradation ability of PAHs by mixed bacterial after optimization

After optimization of environmental factors, a 10-day culture was made in this conditions and the remained phenanthrene extracted and measurements by GC.

RESULTS AND DISCUSSION

Enrichment of Phenanthrene-degrading bacteria and selection of the novel strain

Different bacterial species were isolated from wastewater samples. The clearing zone around isolated colonies on MSM agar plates sprayed with phenanthrene was observed after 2, 4 and 5 days of culture, respectively. Among PAHs degrading bacteria, two strains that have higher degradation of PAHs (when mixed together) were isolated and identifying. Bacteria were labeled with the names of ZF1 and ZF2. Figure 1 shows the colony of the novel bacterium on MSM agar plate covered by phenanthrene.

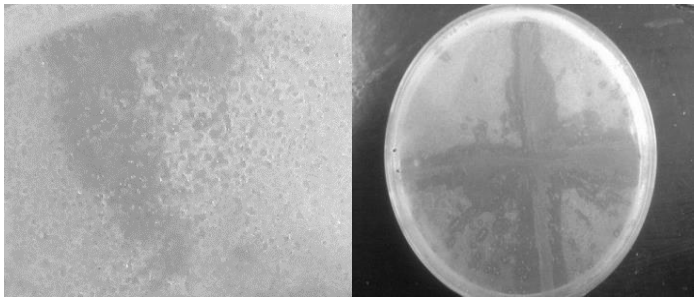


Figure 1 Clear zone on Phe-sprayed MSM agar made by Phe degrading.

Isolation and identification of PAHs degrading strain

Following phylogenetic analysis of 16S rRNA, complementary morphological and biochemical tests, were performed for classification of the ZF1 and ZF2 isolates. Briefly, a 1516 bp nucleotide DNA fragment of 16S rRNA was amplified from the ZF1 isolate and 1436 bp nucleotide DNA fragment of 16S rRNA was amplified from the ZF2 isolate sequenced using specific primers and deposited in NCBI database.
The selected isolates were identified by partial sequencing of the PCR amplified 16S rDNA gene. The obtained sequences were submitted to the BLAST in order to find a homology with other 16S rDNA sequences. Comparing the sequence of the 16S rDNA gene of the isolates with the sequences in GenBank revealed that the ZF1 isolates are similar to *Pseudomonas aeruginosa* and the ZF1 isolate is *Serratia marcescens* with 99% similarity. Also to confirm the identity Strains detected, phenanthrene no topic identification was done. Morphology and biochemical tests with comparing the sequence of the 16S rDNA gene of the isolate revealed that the isolated was a strain of Pseudomonas aeruginosa and Serratia marcescens (Table 1).

Table 1 Morphological and biochemical features of isolates ZF1 and ZF2

Morphological and biochemical features	ZF1	ZF2
Cell morphology	Gram-negative bacilli, non-spore, and non-capsule forming	Gram-negative bacilli, non-spore, and capsule forming
Colony morphology	Circular, smooth-edged, glistening, and yellow–white	Circular, smooth-edged, convex, glistening, and pink
Positive reactions	Oxidase, catalase, mobility, urease, aerobic, nitrate reduction, nitrification, indole production, Utilized d-glucose, Utilized starch	catalase, urease, aerobic, nitrate reduction, Utilized d-glucose, d-fructose, maltose, d-xylose
Negative reactions	Acid-fast, H_2S production, and Utilized d-fructose, maltose, d-xylose	Oxidase, Acid-fast, H_2S production, and indole production, nitrification, Utilized starch
Tolerance range pH	5-10	6-10
Tolerance range temperature	20–50 °C	15–45 °C

Assessment of mixed bacterial growth

Mixed bacterial growth charts (by mines of protein assay) indicated that when mixed bacteria were cultured showed more growth than the two separate bacteria cultures. The low growth of bacteria in separate cultures was due to product intermediate materials that are toxic to bacteria. When bacteria are cultivated in a mixture, reduced intermediate materials production by co-metabolism activity. Analysis of total protein charts showed, 2 mixed culture of bacteria in the first 24 hours after inoculation have low growth rate and increased growth rate in 2 to 8 days after inoculation and the last days reduced growth rate again (Figure 2). Slow growth in last days is due to reducing the carbon and energy source and increasing of waste materials.

The results of GC analysis show, the individual culture of bacteria in MSM included 200 mg/l of phenanthrene can degrade phenanthrene less than 25%, during 10 days. Also, when bacteria were cultured in a mixture, could degrade more than 83% of available phenanthrene (Figure 3, Table 1).

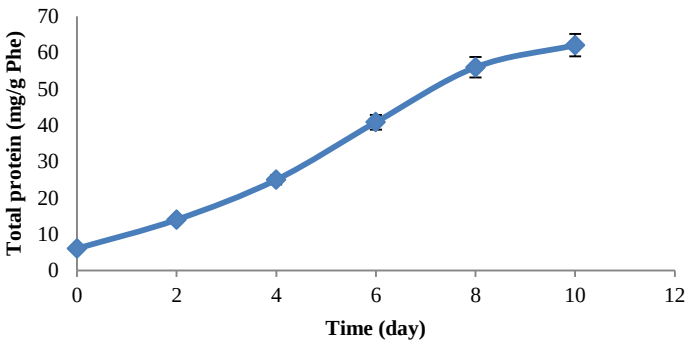
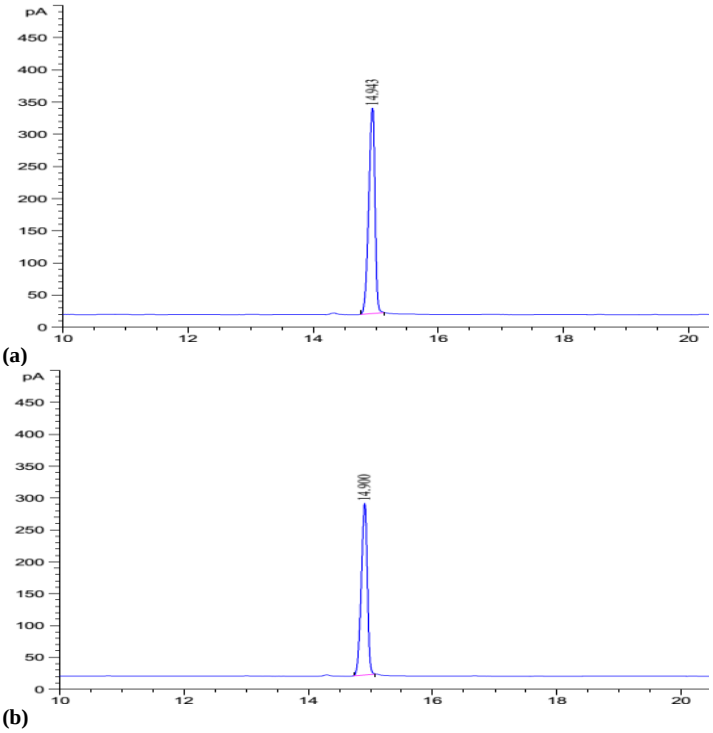
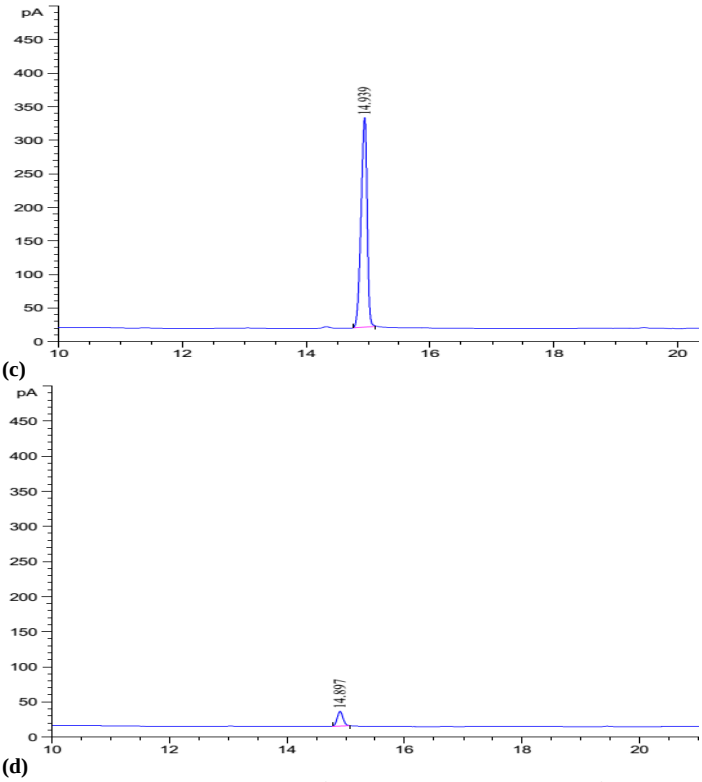


Figure 2 The growth rate for mixed culture with protein assay during 10-day incubation at 30 °C, pH 7 and 120 rpm



(a)

(b)



(c)

(d)

Figure 3 Obtained Chromatograms from remaining phenanthrene after 10 days of incubation at 30 °C, pH 7 and 120 rpm in 100 ml MSM containing 200 mg/l phenanthrene; **(a)** bacteria-free medium (control); **(b)** ZF1 bacteria; **(c)** ZF2 bacteria; **(d)** mix of 2 bacteria (ZF1 and ZF2).

Determining the influence of environmental factors on bacterial grows in phenanthrene

Temperature

Usually degradation of PAHs increases with the increase of the temperature. The increase of temperature will be effective until the proteins and membranes of microorganisms unharmed (**McGenity, 2010**). According to the diagram 2, observed that the mixed bacteria grow and degrade phenanthrene in the range of 25 °C to 45 °C. Because of more production of protein at 35 °C, the temperature considered as optimum for Phenanthrene degradation (Figure 4). The results of analysis of variance and mean comparison showed that the third treatment (35 °C) was statistically significant at the 1% level with other treatments (F value= 8.042, Prob= 0.0085, CV= 5.80%).

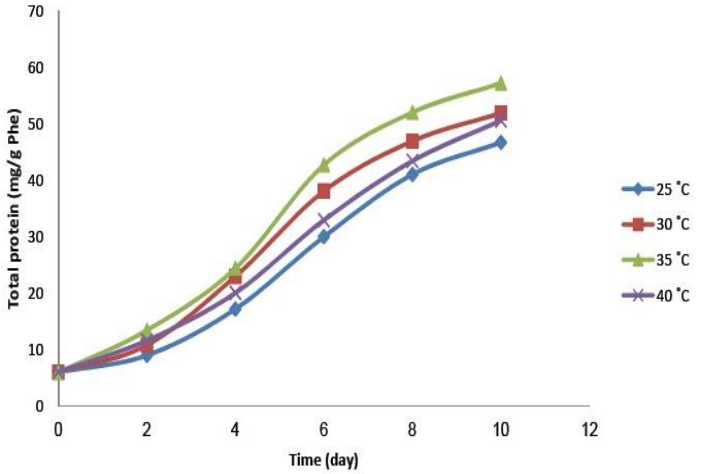


Figure 4 Effect of different temperatures on the growth 2 mix of bacteria at 100 ml MSM containing 200 mg/l phenanthrene during 10 days of incubation at pH 7, 1 g/l of NH4Cl and 120 rpm.

pH

Choosing the optimal pH for the growth of two bacterial mixed was performed by considering optimal temperature (35 °C). The results showed a mixture of 2 bacteria was able to grow and degrade phenanthrene at pH 6 to 9. Diagram 6 showed that the highest amount of bacteria protein production occurred at pH 8 and therefore selected as the best pH for phenanthrene degradation (Figure 5). The results of analysis of variance and mean comparison showed that the third treatment (pH 8) was statistically significant at the 1% level with other treatments (F value= 13.940, Prob= 0.0015, CV= 4.34%).

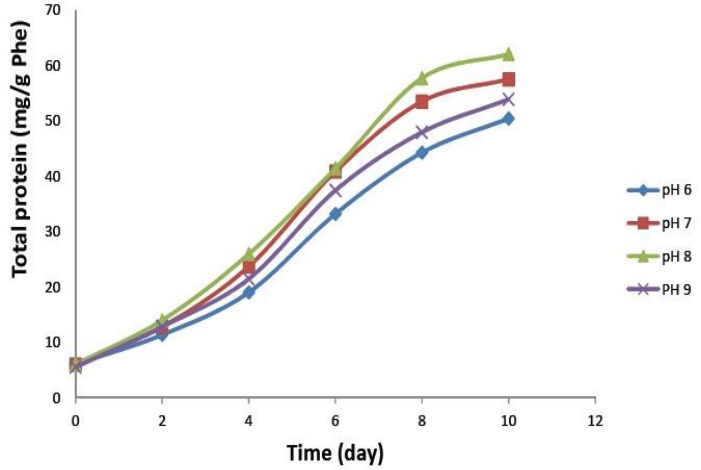


Figure 5 Effect of different pH on the growth 2 mix of bacteria at 100 ml MSM containing 200 mg/l phenanthrene during 10 days of incubation at 35 °C, 1 g/l of NH_4Cl and 120 rpm.

Nitrogen source

Choosing the optimal concentrations of NH4Cl for the growth of two bacteria was performed by considering optimal temperature (35 °C) and optimal pH (pH 8). The mixed bacteria were able to grow on all levels of NH4Cl concentrations. The highest growth of bacteria was obtained in the highest concentration of NH4Cl. The results showed that mixtures of two bacteria growth in 0.5 mg/l concentration were lower than other concentration but little difference was seen in the rate of protein synthesis at 1 g/l, 1.5 g/l and 2g/l concentrations of NH4Cl. So, the lowest concentration (1 g/l) of NH4Cl was chosen as the optimal concentration (Figure 6). The results of analysis of variance and mean comparison showed that1 g/l, 1.5 g/l and 2g/l concentrations of NH4Cl were significant difference at the level of 1% with 0.5 mg/l concentration of NH4Cl (F value= 11.989, Prob= 0.0025, CV= 2.40%).

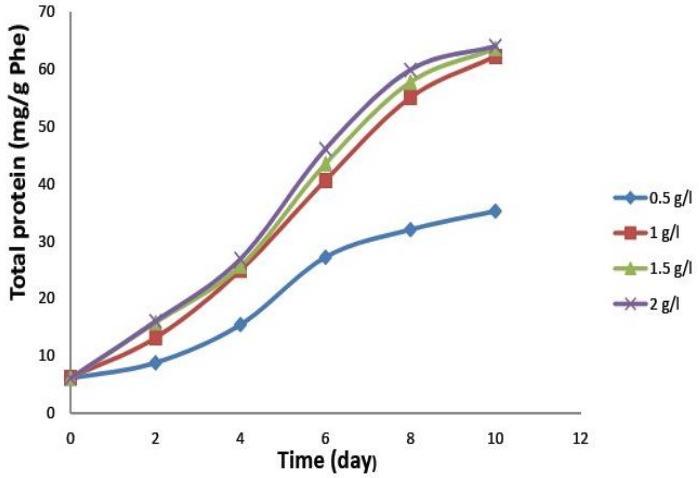


Figure 6 Effect of different NH_4Cl concentrations on the growth 2 mix of bacteria at 100 ml MSM containing 200 mg/l phenanthrene during 10 days of incubation at 35 °C, pH 8 and 120 rpm.

PAHs degradation in optimum growing conditions

To determine the performance of mixture 2 bacteria in the optimum condition the mixture of 2 bacteria added to 100 ml MSM containing 200 mg/l phenanthrene, 250 mg/l fluorene, 250 mg/l anthracene and 150 mg/l pyrene until the OD600 nm reaches up to 0.15. Culture media were incubated in optimal conditions (pH 8, 35 °C and 120 rpm) for 10 days. This mixture could degrade more than 97% of the phenanthrene (Figure 7), 94% of the fluorine (Figure 9), 94% of the anthracene (Figure 10), 45% of the pyrene (Figure 11).

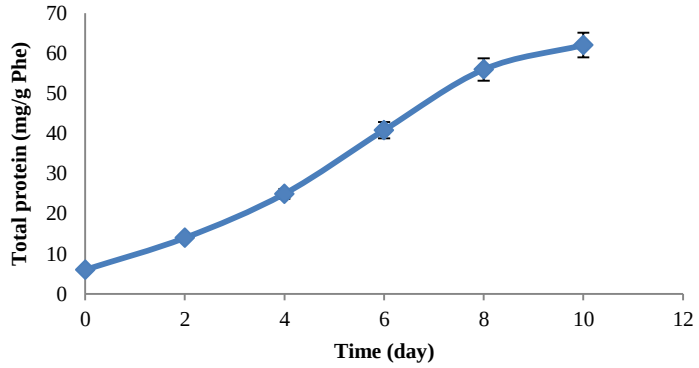


Figure 7 The growth rate for mixed culture with protein assay during 10-day incubation in optimum conditions (pH 8, 35 °C and 120 rpm).

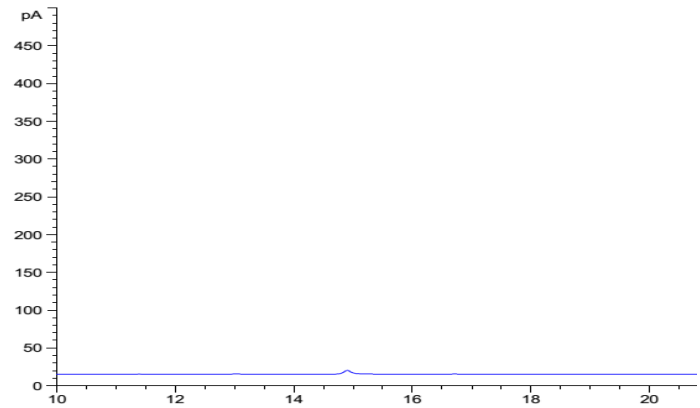


Figure 8 Remaining phenanthrene peak at 100 ml MSM containing 200 mg/l phenanthrene with a mix of 2 bacteria (ZF1 and ZF2) in optimum conditions (pH 8, 35 °C and 120 rpm).

Polycyclic aromatic hydrocarbons are one of the most important oil contaminants and also these compounds are considered a serious threat to humans and other organisms (**Hassanshahian et al., 2012**). In this study, examined the ability of two mixture isolated bacteria and purified isolated strains of Arak petrochemical wastewater to degradation PAHs compounds such as fluorine, phenanthrene, anthracene and pyrene. The results showed a mixture of two bacteria were abler to degradation of PAHs compounds compared to a pure strain of bacteria. This

would due to the increased ability of bacteria enzymes and co-metabolic processes in mixed cultures compared to a pure strain of bacteria (**Casellas et al., 1998; Sugiura et al., 1997**). The bacteria enter the environment when they are alone, they began to break down phenanthrene but after a limited amount of growth stop their decomposition and growth that stopping the growth of bacteria probably due to the toxicity of the intermediates materials. The ability of single bacterial enzyme was low and not able to break down this intermediates material and these materials are increased in the environment and stopping growth. In mixture growth of bacteria due to increasing variety of enzymes involved in the degradation of intermediate composed, these materials degrade and disappear. As a result, if the compounds that were not decomposed by one bacteria established in the environment, these compounds are degraded by enzymes of secondary bacteria and destroyed its toxicity for the first bacteria. Similar results were obtained in **Shahriari** study (**Shahriari et al., 2014**). The low growth of bacteria in separate cultures due to product intermediate materials that are toxic to bacteria. When bacteria are cultivated in a mixture, reduced Intermediate materials production by co-Metabolism activity. In this study, 2 strains of bacteria isolated with the ability to degradation of phenanthrene and identified by morphological and biochemical tests. According to Han et al. 2006 homology with 99% similarity expressing identify in strain level. homology with 97% similarity to less than 99% expressing identify in genus level and homology with 93% similarity to less than 97% expressing identify the new genus and new species (Han et al. 2006). According to the above cases ZF1 bacteria with 99% similarity to *Pseudomonas aeruginosa*, and ZF2 bacteria with 99% similarity to *Serratia marcescen*, are probably strains of *Pseudomonas aeruginosaand Serratia marcescen*. Factors that affect the rate of biodegradation include temperature, salinity, pH, foodstuffs, oxygen, microorganism's species and physical and chemical composition of hydrocarbons (**Agarry et al., 2012; Wilson et al., 1993**). The optimum condition for the growth of bacteria was causing to achieve higher performance in a mix of 2 bacteria and increasing degradation of PAHs compounds. In this study, using the classic method of changing one factor at a time, optimization factors were applied for optimal growth of the 2 mix of bacteria. The application of optimized condition for 3 factors including temperature, pH and nitrogen source caused degradation rate of phenanthrene up to 97% that this amount improved compared with pre-determined optimize condition (83%). Among the applied factors, the most important factors are the temperature and acidity and the effect of different concentrations of NH4Cl as a nitrogen source was minimal. Generally, temperature plays an important role in controlling the features and metabolism rate of petroleum hydrocarbons by microorganisms. The temperature causes changing of demographic structure of bacteria, changing the rate of metabolism and also change the chemical and physical properties of contaminants compounds (**Atlas, 1981**). The solubility of hydrocarbons which are low solubility in water such as PAHs compounds is dependent on the temperature. The temperature increase caused increasing the solubility of these compounds in the environment and therefore increase the amount of degradation. Also, most of the mesophilic bacteria have maximum performance in degradation of PAHs in the temperature range of 30 to 40 °C (**Leahy et al., 1990**). One of the other effective factors in PAHs degradation is pH environment. PH environment causes changes in the prevalence of bacteria, enzyme activities, movement of intracellular and changing food solubility (**Lin et al., 2010**). In this study, the optimal pH for growth of 2 bacteria was considered pH 8. Several reports, based on degradation of PAHs are performed in the pH range of 2 to 10 but observed that microorganisms are active in the neutral pH range (**Jain et al., 2011**). Nutrients are the most important factors in the degradation of PAHs compounds. Lack of nutrients can reduce the number and bacteria performance in PAHs degradation (**Leys et al., 2005**). This study showed that mixtures of 2 bacteria growth in 0.5 mg/l concentration were lower than other concentration but little difference was seen in the rate of protein synthesis at 1 g/l, 1.5 g/l and 2 g/l concentrations of NH4Cl. So, the lowest concentration (1 g/l) of NH4Cl was chosen as the optimal concentration. In this study, in addition to phenanthrene, other PAHs compounds such as fluorine, anthracene and pyrene were analyzed. The results showed that isolated mixed bacteria have a high performance to degradation of these compounds. So that they could degrade 98% of fluorine, 94% of anthracene and 45% of pyrene during 10 days.

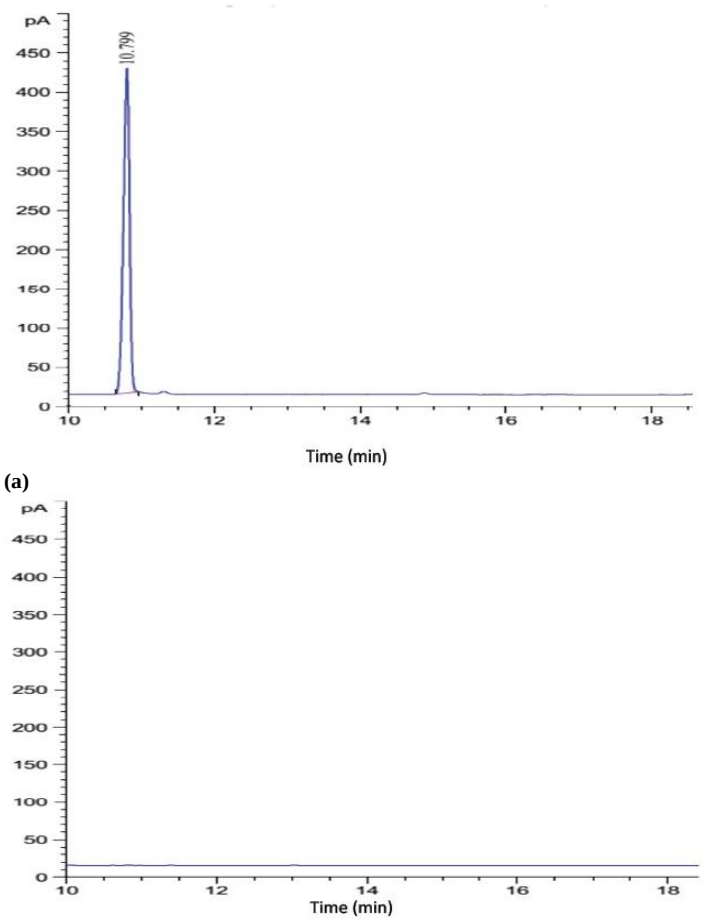


Figure 9 Obtained chromatograms from remaining fluorene after 10 days of incubation at 30 °C, pH 7 and 120 rpm in 100 ml MSM containing 250 mg/l fluorene; **(a)** Without bacteria(control); **(b)** Mix of 2 bacteria (ZF1 and ZF2).

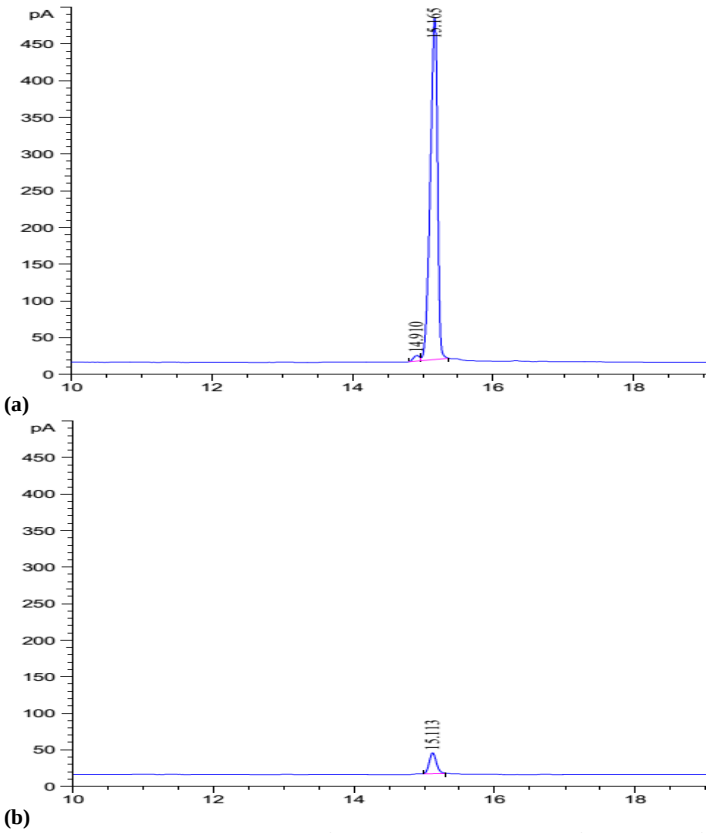


Figure 10 Obtained chromatograms from remaining anthracene after 10 days of incubation at 30 °C, pH 7 and 120 rpm in 100 ml MSM containing 250 mg/l anthracene; **(a)** Without bacteria(control); **(b)** Mix of 2 bacteria (ZF1 and ZF2).

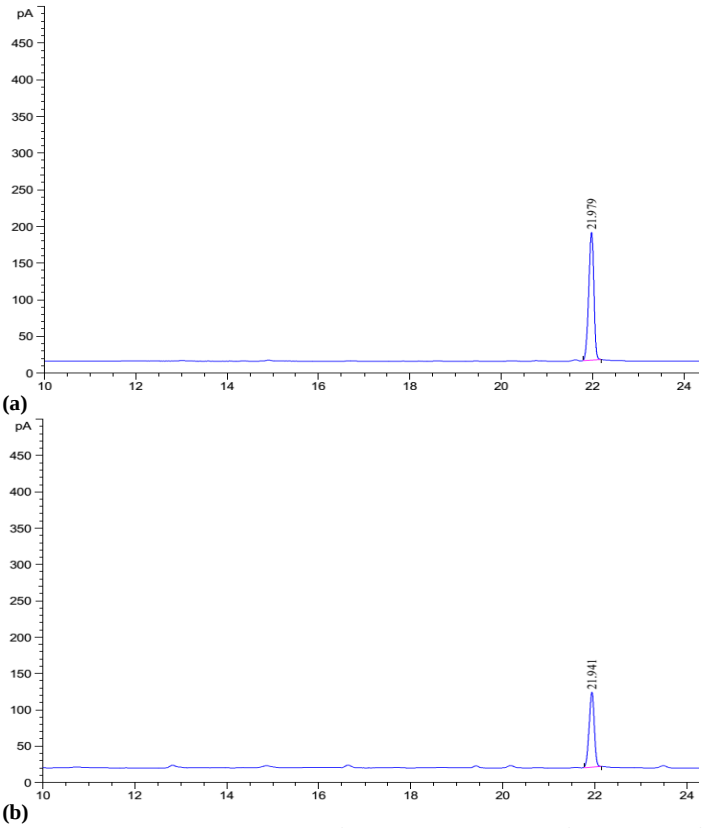


Figure 11 Obtained chromatograms from remaining pyrene after 10 days of incubation at 30 °C, pH 7 and 120 rpm in 100 ml MSM containing 250 mg/l anthracene; **(a)** Without bacteria (control); **(b)** Mix of 2 bacteria (ZF1 and ZF2).

Table 2 The amount of pure strain and mix isolated strains biodegradation after 10 days of incubation at 100 ml MSM containing 200 mg/l phenanthrene at 30 °C, pH 7 and 120 rpm.

bacteria	Phe biodegradation percent
ZF1	13/7 ± 2
ZF2	5 ± 1/2
mix of 2 bacteria (ZF1 and ZF2)	83/6 ± 4

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REFERENCES

Agarry, S. E, Owabor, C. N., Yusuf, R. O, (2012). Enhanced bioremediation of soil artificially contaminated with kerosene optimization of biostimulation agents through statistical experimental design. *Petrol. and Environ., 3*(3): 1-8. http://dx.doi.10.4172/2157-7463.1000120

Anderson, T. A., Kruger, E.L., Coats, J.R., (1994). Enhanced degradation of a mixture of three herbicides in the rhizosphere of an herbicide-tolerant plant. *Chem. Phe., 28*: 1551–1557. http://dx.doi.10.1016/0045-6535(94)90248-8

Atlas, R.M, (1981). Microbial degradation of petroleum hydrocarbons. An environmental perspective. *Microbiological Reviews, 45*: 180-209.

Bagi, A., (2013). Effect of low temperature on hydrocarbon biodegradation in marine environments. *Mar. Environ. Res., 28*(89): 83-90

Bayoumi, R.A., (2009). Bacterial bioremediation of polycyclic aromatic hydrocarbons in heavy oil contaminated soil. *Journal of Appl. Sci. Res., 5*: 197-211.

Casellas, M., Grifoll, M., Sebate, J., Solanas, A. M., (1998). Isolation and characterization of a fluorenone-degrading bacterial strain and its role in synergistic degradation of fluorene by a consortium. *Canadian Jour. of Microbiol., 44*: 734-742. http://dx.doi.10.1139/cjm-44-8-734

Churchill, S.A., Harper, J.P., Churchill, P.F., (1999). Isolation and characterization of a Mycobacterium species capable of degrading three- and four-ring aromatic and aliphatic hydrocarbons. *Appl. Environ. Microbiol., 65*: 549–552.

Coral, G., Karagoz, S., (2005). Isolation and characterization of phenanthrene-degrading bacteria from a petroleum refinery soil. *Ann. Microbiol., 55*: 255-259. http://dx.doi.org/10.1080/10406638.2015.1037006

Coulon F, McKew BA, Osborn AM, McGenity TJ, Timmis KN, (2007). Effects of temperature and biostimulation on oil-degrading microbial communities in temperate estuarine waters. *Environ. Microbial., 9*: 177–86. http://dx.doi.10.1111/j.1462-2920.2006.01126.x

Erfan-Manesh, M. and Afyouni, M., (2008). Environment, Water, Soil and Air Pollution. *Publications of Arkan Danesh.*

Han, X. Y. (2006) Bacterial identification based on 16S ribosomal RNA gene sequence analysis, in: Y.W. Tang, AND C. W. Stratton. *Adv. Tech. in diag. Microbiol. Springer*., 323- 326.

Hassanshahian, M., Emtiazi, Cappello, S., (2012). Isolation and characterization of crude oil degrading bacteria from the Persian Gulf and the Caspian Sea. *Mar. Pollut. Bulletin., 64*: 7-12. http://dx.doi.10.1016/j.marpolbul.2011.11.006

Heitkamp M.A., Cerniglia C.E., (1988). Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated form sediment blow an oil field. *Appl. Environ. Microbial., 54*: 1612-1614.

Jain, P. K., Gupta, V. K., Gaur, R. K. Lowry, M., et al. (2011). Bioremediation of petroleum oil contaminated soil & water. *Environ. Toxicol., 5*(1): 1-26. http://dx.doi.10.3923/rjet.2011.1.26

Janošek J., Bittner M., Hilscherová K., Bláha L., et al. (2007). AhR-mediated and antiestrogenic activity of humic substances. *Chem. Phe., 67*: 1096–1101. http://dx.doi.10.1016/j.chemosphere.2006.11.045

Johnsen, A.R., Wick, L.Y., Harms, L., (2005). Principle of microbial PAH-degradation in soil. *Environ. Pollut., 133*: 71-84. http://dx.doi.10.1016/j.envpol.2004.04.015

Kasai, Y., K. Shind, S. Harayama and N. Misawa, (2003). Molecular characterization and substrate preference of a polycyclic aromatic hydrocarbons dioxygenase from Cycloclasticus sp. StrainA5. *Appl. Environ. Microbial., 69*: 6688-6697. http://dx.doi.10.1128/AEM.69.11.6688-6697.2003

Kelly, I., Cerniglia, C.E., (1991). The metabolism of phenanthrene by a species of Mycobacterium. *J Ind. Microbiol., 7*: 19–26.

Larkin, M.J., Kulakov, L.A., Allen, C.C.R., (2005). Biodegradation and *Rhodococcus-masters* of catabolic versatility. *Curr. Opin. Biotech., 16*: 282–290.

Leahy, J.G., Colwell, R.R., (1990). Microbial degradation of hydrocarbons in the environment. *Microbiol. and Molecul. Biolog. Rev., 54*: 305-315.

Leys, N.M., Bastiaens, L., Verstraete, W., Springael, D., (2005). Influence of the carbon/nitrogen/phosphorus ratio on polycyclic aromatic hydrocarbon degradation by Mycobacterium and Sphingomonas in soil. *Amab., 66*: 726-736. http://dx.doi.10.1007/s00253-004-1766-4

Lin, C., Gan, L., Chen, Z. L., (2010). Biodegradation of naphthalene by strain Bacillus fusiform is (BFN). *Jour. of Hazard. Mater., 182*: 771-777. http://dx.doi.10.1016/j.jhazmat.2010.06.101

McGenity, T.J., (2010). Halophilic hydrocarbon degraders. In: K.N. Timmis (ed). Handbook of hydrocarbon and lipid Microbiology. *Springer-Verlag, Berlin, pp*: 1939-1951.

Onruthai P., Kwankeaw T., Kenichi I., Toshio O., (2012). Isolation of novel phenanthrene degrading bacteria from seawater and the influence of its physical factors on the degradation of phenanthrene. *Sci. Asia, 38*: 36–43. http://dx.doi.10.2306/scienceasia1513-1874.2012.38.036

Rock, S., (1997). Phytoremediation. In: Freeman, H. (Ed.), Standard Handbook of Hazardous Waste Treatment and Disposal. 2nd ed., McGraw Hill Inc., *New York. PP*., 93-112.

Samanta, S.K., Singh, O.V., Jain, R.K.., (2002). Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. *Trends Biotech., 20*: 243–8.

Shahriari Moghadam, M., Ebrahimipour, G., Abtahi, B., Ghassempour, B., (2014). Biodegradation of polycyclic aromatic hydrocarbons by a bacterial consortium enriched from mangrove sediments. *Jour. of Environ. Health Sci. & Engin., 12*: 114. http://dx.doi.10.1186/s40201-014-0114-6

Sugiura, K., Ishihara, M., Shimauchi, T., Harayama, S., (1997). Physicochemical properties and biodegradability of crude oil. *Environ. Sci. Tech., 31*: 45-51. http://dx.doi.10.1021/es950961r

Tian, L., Ma, P., Zhong, J.J., (2002). Kinetics and key enzyme activities of phenanthrene degradation by *Pseudomonas mendocina*. *Proc. Biochem., 37*:1431–7. http://dx.doi.10.1016/S0032-9592(02)00032-8

Vinas, M., Sabaté, J., Espuny, M.J. and Solanas, A.M., (2005). Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. *Appl. Environ. Microbial., 71*(11): 7008–7018. http://dx.doi.10.1128/AEM.71.11.7008-7018.2005

Wilson, S. C., Jones, K. C., (1993). Bioremediation of soil contaminated with polynuclear aromatic hydro- carbons (PAHs): a review. *Environ. Pollut., 81*: 229. http://dx.doi.10.1016/0269-7491(93)90206-4

Yaghmaei, S., Safekordi, A., (2001). Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by some bacteria isolated from coaltar contaminated soil. *Scientia Iranica., 8*: 197.

Zafrriou, O.C., (1977). Marine organic photochemistry previewed. *Mar. Chern., 5*: 497-522. http://dx.doi.10.1016/0304-4203(77)90037-8

Zaidi, B.R., Imam, S.H., (1999). Factors affecting microbial degradation of polycyclic aromatic hydrocarbon phenanthrene in the Caribbean coastal water. *Mar. Pollut. Bull., 38*: 737–42. http://dx.doi.10.1016/S0025-326X(99)00037-5