

Isolation and identification of arsenic eliminating bacteria from Lake Maharloo and evaluation of the pattern of their antibiotic resistance

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Abstract

Introduction: Arsenic is one of the most dangerous heavy metals in the environment that is harmful for organisms such as human beings. Due to this treat economic approach for elimination of arsenic from water and wastewater is necessary. In this research arsenic-resistant bacteria from water and sediments of Lake Maharloo were isolated and identified then their antibiotic resistance was evaluated.

Methods and Materials: Sampling was done from water and sediments of four stations in Maharloo Lake in three seasons. Bacteria were isolated and purified after cultivation and enriching samples in LB broth medium containing 5mg/L arsenic oxide. Isolated bacteria were identified by usual and standard microbiological tests. Then antibiotic sensitivity was determined by antibiogram method and Muller Hinton Agar culture medium.

Results: Bacteria such as *Bacillus* sp, *Vibrio* sp, *Staphylococcus* sp, *Corynebacterium* sp, *Micrococcus* sp, *Pseudomonas* sp and *E. coli* were isolated in different seasons from water and sediments of Lake Maharloo. The maximum and minimum abundance percentage of arsenic-resistant bacteria was found in sediments of spring (56.25%) and winter (12.50%) ($P < 0.05$) respectively. Also The maximum and minimum abundance percentage of arsenic-resistant bacteria in total of water and sediment related to Khoshk river in winter and the middle of lake in autumn ($P < 0.05$) respectively. Antibiotics resistance patterns evaluation revealed that isolated bacteria had the most antibiotic resistance to penicillin and the lowest to amikacin in all seasons.

Conclusions: In this research indigenous arsenic resistant bacteria of Lake Maharloo were identified. Entrance of arsenic metal and different antibiotics to Lake Maharloo have caused an increase in the arsenic resistant bacteria and their antibiotic resistance.

Keywords: Arsenic, Arsenic Resistant Bacteria, Lake Maharloo, Penicillin, *Vibrio alginolyticus*.

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Introduction

Economical and industrial activities increasing along with population growth contribute to spreading of various environmental contaminants. Arsenic is a known dangerous heavy metal that causes skin lesions, acute and chronic venenation, skin, liver and lung cancer and more over it is associated with gangrene, diabetes, hypertension, capillary malady. Inorganic arsenic effects include cell enzymes denaturalization via linking with sulfhydryl groups that it causes cell demolition by increase in reactive oxygen species (ROS) and change in gene adjustment (1-3).

Arsenic has the ability to bind to sulfhydryl groups of proteins and dithiols such as glutaredoxine. On the other hand, arsenat is a chemical analog of phosphate and can inhibit oxidative phosphorylation. It may interfere with DNA repair system or DNA methylation state. Some polluted area habitant bacteria are able to delete arsenic from their environment. These bacteria detoxify metals by methylation and enzyme process (4).

The role of bacteria in arsenic bioremediation were surveyed from Cornoules mining site (Gard, France). One of gram of negative bacteria belonging to *thiomonas* genus were isolated and identified by the ability to oxidize arsenic to arsenat with lower solubility and toxicity (5). Usual physicochemical methods for metal purification from polluted sites are expensive and not compatible with environmental conditions. Therefore biotechnological achievements are lately considerable as a replacement method (6).

Ten arsenic resistant bacteria were isolated from Orbetloo lagoon in Italy and among them *Bacillus* sp. and *Pseudomonas* sp. showed high resistant to arsenic (7).

Ars genotype characterization was studied in arsenic resistant bacteria from arsenic contaminated gold and silver mines in the republic of Korea (2008). It was shown that

these genes have good potential to arsenic elimination from industrial effluents (8).

Takeuchi et al. (2007), concluded that arsenic resistant bacteria were widespread in aquatic environments. *Marinomonas communis* was characterized as a potential candidate for bioremediation of arsenic contaminated water (9).

Therefore acute characterization of arsenic resistant bacteria is the first step to arsenic bioremediation from aques ecosystems and industrial effluents. Lake Maharloo is located in south east of shiraz (Iran) in which heavy metals like arsenic enter to the brine site of lake gently by entrance waters. Different pollutants such as agricultural (pesticides, phosphorous fertilizer and plants dehumidifiers), industrial (metal production, glass, batteries, colored material, medicine, veterinary, food additives) and municipal enter and ultimately these contaminants along with the obtained salt enter the human food chain.

Lake Maharloo surface water is affected by continental agents like rainfalls, temperature and evaporation. Continental balance of lake depends on balance between amount of rain and evaporation. Maharloo district is a closed zone from hydrological aspect and evaporation adjustment provided from rainfalls and aqueous flows. Entrance flows to this lake consist of soft flows via a perennial river with low irrigation (Pole Fasa river), alternative river (Rahdar and Khoshk river), temporary river (Nazar Abad of Sarvestan) and also some temporary streamlets, fountains and spate swages.

The purpose of this research was isolation and identification of arsenic resistant bacteria from Lake Maharloo water and sediments and their antibiogram determination in different seasons.

Methods and Materials

Four sampling stations were determined with regard to the results of previous measures and studies (10) and thorough cognition of

environs agricultural and industrial activities of lake and entrance of Shiraz city effluents. Geographical situations were located by the Geographical Position System (GPS).

Stations geographical characteristic and satellite map of lake is shown in table 1 and figure 1 respectively.

Table1: Geographical characterization of sampling stations

Station	Latitude	Longitude
Shiraz Khoshk River	29° 31' 1.5"	52° 43' 42"
Pole Fasa River	29° 28' 10.1"	52° 42' 10.8"
Nazar Abad River of Sarvestan	19.8° 29' 29"	52° 42' 30"
Middle of Maharloo Lake	29° 29' 15.8"	52° 44' 5.7"

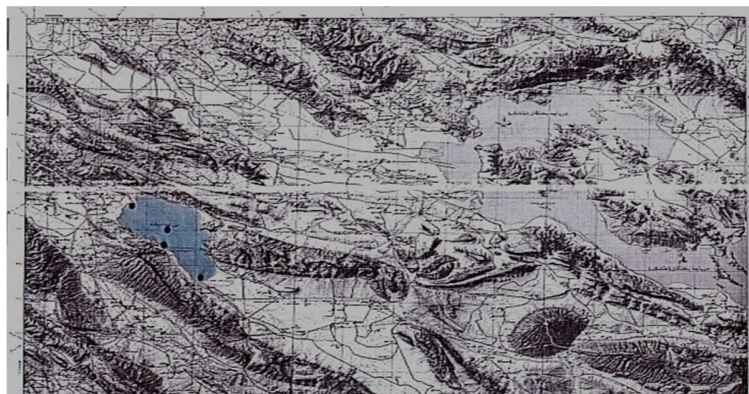


Fig. 1: Satellite map of Lake Maharloo

Sampling was accomplished from water and surface sediments of stations in 3 consecutive seasons, autumn, winter and spring. Triplicate sampling was done in the second month of each season (totally 36 samples from water and sediments).

Sterile plastic dishes were used for sampling (60 ml, made by Sopa Corporation).

Samples were transferred to laboratory next to ice within 4 hours.

Atomic absorption spectroscopy and flame method were used for arsenic measurements (model 650, made by Perkin Elmer Corporation).

Bacterial population was counted with dishes numeration method from different collected samples to sake arsenic resistance bacteria distribution survey.

Dillution 10^{-1} to 10^{-10} were prepared by physiological serum and then cultured with

surface plate method on LB agar containing 5 mg/l arsenic oxide and LB agar without arsenic (control). Cultured plates were incubated in 30° C for 72 hours. After distinct colonies appearing, they were counted and multiplied in obtained volume and dillution number (with positive power) to obtain bacterial number on the basis of CFU/g or CFU/ ml in control and metal containing media.

Isolation of arsenic resistant bacteria was done by primary enrichment and proximate culturing in agar media. Hence 0/1 ml of each sample was spreaded in LB agar containing 10 mg/l arsenic oxide and 3% salt. Medium were incubated in 30° C for 48 hours and then purified cultures were obtained from obtained colonies.

For accurate characterization, various biochemical tests were done such as gram reaction, catalase test, KOH test, oxidase

reaction, lysine decarboxylase, citrate, TSI (Triple Sugar Iron Agar) and SIM (Sulfide Indole Motility). Isolates were identified based on bergy's manual of systematic bacteriology (11).

Oxidase test and different salt concentrations in Tryptic Soy Broth (Merk Germany products) were used for *Vibrio* sp. diagnosis. Different types of vibrio were determined by comparison results with distinguishing features which is shown in table (12). Afterwards antibiotic sensitivity was determined by antibiogram method and Muller Hinton Agar culture medium. Hence amikacin (AN), ampicillin (AM), rifampin (RA), cephalexin (CN), chloramphenicol (C), erythromycin (E), gentamicin (GM), penicillin (P), tetracycline (TE) and tobramycin (TOB) were used. After 2 days, bacterial antibiotic sensitivity was registered by measuring the diameter of the zones of inhibition.

Statistical analysis of results was done by ANOVA and Duncan test and SPSS software. Excel software was used to draw graphs.

Results

Average arsenic concentration based on ppm were obtained 0.016 and 0.34 from water and sediments of Khoshk river station, 0.08 and 0.18 from water and sediments of Pole Fasa station, 0.06 and 0.014 from water and sediments of Nazar Abad river station and

0.11 and 0.24 from water and sediments of central station of lake respectively.

The average percentage of isolated resistant bacteria from Lake Maharloo dissociated from every season are shown in figure 2. The maximum and minimum abundance percentage of arsenic resistant bacteria related to spring and winter were (56.25%) and (12.50%) respectively ($P < 0.05$).

Basis of obtained results, diversity of identified gram negative bacteria were more than gram positive ones (table 2).

The maximum abundance of identified bacterial colonies related to *Vibrio alginolyticus* (winter) and the minimum ones related to *Micrococcus* sp., *Pseudomonas* sp., *E. coli*, *Vibrio mimicus* and *Vibrio cholerae* shown in figure 4.

The results of resistance pattern to antibiotics indicated that all isolates from different seasons and stations are resistant to penicillin. In autumn, maximum antibiotic resistance after penicillin (100%) was seen against ampicillin (83.3%) and amikacin (66.7%) respectively (figure 5).

Maximum antibiotic resistances in winter were obtained against penicillin (100%), tetracycline (61.5%) and amikacin (47%) (figure 6).

According figure 7, maximum antibiotic resistances in spring were determined against penicillin (100%), ampicillin (75%) and amikacin (50%).

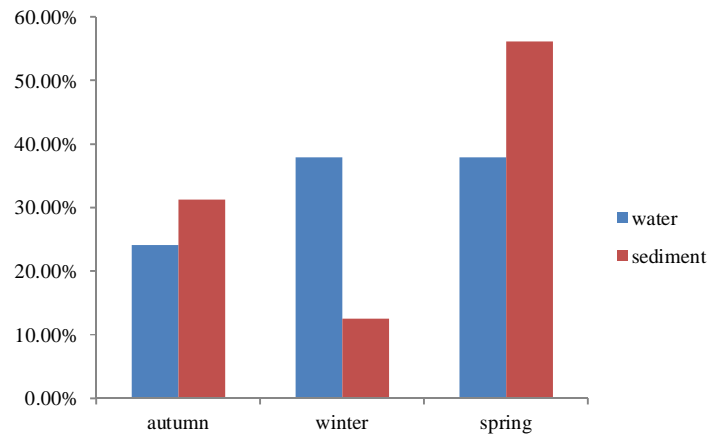


Figure. 2: Abundance percentage of resistant bacteria of Lake Maharloo in different seasons

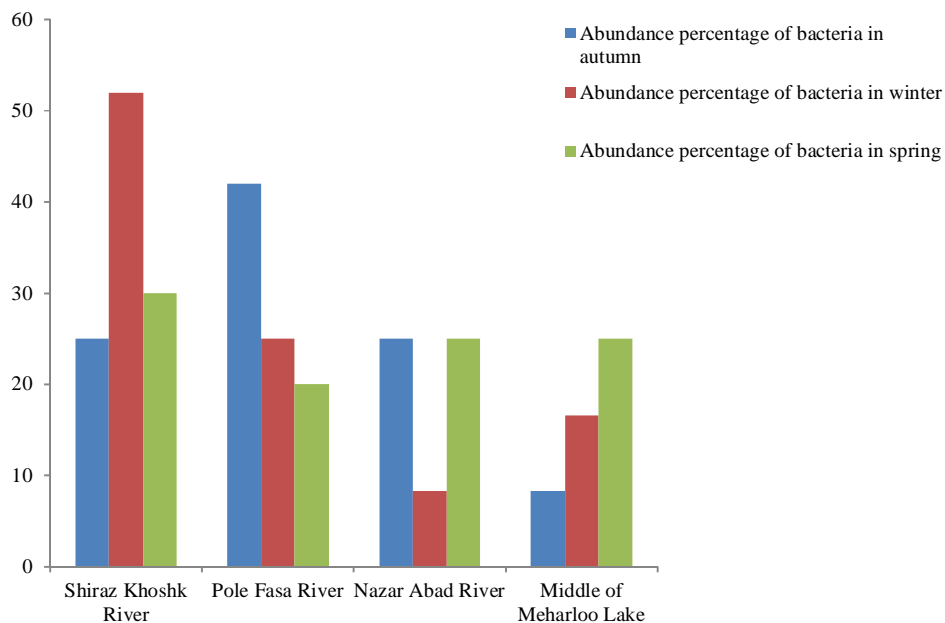


Figure. 3: Abundance percentage of resistant bacteria of Lake Maharloo from different stations

Table 2: Gram positive and gram negative isolated bacteria

gram positive	gram negative
<i>Staphylococcus</i> sp.	<i>Vibrio alginolyticus</i>
<i>Bacillus</i> sp.	<i>Vibrio fluvialis</i>
<i>Corynebacterium</i> sp.	<i>Vibrio mimicus</i>
<i>Micrococcus</i> sp.	<i>Vibrio cholerae</i>
	<i>Escherichia coli</i>
	<i>Pseudomonas</i> sp.

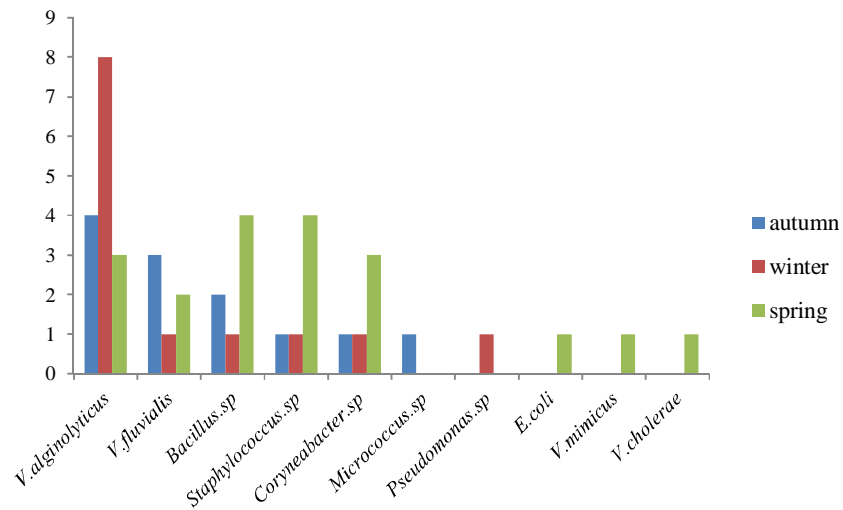


Figure. 4: The number of arsenic resistant bacterial isolated colonies from different seasons

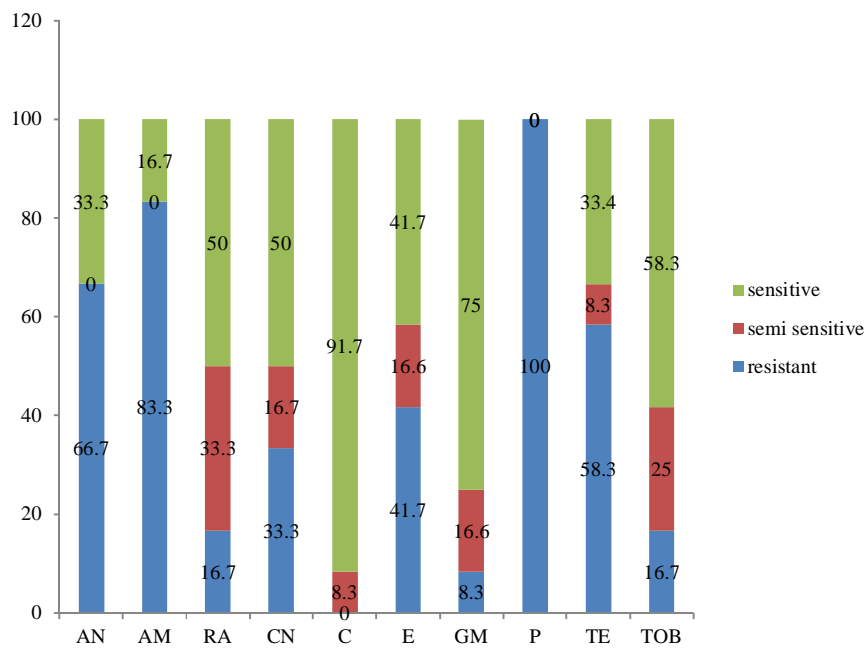


Figure. 5: Resistance percentage of isolated bacteria against tasted antibiotics in autumn

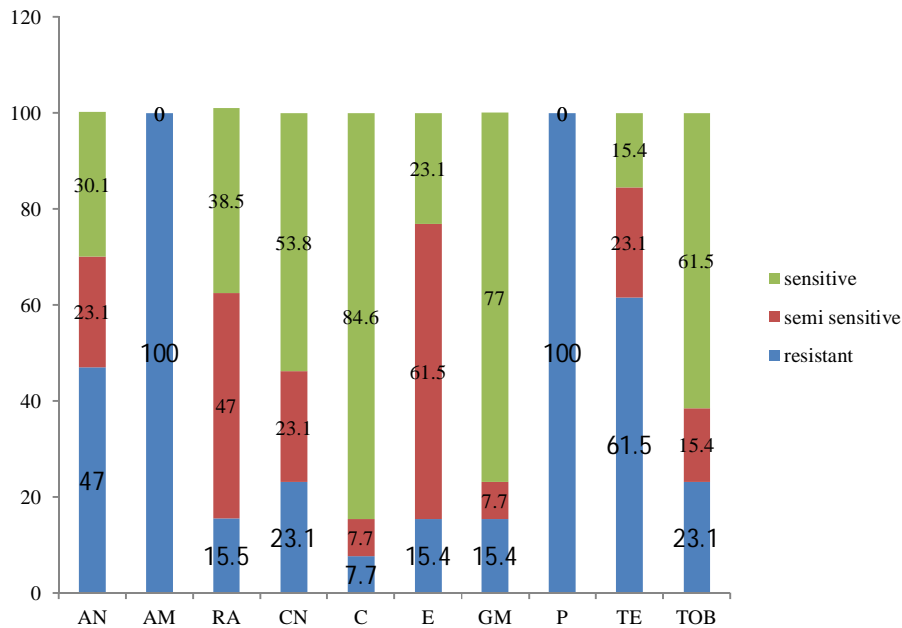


Figure. 6: Resistance percentage of isolated bacteria against tested antibiotics in winter

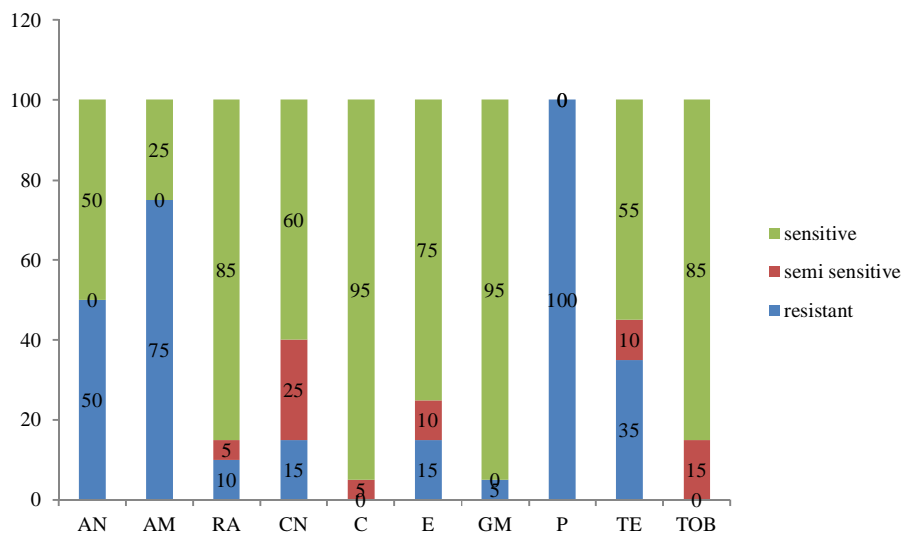


Figure. 7: Resistance percentage of isolated bacteria against tested antibiotics in spring

Discussion

Arsenic cause growth cease and death of many bacteria in mediums containing arsenic. So the number of arsenic resistant bacteria were lower than bacteria in control medium (without arsenic). The maximum abundance of bacteria was observed in spring. It can be related to winter

precipitations that enter large amount of pollutants via rivers to the lake and microbial load increase. Comparison of abundance percentage isolated bacteria demonstrated that most arsenic resistant bacteria were obtained from Khoshk river due to entrance of urban and industrial

wastes containing arsenic and high levels of phosphorous and nitrogen. This subject matter confirmed previous research on water and sediments of Lake Meharloo (10).

In the current study, different gram positive and gram negative bacteria were identified as arsenic resistant bacteria. Gram negative bacteria were more diverse because of differences in cell wall. Gram negative bacteria possess two layer or sometimes several distinct cell wall layers. Outside of gram negative bacteria cell wall is surrounded by a membrane called outer membrane. There is a space between outer membrane and cell wall called preplasmic. Toxin and bacterial enzymes accumulate in preplasmic with high concentration (13). There are many grams positive and gram negative bacteria resistant to arsenic reported in past years.

Chitpirom et al (2009) isolated and identified arsenic resistant bacteria from waste and agricultural soils collected in Thailand. On the basis of the morphological, physiological and biochemical characteristics and 16S rRNA gene sequence analyses, 27 isolates were identified as *klebsiella*, *acinetobacter*, *pseudomonas*, *comamonas* and *entrobacter* (15).

In a nother research Escalante et al (2009) isolated arsenic resistant bacteria. They introduced forty nine bacterial strain as arsenic resistant bacteria. *Pseudomonas* sp. isolated from Lake Maharloo in the current study was a similar bacteria with the mentioned research.

Anyanwa and Ugwa (2010) isolated arsenic resistant bacteria from a sewage treatment plant. The isolates were identified as strains of *Pseudomonas aeruginosa*, *Bacillus* sp., *Flavobacterium* sp., *Escherichia coli*, *Klebsiella* sp. and *Staphylococcus aureus* (17). The majority of these bacteria were isolated in the present study.

Lake Maharloo indigenous bacteria such as *Vibrio alginolyticus* and *Vibrio fluvialis*

were identified from all stations in every 3 seasons.

Bacteria transfer plasmids and transposons containing arsenic resistant gene for survival in contaminated sites, therefore contamination brings about resistant bacteria proliferation (18). In some bacterial strains, resistance determinant agents are on plasmids (19-21). Bioremediation by arsenic resistant bacteria and some arsenic detoxification process have been studied by chromosomes and ars operons encoding plasmid in *E.coli* (8). Chromosomal, plasmid and transposon resistance was reported in different bacteria (22).

There are self- induced operon by arsenite and arsenate in *E.coli* and *Staphylococcus aureus*. Plasmid of resistance to arsenic, pI258, has 3 gene in *Staphylococcus aureus*: ars A (repressor regulatory encoding protein), ars B (aflox encoding protein) and ars C (23-25). Many cyt19 homologous bacterial genes called arsM were discovered by Microbial genome experiments and their protein products was named ArsM (S-adenozyl methyl transferase As(III)) and arsR genes control ars oprons. Ions such as Fe^{2+} , Mn^{2+} , S^{2-} or H_2 can provide reclamation power for microbial metabolisms (26).

Since Lake Maharloo bacteria are halophile, presence of sodium and potassium which are essential elements for microorganisms growth and enzymes activities in halophiles help them tolerate heavy metals like arsenic (27).

In the present study, antibiotic resistance pattern evaluation indicated all isolates were resistant against penicillin. Langae et al. (2000) showed too that expression of Beta-lactamase enzyme (penicillinase) is the origin of this tolerance. This enzyme hydrolysis penicillin Beta-lactam before connection with transpeptidase. Resistance indexes to penicillin and heavy metals like mercury, arsenic and cadmium are common in some cases and most of their genes are on one plasmid (28). In other words, resistance

levels depend on presence or absence of plasmid. Almost all bacterial tolerance to heavy metal is a plasmid trait that it can be eliminated with plasmid deletion. Beside plasmids, chromosomes and the other agents like transposons may also contribute in antibiotic resistance (29). Many reports showed that heavy metals pollution in natural environments has an important role in antibiotic resistance (30,31). In a study with isolation of 15 *entrobacter* strain from polluted costal water of turkey, indicated that resistant strains have heavy metal resistance plasmid. Approximate relevance between plasmid and resistant against antibiotics and heavy metal were proved in a study in Mugla university of turkey on 22 *staphylococcus* strains (32). Present results also confirmed the role of arsenic pollution in antibiotic resistance.

According to results, resistance to penicillin was indicated by all bacteria and ampicillin was in second grade. High resistance to these antibiotics may be due to the entrance of farms, domestic, hospital and livestock effluents to the lake by different rivers. In autumn the most resistant antibiotic bacteria was *Vibrio alginolyticus*. This bacterium resisted to several types of antibiotics like AN, AM, RA, CN, P, TE. These results were the same for *Vibrio alginolyticus* and *pseudomonas* in winter. With regard to the number and diversity of isolated bacteria in spring, table of antibiotic resistance pattern has fluctuation. A type of *Staphylococcus* sp. resisted to AM, AN, CN, GM, P and TE antibiotics that was a special result in comparison with the previous seasons. *Vibrio alginolyticus* also showed resistance to 3 types of antibiotics, AM, CN, and P. This bacterium is a type of halophile *vibrio* that grows in high salinity levels. This is human pathogenic organisms that can cause digestion system, ear and eyes infections (33). Abundance of this bacterium in comparison with the other isolated bacteria

and their antibiotic resistance is due to high salinity of Lake Maharloo.

Conclusions

Characterized bacteria in this research are endemic to water and sediments of Lake Maharloo which can be used in industrial refineries. These bacteria are able to remove environmental pollutions without any genetic manipulation. Entrance of arsenic metal and different antibiotics to Lake Maharloo have caused an increase in the arsenic resistant bacteria and their antibiotic resistance.

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