Polycyclic Aromatic Hydrocarbons Degradation by Aquatic Bacteria Isolated from Khazar Sea, the World’s Largest Lake

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Abstract

Background: Aquatic microorganisms have an important role in the bioremediation of environmental pollutants. Polycyclic Aromatic Hydrocarbons (PAHs) are described as dangerous pollutants that can bind covalently to the nucleic acids, causing mutations. Therefore, they have carcinogenic and toxic properties. Also, are involved in diseases such as asthma, lung dysfunction, and chronic bronchitis. This study aimed to isolate and characterize aquatic biodegrading bacteria from the world’s largest lake, Khazar, with the ability to use PAHs as only carbon source.

Methods: Samples were taken from the estuary of Siah Rud River (Mazandaran province, Iran) and Fereydunkenar beach leading to isolation of twenty-three bacteria on marine agar and sea water media. The isolates were cultured on separate ONR7a medium, each supplemented with only one PAH; as the sole carbon source; including naphthalene, phenanthrene, and anthracene.

Results: Eleven bacterial isolates were able to grow on supplemented media: TBZ-E1, TBZ-E2, TBZ-E3, TRZ-S12, TRZ-S16, TBZ-E20, TRZ-SF2, TBZ-F1, TBZ-F2, TBZ-F3 and TBZ2. These isolates belong to Alteromonas, Marivivens, Pseudoalteromonas, Vibrio, Sherewella, Photobacterium, Mycobacterium and Pseudomonas genera. The qualitative analysis showed that the consortium of isolates TBZ-F1, TBZ-F2, TBZ-F3, TBZ-SF2, and TBZ2 displayed the highest degradation rate for phenanthrene and naphthalene. Naphthalene, phenanthrene, and anthracene were potently degraded by TBZ2 and TBZ-SF2 and accordingly were subjected to measure degradation potential of mentioned PAHs.

Conclusion: The bacterial isolates of Caspian lake have a critical duty in biodegradation of PAHs. These isolates are representative samples of the bacterial population of this lake, participating in the purification process of this habitat.

Introduction

Aquatic microorganisms are very important due to their roles in materials decomposition, food chains, and biogeochemical cycling.1 The beneficial effects of bacteria derive from their role in the carbon, oxygen, and/or nitrogen cycles, which are important for the organism’s survival and their associations with other living systems. Therefore, isolation and identification of effective microorganisms are needed to determine how they interact in an ecosystem.2 Since 1900, all of marine sciences have developed greatly. Unfortunately, most of marine bacteria are left unknown and also there is not enough information about known bacteria based on their applications.3 It can be due to their small sizes, limited range of morphologies and more importantly the difficulties in obtaining pure cultures that can be representative of natural populations.4 Today, molecular approaches such as 16s rRNA gene sequencing are used as genetic markers for the dominant bacterial species in natural microbial populations.5 PAHs are a group of priority pollutants present at high concentrations in many industrial contaminated sites.6 They enter the environment from numerous routes including fossil fuel or other organic material burning, the accidental spilling of processed hydrocarbons and oils, coal liquefaction and gasification, or organic oil seepage and surface run-offs from forest/brush fires and natural geologic processes.7 PAHs have widespread environmental
distribution and have potential for causing cancer in human. Some of low-molecular-weight PAHs are acutely toxic and higher-molecular-weight PAHs are genotoxic. Based on toxicity, carcinoogenicity, and widespread distribution, the U.S. Environmental Protection Agency has listed 16 PAHs as priority pollutants. PAHs are considered more stable and less degradable than many other organic compounds. Given the harmful effects of PAHs along with their stability, it is very important to develop ways to remove PAHs from the environment. Bioremediation is the utilization of microorganisms to convert hazardous organic materials into harmless substances, like carbon dioxide and water. Bioremediation has a lot of advantages over thermal and some physicochemical techniques, such as its low cost. For all these reasons, isolation and characterization of bacteria with the ability to degrade PAHs is indeed in demand. This study aimed to isolate and identify aboriginal aquatic bacteria which are adaptive in the most environmental pollution region of Khazar (Caspian) sea with the ability to use PAHs as only carbon sources. In addition, most important question is whether the native environmental bacteria have the ability to remedy of lakes pollution?

**Materials and Methods**

**Water sample collection**

Two water samples were collected from the estuary of Siah Rud River (36°39′05.2″N 52°19′15.9″E) (Mazandaran province, Iran) and Feraydunkenan Beach (36°41′50.1″N 52°33′28.9″E) (Fereydunkenan County, Mazandaran Province, Iran). The samples were then transported to the laboratory of pharmaceutical biotechnology, Faculty of Pharmacy, Tabriz University of Medical Sciences. Isolation of aquatic bacteria was performed on marine agar and sea water media. Marine agar medium contains (per liter): peptone, 5.0 g; yeast extract, 1.0 g; Fe (II)-citrate, 0.1 g; NaCl, 19.45 g; MgCl₂ (dried), 8.8 g; Na₂SO₄, 3.24 g; CaCl₂, 1.8 g; KCl, 0.55 g; NaHCO₃, 0.16 g; KBr, 0.08 g; SrCl₂, 34.0 mg; H₂BO₃, 22.0 mg; sodium silicate, 4.0 mg; NaF, 2.4 mg; (NH₄)₂PO₄, 3.5 g. Artificial sea water consists of NaCl, 28.13 g; KCl, 0.77 g; CaCl₂, 2H₂O, 1.6 g; MgCl₂6H₂O, 4.80 g; NaHCO₃, 0.11 g; MgSO₄.7H₂O, 3.5 g. About 10 μL aliquots from the samples were spread onto plates. The plates were incubated at 30 °C for a week. To obtain a pure culture, the colonies were cultured in marine broth and stored at -70 °C in marine broth supplemented with 30% (v/v) glycerol. Gram staining was performed and the result was checked by KOH lysis test technique.

**Biochemical tests**

Biochemical tests were performed for isolates: TBZ-SF2, TBZ-F1, TBZ-F2, TBZ-F3 and TBZ2 using biomerieux API 20E and API 20NE kits. Assimilation tests were performed by the aid of API 20NE kit and fermentation/oxidation tests of some sugars were done by API 20E kit. Additionally, Oxidase and Catalase tests, Growth at various NaCl concentrations, temperatures from 5 up to 50 °C at intervals of 5 °C and pH 4.0 to 12.0 (using increments of 1 pH unit) were also carried out on lab-made marine broth medium. To determine the optimal growth on NaCl concentration, temperature and pH, samples were measured by UV absorbance at 600-nanometer wavelength (Optical Density (OD) in 600 nm) after 48 hours. The pH values were adjusted using buffer system including 0.1 M citric acid/0.1 M sodium citrate for pH 4.0-5.0; 0.1 M KH₃PO₄/0.1 M NaOH for pH 6.0-8.0; 0.1 M NaHCO₃/0.1 M Na₂CO₃ for pH 9.0-10.0; 0.05 M and Na₂HPO₄/0.1 M NaOH for pH 11.0-12.0.

**16S rRNA gene sequences**

To conduct genetic characterization, DNA was extracted from isolates according to the method described by Corbin et al. (2001) with some modifications. For phylogenetic analysis based on the 16S rRNA gene sequence, the 16S rDNA was targeted for amplification by polymerase chain reaction (PCR) in the presence of forward 16F 5′- AGAGTTTGATCCTGTCAGCAG-3′, and reverse 16R for Gram-negative bacteria; 5′- ACGGCTACCTTGTTACGACTT-3′ and reverse 16R for Gram-positive bacteria; 5′- TACCTTGTTAGGACCTCAC-3′ primers. The program for PCR was: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 55 sec and extension at 72°C for 85 sec and completed by the final extension at 72°C for 10 min. The amplified fragments were purified with Roche kit (Germany) followed by sequencing with forward and reverse primers by Macrogen Company (Korea). The 16S rDNA sequencing results were analyzed using Chromas Pro software (ChromasPro 2.1, Technelysium Pty Ltd, Tewantin QLD, Australia). Phylogenetic trees were constructed using the neighbor-joining method in MEGA version 7 software package.

**Qualitative bio-degradation Tests**

Stock concentrated-solutions of each PAH (naphthalene, phenanthrene, and anthracene) (Merck-Schuchart, Germany) were prepared in acetone with a concentration of 1 mg/ml and sterilized using 0.22 μm filters. The capability of isolates to use of PAHs as the sole sources of carbon, ONR7a medium with naphthalene, phenanthrene, and anthracene) (Merck-Schuchart, Germany) were prepared in acetone with a concentration of 1 mg/ml and sterilized using 0.22 μm filters. The capability of isolates to use of PAHs as the sole sources of carbon, ONR7a medium with naphthalene, phenanthrene, and anthracene was prepared. For this purpose, ONR7a medium was prepared based on three solutions, containing: solution “1” with the volume of 500 ml is consisted of NaCl, 22.79 g; Na₂SO₄, 3.98 g; KCl, 0.72 g; NaBr, 83.00 mg; NaHCO₃, 31.00 mg; H₂BO₃, 27.00 mg; NaF, 2.60 mg; NH₄Cl, 0.27 g; Na₂HPO₄.7H₂O, 89.00 mg;
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TAPS0, 1.30 g, 12 g of agarose (Sigma) (pH=7.6), solution “2” with the volume of 450 ml is consisting MgCl2, 6H2O, 11.18 g; CaCl2, 2H2O, 1.46 g; SrCl2, 6H2O, 24.00 mg and solution “3” with the volume of 50 ml composed of FeCl3, 4H2O, 2.00 mg/ml. The acetone stock solutions were diluted with cool ONR7a medium before solidification and the final concentration of each PAH was 200 ppm.15,18 Besides, pure ONR7a medium was tested as negative control plates and plates with glucose (200 ppm) were served as positive controls.

Quantitative bio-degradation Tests

Based on qualitative analysis, the isolated TBZ1 and TBZ2 strains showed to have high degradation potential. Therefore, quantitative biodegradation tests were carried out in ONR7a broth medium containing 50 ppm of naphthalene, phenanthrene or anthracene for these isolates. For this aim, 2 ml of bacterial suspension with OD=0.6 in 600 nm were added in 17.8 ml of ONR7a broth medium along with 200 μl of PAH stock solution as sole carbon source. Two series of control Erlenmeyer flasks were prepared for this study: the bacterium-free media and the PAH-free culture media.

The flasks were incubated on 100 rpm shaking incubator at 30 ºC for 6 days. Spectroscopic scanning of the samples were carried out by using spectrophotometer (UNICO UV-1200) with λ max of 311 nm for naphthalene, 316 nm for phenanthrene and 345 nm for anthracene.19 Before spectroscopic measurement, PAHs were extracted from culture medium in 5 ml of cyclohexane.20 PAH residual concentration was calculated based on calibration curve equation. All experiments were done at least in triplicates. To make sure that the decline in PAH concentration is completely contributed to bacterial growth due to PAHs consumption and biodegradation, not to cell surface adsorption or accumulation, bacterial growth was checked by OD measurement at 600 nm during examinations.

Statistical analysis

Results of quantitative tests and measurements were analyzed by SPSS Statistics 23 based on ANOVA and Tukey’s post hoc tests and Excel 2016 softwares.

Results

Twenty three bacterial isolates were obtained from water samples of Siah Rud River and Fereydonkenar beach which were isolated from sea water medium and marine medium. Among these, 13 isolates were able to clarify insoluble PAHs from ONR7a broth medium. These isolates were picked for 16S rRNA sequencing. To find out the phylogenetic position of each isolate, the 16S rRNA gene sequences were analyzed based on NCBI and EzTaxon data banks and finally, the respective phylogenetic tree was constructed (Figure 1). These isolates contain TBZ-E1 (MK480663) (TBZ-E2 = TBZ-E4), TBZ-E3, TBZ-S12 (MK480664), TBZ-S16 (MK480662), TBZ-E20, TBZ-SF2, TBZ-F1 (MK479299), TBZ-F2 (MK480612) (=TBZ1), TBZ-F3 (MK480666) and TBZ2 (KX712072). The results indicated that strain TBZ2 belongs to the genus Pseudomonas and its closely related type strain is P. oleovorans subsp. Lubricantis RS1 (98.65% similarity).21 TBZ2 is recently introduced as Pseudomonas khazarica as a novel species.22

Strain TBZ-SF2 showed 100% similarity in 16S rRNA sequence with S. haliotis JCM 14758.23 Strain TBZ-F1 belongs to the genus Shewanella and its closely related type strain is S. algae CMC 21037 (99.66% similarity).24 Strains TBZ-F2 and TBZ1 are members of the genus Photobacterium and their closely related type strain is P. ganghwaense DSM 22954 (99.65% similarity).25 Strain TBZ-F3 belongs to the genus Microbacterium and its closely related type strain is M. esteraromaticum DSM 8609 (99.88% similarity).26 Strain TBZ-E1, TBZ-E2, and TBZ-E4 belong to the genus Alteromonas and their closely related type strain is A. litorea (99.86%). Strain TBZ-E3 belongs to the genus Marivivens and its closely related type strain is M. donghaensis AM-4 (100% similarity).27 Strain TBZ-S12 belongs to the genus Pseudoalteromonas and its closely related type strain is P. flavigula NCIMB 2033 (99.86% similarity).28 Strain TBZ-E20 belongs to the genus Pseudomonas and its closely related type strain is P. chengduensis MBR (100% similarity).29 (Table 1)

Based on qualitative biodegradation tests, five bacterial isolates had the remarkable ability to use naphthalene, phenanthrene or anthracene as sole carbon sources. These strains were subjected to biochemical API 20E and API 20NE tests. Besides, Lab-made tests including oxidase and catalase tests, growth at different temperatures, pHs and concentrations of NaCl were performed and the results of these tests are summarized in Table 2 and Table 3, respectively.

Qualitative Biodegradation Tests demonstrated that stains TBZ-F2 and TBZ2 are more efficient than others in terms of consuming and degradation of naphthalene, phenanthrene or anthracene. So these strains were subjected to quantitative biodegradation tests. Bacterial growths for two isolates were analyzed in 6 days based on OD measurement in λ = 600nm. Growth curves are shown in Figures 2 and 3, and the results of naphthalene, phenanthrene, and anthracene bio-degradation tests are shown in Tables 2 and 3, respectively.

Discussion

Aromatic compounds comprise almost 65% of chemical wastes. Most of the aromatic compounds possess mutagenic and toxic properties.31 PAHs are very stable pollutants...
Figure 1. Neighbor-joining phylogenetic tree derived from 16S rRNA gene sequence data showing the positions of 13 isolated bacteria including TBZ-E1 (TBZ-E2 = TBZ-E4), TBZ-E3, TBZ-S12, TBZ-S16, TBZ-E20, TBZ-SF2, TBZ-F1, TBZ-F2 (=TBZ1), TBZ-F3 and TBZ2 among related bacteria. Numbers at branch points are bootstrap percentages based on 500 replicates. Bar, 0.02 changes per nucleotide position.
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Table 1. Closest known bacteria to various isolates from Caspian Sea based on 16S rRNA gene sequence.

| Isolate       | Number of isolates | Accession number | Nearest relative                        | Genus               | Similarity (%) |
|---------------|--------------------|------------------|-----------------------------------------|---------------------|----------------|
| TBZ2          | 1                  | KX712072         | P. oleovorans subsp. Lubricantis        | Pseudomonas         | 98.65          |
| TBZ-SF2       | 1                  | HM016086         | S. haliotis                             | Shewanella          | 100.00         |
| TBZ-F1        | 1                  | MK479299         | S. algae                                | Shewanella          | 99.66          |
| TBZ-F2, TBZ1  | 2                  | MK480612         | P. ganghwense                           | Photobacterium      | 99.65          |
| TBZ-F3        | 1                  | MK480666         | M. esteralaromaticum                    | Microbacterium      | 99.88          |
| TBZ-E1, TBZ-E2, TBZ-E4 | 3 | MK480663         | A.litorea TF-22                         | Alteromonas         | 99.86          |
| TBZ-E3        | 1                  | KT282004         | M. donghaensis AM-4                     | Marivivens          | 100            |
| TBZ-S12       | 1                  | MK480664         | P. flavipulchra NCIMB 2033              | Pseudoolteromonas   | 99.86          |
| TBZ-S16       | 1                  | MK480662         | V. diazotrophicus NBRC 103148           | Vibrio              | 99.86          |
| TBZ-E20       | 1                  | EU307111         | P. chengduensis MBR                     | Pseudomonas         | 100            |

Table 2. Comparison of the characteristics of isolated bacteria based on API 20E and API 20NE.

| Characteristics | Isolate TBZ2 | Isolate TBZ-SF2 | Isolate TBZ-F1 | Isolate TBZ-F2 | Isolate TBZ-F2 (=TBZ1) |
|-----------------|--------------|-----------------|----------------|---------------|-----------------------|
| Hydrolysis of:  | Arginine     | +               | -              | -             | +                     |
|                 | Esculin      | -               | +              | +             | +                     |
| Production of:  | Indole       | -               | -              | -             | +                     |
|                 | Acetoin¹     | -               | -              | -             | -                     |
|                 | H₂S          | -               | +              | -             | -                     |
| Enzymes:        | Urease       | -               | -              | -             | +                     |
|                 | Tryptophane deaminase | +           | -              | -             | -                     |
|                 | Gelatinase   | ±²              | +              | +             | -                     |
|                 | Lysine decarboxylase | -         | -              | -             | -                     |
|                 | Ornithine decarboxylase | -       | +              | +             | -                     |
|                 | β-Galactosidase (Ortho-Nitro Phenyl-βDGalactopyranosidase) | -       | -              | -             | +                     |
|                 | β-galactosidase (Para-Nitro Phenyl-βDGalactopyranosidase) | -       | -              | -             | ±                     |
| Fermentation / Oxidation of: | D-Glucose | +               | -              | -             | +                     |
|                 | D-Mannitol   | +               | -              | -             | +                     |
|                 | Inositol     | -               | -              | -             | +                     |
|                 | D-Sorbitol   | -               | -              | -             | ±                     |
|                 | L-Rhamnose   | -               | -              | -             | +                     |
|                 | D-Sucrose    | -               | -              | -             | +                     |
|                 | D-Melibiose  | -               | -              | -             | +                     |
|                 | Amygdalin    | -               | -              | -             | ±                     |
|                 | L-Arabinose  | -               | -              | -             | +                     |
| Assimilation of: | Glucose     | +               | -              | -             | ±                     |
|                 | Arabinose    | -               | -              | -             | ±                     |
|                 | Mannose      | +               | -              | -             | ±w¹                   |
|                 | Mannitol     | +               | -              | -             | w                     |
|                 | N-Acetyl-Glucosamine | -    | +              | +             | w                     |
|                 | Maltose      | -               | -              | -             | w                     |
|                 | Potassium Gluconate | +         | -              | -             | +                     |
|                 | Capric acid  | +               | +              | -             | +                     |
|                 | Adipic acid  | +               | +              | -             | -                     |
|                 | Malate       | +               | +              | -             | ±                     |
|                 | Trisodium Citrate | +        | -              | -             | ±                     |
|                 | Phenylacetic acid | -         | -              | -             | +                     |

¹ Voges–Proskauer or VP test, ² ± means doubtful, ³ weakly positive
Table 3. Comparison of the characteristics of isolated bacteria based on lab-made tests.

| Characteristics | Isolate TBZ2 | Isolate TBZ-SF2 | Isolate TBZ-F1 | Isolate TBZ-F3 | Isolate TBZ-F2 (= TBZ1) |
|-----------------|--------------|-----------------|---------------|---------------|------------------------|
| Oxidase test    | +            | +               | +             | +             | +                      |
| Catalase test   | +            | +               | +             | +             | +                      |
| Temperature range (°C) | 10-55°C (optimum 30 °C) | 10-42°C (optimum 37 °C) | 10-40°C (but not at 4 °C) | 4-37°C (but very week at 4 °C, optimum 30°C) | 10-45°C (optimum 35°C) |
| pH range        | 4-10 (optimum 6-7) | 5-11 (optimum 7) | 5-11 (optimum 7.5) | 5-10 (optimum 7-7.5) | 5-11 (optimum 8-9) |
| NaCl concentration (%) | 0-8.5% (w/v) (optimum 2-2.5%) | 0-10% (w/v) (optimum 4%) | 0-6% (w/v) (optimum 2-2.5%) | 0-6.5% (w/v) (optimum 2-2.5%) | 1-7% (w/v) (optimum 2%) |

Figure 2. Growth curve for isolate TBZ-F2 (=TBZ1) based on OD measurement in λ = 600 nm in presence of naphthalene, phenanthrene or anthracene as sole carbon source and without carbon source in control group.

Figure 3. Growth curve for isolate TBZ2 based on OD measurement in λ = 600 nm in presence of naphthalene, phenanthrene or anthracene as sole carbon source and without carbon source in control group.

due to having benzene rings. In fact, by increasing the number of benzene rings, the stability of PAHs increases and microbial degradation become more difficult.32 Five isolates were able to use PAHs as sole carbon source and consequently, were subjected to PAH degradation potential analysis. These isolated strains belonged to the genera *Shewanella*, *Photobacterium*, *Mycobacterium*, and *Pseudomonas*.

Based on other studies on PAH degrading microorganisms, most of anaerobic nitrate-reducing and sulfate-reducing bacteria have the potential to degrade PAHs. The greatest number of PAH-degrading bacteria belong to the genus *Pseudomonas*.33 Kim *et al.* compared genome sequences of two pyrene-degrading *Mycobacterium* strains, isolated from Han river.44 In a study, Tahseen *et al.* enhanced *Pseudomonas putida* degradation potential of crude oil by gamma-ray irradiation.53 Rathour *et al.* demonstrated that a bacterial strain belonging to genus *Shewanella*, isolated from Pongong lake is capable of pyrene degradation.56 Liu *et al.* introduced *Photobacterium* strains as polycaprolactone (PCL) degrading isolates of Yangtze river.37 Multiple studies reported the sensitivity of *Photobacterium phosphoreum* to PAHs. Therefore, *P. phosphoreum* is used for toxicity prediction of PAH contaminations in luminescence inhibition test (Microtox test).38 In the present study, *Photobacterium ganghwence* is introduced as PAH-degrading bacteria. Non-toxic effect of these materials on bacterial growth may be due to low molecular weights and simple structures of these compounds.59 Although, more researches are required to identify producing metabolites from these PAHs and toxicity of them. Arbabi *et al.* performed bio-treatment of phenanthrene with two indigenous bacterial mixed cultures, isolated from petroleum contaminated sites in Iran, for 20 weeks in the solid-phase reactor and could effectively remove phenanthrene.60 Mardani *et al.* used genetically manipulated *Pseudomonas putida* for oil biodegradation in spiked soil and could significantly increase biodegradation of PAHs in the recombinant group.41 Tarhriz *et al.* isolated and introduced *Tabrizicola aquatica* as the novel genus of aquatic bacteria from qurugol Lake in the mountains region of Azerbaijan which can adsorb heavy metals.42 Elyasifar *et al.* isolated halophilic bacterial belonged to *Bacillus subtilis* and *Virgibacillus olivae*, from Dagh Bajarmand and Haj Aligholi salt deserts in Iran with potential to produce antibacterial and antifungal agents.43 Several aerobic naphthalene-degrading bacteria account for 86.9% of the hydrocarbon-degrading microorganisms found in gasoline-contaminated aquifers.45 For the first time, Ben Said *et al.* described the ability of *Shewanella oneidensis* species to degrade fluoranthene and pyrene as high molecular weight and, phenanthrene as a low molecular weight PAH.45 Several *Mycobacterium* species

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Figure 4. Naphthalene, phenanthrene or anthracene degradation curve by isolate TBZ1 in comparison with control groups.

Figure 5. Naphthalene, phenanthrene and anthracene degradation curve by isolate TBZ2 in comparison with control groups.
are suggested as degradants of high-molecular-weight PAHs.\textsuperscript{19,46} Also, genomic studies on these species were done to understand mechanisms of bacterial PAH degradation, for example, \textit{Mycobacterium gilvum} PYR-GCK.\textsuperscript{18}

\section*{Conclusion}

The Khazar (Caspian) Sea as the largest inland mass of water is exceptional scientific interest. The human-made changes, notably those resulting from the construction of dams, shipping, fisheries and the production of petroleum and natural gas in the Khazar (Caspian) basin cause to change its complex geologic and climatic evolution. Our funding showed that the native bacterial population of Khazar (Caspian) Lake have an efficient role in biodegradation of PAHs. By separating the adaptive bacteria in the most environmental pollution region of Khazar (Caspian) sea with the ability to use PAHs as only carbon source from Khazar (Caspian) Lake, it has been inferred that part of the purification process of the lake is performed by these bacteria.

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\section*{Conflict of Interest}

The authors claim that there is no conflict of interest.

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