

IMPACT ASSESSMENT OF HEAVY METALS POLLUTION IN THE AIR ON BACTERIAL MICROFLORA OF ELM TREES

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Abstract: Heavy metals are one of the most important environmental pollutants in all over the world. Heavy metals not only are seen in the soil and water but also can be found in the air. In recent years, the concentration of these metals has been increased in the atmosphere of many cities due to human activities. Examining the qualitative and quantitative changes of the endophyte microorganisms is one of the best markers to investigate the long-term impacts of these pollutants. This research was conducted in order to identify the effect of cadmium, lead and mercury on the population of the endophyte bacteria inside the elm trees in Tabriz. The plant samples including leaf and stem were collected during two years (2015-2016) in two different regions of the city randomly and their endophyte bacteria were isolated. One of the selected sampling site was the central region of the city with the highest pollution and the other one was marginal with less pollution in Tabriz. The experiments showed that there was a significant difference between the bacterial micro flora in these two regions. While, in the elm trees of the marginal region with less pollution the dominated bacteria were Gram negative bacteria, mostly from *Pseudomonas* genus, though in the polluted area, Gram positive bacteria, particularly *Actinomyces* and *Bacillus* were dominated.

Key words: Air pollution, Bacteria, Cadmium, Lead, Mercury

1. INTRODUCTION

Lead, mercury and cadmium are the most important and frequent heavy metals in the nature which is mostly released by both natural and anthropogenic sources (Jung, 2008). Various experiments showed that different types of these heavy metals, though a few, are existed in the earth atmosphere mostly as a result of man's activities (Barry & Chorley, 2009). These compounds are seen in the air; however, they are not in gas state and mainly they can be found as microscopic suspended particles in the air (Ozsoy & Ornektekin, 2009). The smoke of factories, power plants and automobiles are the main sources for high concentration of these metals in the atmosphere. Presence of low concentration of these metallic compounds in the atmosphere is considered natural and they are not toxic to human and other creatures. However, in some areas, especially in urban parts (metropolitans), the concentration of these compounds is so high that they have been converted into an environmental threat for

life and health of humans (Seinfeld & Pandis, 2016).

Heavy metals exposure affects virtually various creatures in different ways and lead to disorder in their natural physiology. The destructive effects of these compounds, in addition to large creatures, have also been proved to tiny microorganisms and as a result, the population of these organisms has been changed quantitatively and qualitatively. These micro-flora changes are not visible by unarmed eyes; while a trivial alternation in the quality and quantity of microorganisms in the nature, might cause some variation in the ecosystem and consequently a considerable impact on the other organisms (Brighigna et al., 2000).

The current research was conducted for investigation and comparison of the bacterial population in the urban trees under two different conditions. In order to specify the impact of these heavy metals on the bacterial population, two different conditions including polluted and relatively healthy ones were studied. Due to high frequency of elm trees in Tabriz and also their long life and

stability of micro-ecosystem inside the stem and branches, they were selected as an appropriate one for examining the bacterial population in contaminated and healthy conditions. Thus, the objective of this study was evaluation of the population of endophyte bacteria inside the elm trees of Tabriz region in order to specify the long-term impact of heavy metals on the natural population of these bacteria.

2. MATERIAL & METHODS

2.1. Experiment site

For collecting samples, the trees located around the two air pollution measuring stations of Tabriz were examined. The first station was located in the central and highly crowded and polluted part of the city (Raste Kocheh) and the second station was located in the margin of the city near the relatively less crowded part (Hakim Nezami). Based on previous studies, it was shown that the concentration of three heavy metals of lead, cadmium and mercury in the first station was significantly higher than the second station during the year 2016 (Sabourmoghaddam, 2017).

2.2. Bacterial isolates collection

Totally, 10 elm trees were chosen randomly around each station and from each trees, 10 healthy branches along with leaves were selected randomly and placed in plastic bags and transferred to the laboratory under cold conditions. The common cultures such as Starch Casein Agar (SCA), Nutrient Agar (NA), King's B and Mathur's medium were used for isolating the endophyte bacteria. In order to culture bacteria, at first, different parts of the plant including stems, main branches and leaves were divided into small pieces. Afterward, the tissues were washed by tap mild water and disinfected three times. To validate surface sterilization, Cao et al., 2004 method was employed. For this purpose, surface-sterilized elm samples were washed three times in sterile water, and then soaked inside the water and

stirred for a minute. An aliquot of 0.5 ml suspensions were then cultured on NA media, incubated at 30 °C, then checked for microbial growth to confirm that all bacterial isolates are endophyte.

2.3. Quantification of bacterial population

For examining the quantity of bacterial population inside the trees, dilution method was employed (Hasanzadeh, 1995). One gram of each plant samples collected from elm trees in both stations was chopped in the porcelain mortar separately and the obtained suspension was poured into other pipe with nine milliliter sterile distilled water and dilution was done for eight times (10^8). From each diluted solution, 250 microliters were cultured in NA media, then the cultured petri-dishes were placed in 37°C for 24 hours to grow the bacterial colony. After the emerge of the bacterial colonies, the number of them were counted using colony counter.

2.4. Identification of bacterial isolates

The endophyte bacteria were identified using biochemical and molecular methods. For molecular detection and division of different bacterial groups, the universal primer of four main bacterial groups including Pseudomonas, Enterobacteriaceae, Bacilli, Actinomycetes were used (Table 1). One percent agarose gel was used for visualizing PCR products. The existence or nonexistence of the mentioned bond was studied under UV light.

3. RESULTS

In total of the elm trees, around the two air pollution measuring sites, 50 leaf, branch and wood samples were collected and transferred into the laboratory. The collected samples were disinfected and cultured in different media and a total of 220 different bacterial colonies were selected and purified in terms of shape, size and color of the colony (Fig. 1). The bacterial isolates were placed in glycerol in -20°C after purification and kept for next experiments.

Table 1: Universal primers and their sequences used in this study

Universal Primer name	Sequence (5'-3')	Related organisms	References
ACT235f ACT878r	CGCGGCCTATCAGCTTGTTG CCGTACTCCCCAGGCGGGG	Actinobacteria	Farris & Olson. (2007)
463F 463r	CTAAAACCTCAAAGGAATTGACG AATACGTTCCCGGCCTT	Bacilli	Kadyan et al. (2013)
PsEG30F PsEG790R	ATYGAAATCGCCAARCG CGGTTGATKTCCTTGA	Pseudomonas	Mulet et al. (2009)
wecE1 wecE2	AGGGCGTGATGTCCACTTAC GAAGAACTGGCTGCGGTTAG	Enterobacteriaceae	Maynard et al. (2005)



Figure 1. Different types of bacterial colonies isolated from elm trees

The results of colony counting showed that there was no significant difference in the endophyte bacterial population between two sites. In both sites, for each gram of plant sample, about 17-30 bacterial colonies were obtained. Depending on the type of tissues, the quantity of colonies was different; in green parts, the numbers of colonies were 30 percent

higher than the numbers of them in the stem parts of trees. Although the number of bacteria counted in the spring was slightly more than those that counted in the winter, but there were no significant differences between samples of various seasons.

According to the results of KOH, from 220 isolated bacterial samples, 144 isolates (65%) were

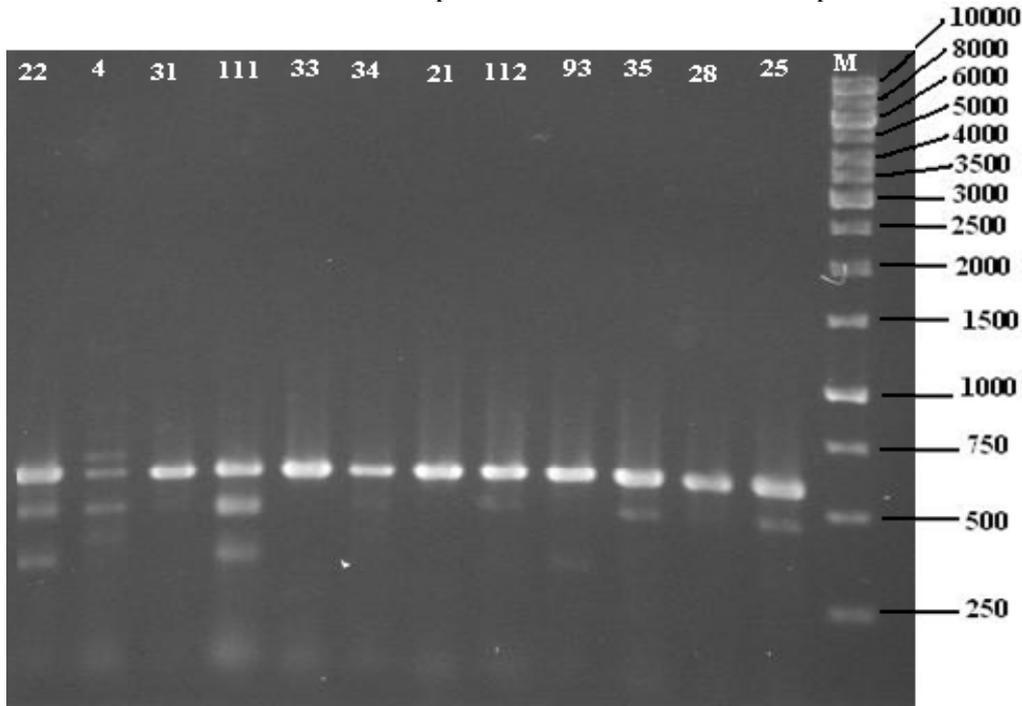


Figure 2. PCR products from Actinobacteria isolates collected from the elm trees

Gram Positive and the rest of them (76 isolates, 35%) were Gram Negative. Likewise, based on the results of O/F test, 170 isolates (77%) were obligated aerobic and the remainders (50 isolates, 23%) were facultative anaerobic.

PCR test was performed on all 220 bacterial isolates. Only 86 isolates out of 220 purified bacterial isolates, produced the expected bands and the rest of them did not generate any band. These results reveal that those bacteria that didn't show any bands are likely to belong to the different bacterial groups other than four tested groups and therefore they require exclusive experiments that it was impossible to do due to limited financial resources.

Among 86 positive isolates, 35 isolates produced 643bp band using Actinobacteria universal primer (ACT235f and ACT878r), which indicating that these 35 isolates are belong to the Actinomyces. Of these 35 isolates (Fig. 2), 19 isolates were collected from site 1 and the rest of them were belong to the site 2. 23 isolates from 86 isolates, produced a short band almost 463 nucleotides by (463F and 463R) universal primer (Fig. 3), which confirms that all these 23 isolates belong to the Bacillus Positive Gram bacteria. Of these 23 isolates, 19 isolates were collected from site 1 (polluted area) and the rest of them were from site 2 (relatively healthy area). All these 23 and 35 isolates were previously identified as

Gram Positive in biochemical tests which matches with the result of PCR. Except for two cases, other 56 isolates were determined obligate aerobic in OF tests.

Among the 28 remained isolates, 10 isolates were identified as *Pseudomonas* by universal primer (PsEG30F and PsEG790R) and the rest of them (18 isolates) were specified as Enterobacteriaceae by universal primer (wecE1 and wecE2). Of 10 positive isolates of *Pseudomonas*, 3 isolates were related to site 1 and 7 isolates were collected from site 2. Amongst 18 Enterobacteriaceae family, 8 isolates have been collected from site 2 and 10 isolates were belonged to site 1.

4. DISCUSSION

The endophyte bacteria have relatively fixed population in their ecological nests, compared to the external saprophytes or epiphytes and they are less influenced by the environmental conditions in short-term; therefore, any changes in their population can be done under long-term influences of the environmental factors (Lodewyckx et al., 2002). Hence, investigation of the endophyte bacteria population can provide a criterion or marker for the environmental changes that are not seen apparently. For instance, Sumampouw & Risjani (2014) showed that bacteria are the best markers for identification of the environmental

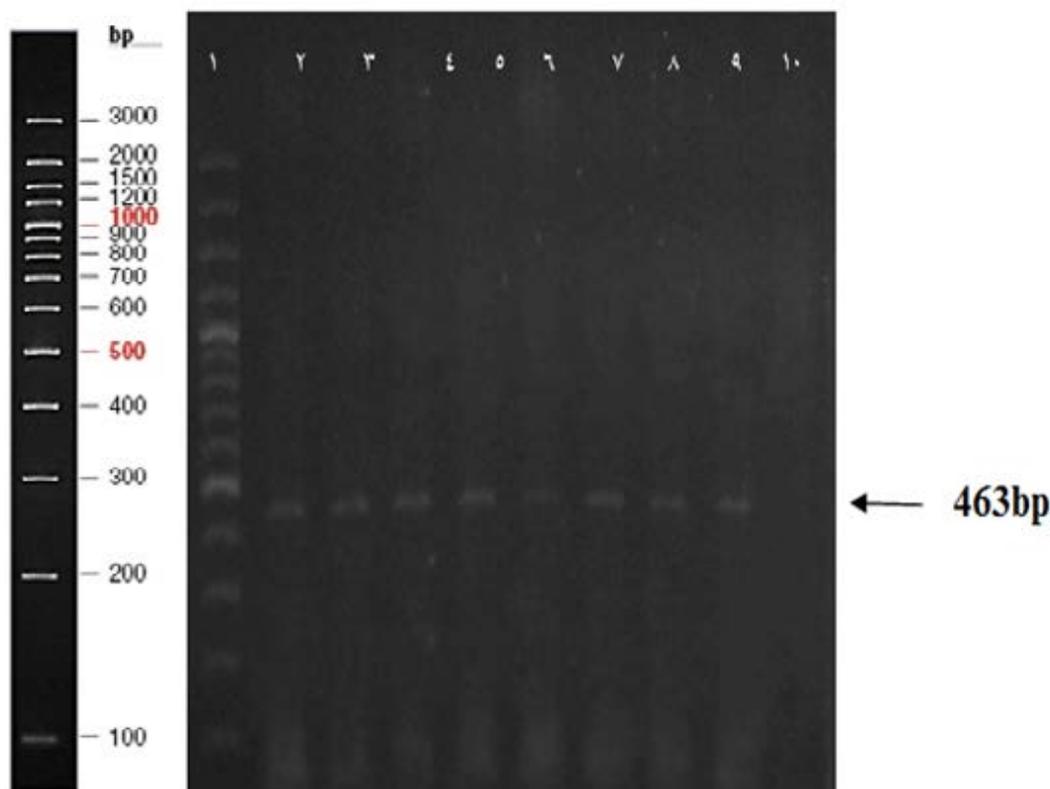


Figure 3: PCR products from Bacilli isolates collected from elm trees

pollutions and by continuous analysis of the different bacterial population, the type and level of changes even in insignificant amount can be specified. The analysis of epiphyte and endophyte bacterial populations on the trees is important. However, the epiphyte bacterial population changes rapidly (even less than some hours) due to raining or temperature fluctuations and this trend makes difficult to analyze the data. While, the endophyte bacteria change rarely even in extreme temperature changes and humidity over some months (Kinkel et al., 2000). Thus, the focus of this research was on endophyte bacteria rather than epiphyte bacteria.

The results of various studies showed that heavy metals are harmful for bacteria. Since the number of soil bacteria is high and the accumulation of pollutants is higher in soil in comparison to the air, most of the researches were conducted on the soil bacteria. Different research showed that the soil of the most regions in the world is contaminated by high concentration of heavy metals. In a comprehensive research, Wuana & Okieimen (2011) found that heavy metals are the most important soil pollutants in most countries and their higher concentration has been reported in some areas of the world.

Various research reveals that the metals such as lead, mercury and cadmium have a significant dreadful impact on the soil total bacteria (Baath, 1989) and increases of these pollutants decreases the bacterial population. For instance, Gad El-Rab et al., (2006) showed that cadmium has a dreadful effect on the different bacterial population. It decreases the growth and multiplication of bacteria and reduces their population in the environment by impact on the bacterial DNA multiplication.

In this research, although the bacterial population was slightly more in healthy region quantitatively, but there was no significant difference in both site. Probably, with increasing of pollution in the environment and the reduction in population of some bacterial groups, in contrary to soil, other bacterial groups are replaced in the trees and causes the fixation of the bacteria population inside the tree.

However, the impact of heavy metals on the different bacterial groups were not the same and as antibiotics, the bacteria showed different sensitivity to heavy metals (Fließbach et al., 1994). Among the different bacterial groups, Gram positive bacteria were more tolerant to the heavy metals (Sandaa et al., 1999). In the contaminated environments, with increasing of the environmental pollution, Gram positive bacteria, especially groups with high GC in genome (actinomyces) were dominated. In this research, most of the identified bacteria in the contaminated area were Gram positive, particularly

Actinomyces and in the healthier region, the number of Grams positive and negative were equal.

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