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Türk Hijyen ve Deneysel Biyoloji Dergisi

Environmental risk assessment under the pollutants exposure with using four lichen species and molecular assay in cement plant, Aşkale-Erzurum (Turkey)

Aşkale-Erzurum Çimento fabrikası etrafında kirleticilere maruz kalmış dört farklı liken türü kullanılarak yapılan moleküler boyutta çevresel risk değerlendirmesi

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ABSTRACT

Objective: The aim of the study is to determine the genotoxic effects of various environmental pollutants around cement factory in Aşkale-Erzurum. It was studied four lichen species which include *Pseudevernia furfuracea*, *Lobaria pulmonaria*, *Cetralia islandica* and *Usnea longissima*.

Methods: The main observation or changes in the protein assay and RAPD patterns included appearance of new bands and/or disappearance of normal bands compared with the control samples.

Results: Although significant amount of decrease in protein content of the samples exposed to pollutants has been observed 50 m away from cement factory, no changes was detected in the protein content of liken samples 100 m and 200 m away from cement factory. Among the four studied species, *P. furfuracea* revealed to have the highest level of band appearance and disappearance. Following the exposure to the pollutants of 1, 2 and 3 district situated at a distance of 50, 100, 200m to the cement factory, P. furfuracea with a control bands were observed respectively. Moreover 31, 13 and 15 bands from the control species disappeared in sites 1, 2 and 3 in *P. furfuracea* samples. Furthermore, the highest polymorphism value was obtained (P% = 86,6%) in *U. longissima* and *L. pulmonaria* by the OPC04 primer, and the lowest polymorphism was

ÖZET

Amaç: Aşkale-Erzurum çimento fabrikasının etrafında çeşitli çevresel kirleticilerin genotoksik etkisinin belirlenmesi amacıyla *Pseudevernia furfuracea*, *Lobaria pulmonaria*, *Cetralia islandica* ve *Usnea longissima* isimli dört liken türü kullanılmıştır.

Yöntemler: Çevresel kirleticilere maruz kalmış örnekler ile kontrol örneklerinin protein boyutunda ve moleküler boyutta da RAPD bantlarında yeni bant oluşumu ve/veya bant kaybolması olup olmadığı kontrol edilmiştir.

Bulgular: Çimento fabrikasına 50 m uzaklıkta kirleticilere maruz kalan örneklerde protein içeriğinde belirgin bir düşüş gözlenmiş olmasına karşın çimento fabrikasına 100 m ve 200 m uzaklıktaki liken örneklerinde protein içeriğinde herhangi bir değişim gözlemlenmemiştir. Çalışılan dört tür arasında P. furfuracea bant görünümü ve bant kaybolma oranı en yüksek olan türdür. Çimento fabrikasına 50, 100 ve 200m. uzaklıkta bulunan 1., 2. ve 3. bölgeler kirleticilere maruz kaldıktan sonra *P. furfuracea*'da (kontrol bant sayısı 83) sırasıyla 19, 45 ve 51 bant gözlenmiştir. Buna ek olarak yine 1., 2. ve 3. bölgelerdeki *P. furfuracea* örneklerinde sırasıyla 31, 13 ve 15 bant kaybolmuştur. Ayrıca, en yüksek polimorfizm değeri OPC04 primeri ile *U. longissima* ve *L. pulmonaria* (P%= % 86.6) liken türlerinde ve en düşük polimorfizm oranı (P%= 45.4%) OPC01 primeri ile

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yielded (P%= 45,4%) in *L. pulmonaria* by the OPC01 primer. According to this study site 1, which is the nearest site to the cement factory (50m), has the highest appearance and disappearance band. As the samples from site 1 revealed the lowest level of GTS values might led to a high level of genotoxic effect in the four lichen species.

Conclusion: This study provides preliminary evidence to the biological effects and genotoxicological consequences caused by various environmental contaminants with the use of four different lichen species collected from around cement factory. The use of indicator organisms as a biomarker in the early detection of genotoxic agents showed reliable sensitivity in terms of estimating the level of damage caused by air pollution.

Key Words: lichen, genotoxicity, risk assessment, pollutant

L. pulmonaria'da elde edilmiştir. Çalışma sonucunda elde edilen bulgulara göre; çimento fabrikasına en yakın (50 m) yer olan bölge 1'de en yüksek bant artışı ve bant kaybolması tespit edilmiştir. Genetik Kalıp Stabilitesi (GKS) değerlerinin düşük seviyede ortaya çıktığı bölge 1'de dört liken türünde genotoksik etki yüksek düzeyde belirlenmiştir.

Sonuç: Bu çalışma, çimento fabrikası etrafında toplanan dört farklı liken türünün kullanılması ile çeşitli çevresel kirleticilerin neden olduğu genotoksik ve biyolojik etkinin ön belirteci olarak bilgi vermektedir. Genotoksik ajanların erken uyarı durumunun belirlenmesinde biyomarkır olarak indikatör organizmalar ile birlikte kullanılmasının, hava kirliliğinin yol açtığı hasar düzeyinin yorumlanmasında güvenilir olduğu görülmüştür.

Anahtar Kelimeler: liken, genotoksisite, risk değerlendirmesi, kirletici

INTRODUCTION

Air pollution represents a serious threat to both the environment and to human health. Biomonitoring helps us to understand the possible ecotoxicological impacts of such contamination by providing valuable information on environmental pollution and improving the process of risk assessment through measurement of the physiological responses of individuals (1). Millions of tons of toxic pollutants such as; ozane, particular matter, carbon monoxide, nitrogen oxides, sulfur dioxide and lead are released into air each year. Mobiles (cars, buses, trucks, etc.) and industrial sources (factories, refineries, power plants, etc.) are the major reasons of such kind of contamination. Furthermore polycyclic aromatic compounds (PACs), heavy metals and halogenated aliphatic hydrocarbons, have been shown to be genotoxic to the living organisms (2). Polycyclic aromatic hydrocarbons (PAHs) are capable of making covalent interactions with nucleophilic centres of DNA (3). They also cause base pair substitutions, frameshift mutations, deletions, S-phase arrest, strand break age and a variety of chromosomal alterations (4-6). Further studies have pointed out that in humans long-term exposure to air pollution is one of the factors involved in the development of cancer (7-9).

Lichens have a high surface ratio and ion exchange properties, and lack variability in morphology throughout thegrowing season. All this may explain why lichens are sensitive to environmental pollution and have been widely used as biomonitors of environmental pollution (10-14). Especially epiphytic lichens are effective airpollution biomonitors (15), because they rely on atmospheric dry and wet deposition for their mineral nutrition (16) and respond to environmental pollution by changing frequency (17). In heavily polluted areas such as urban areas lichens are often absent. In such cases transplant techniques have been used to monitor air pollution: one of these techniques consists exposing bags containing lichen in the studied area, to measure concentrations of contaminants in the

lichen samples (14, 16, 18-24).

A number of researchers have shown the genotoxic potential of 2,4-D that were investigated by using different test systems including chromosome aberration assay, micronucleus and comet assay technique that monitor genotoxic effects on plants (25-28). The advantage of measuring the direct effects of genotoxins on DNA mainly depends on its sensitive and non-time consuming properties (25). The developmentof several PCR-based techniques, provides many advantages in the analysis of genetic toxicology (29). The random amplified polymorphic DNA (RAPD) method; a PCR-based technique which is simple, fast and capable of detecting not only point mutations but also temporary alteration of DNA that may not finally manifest themselves as mutation in future and allow detection of low doses of pollutants. The purpose of genotoxicity testing is to determine if a substrate will influence genetic material or may cause cancer. Ames test, in vitro toxicology test, in vivo tests and Comet assays are one of the most common tests for genotoxicity. Many studies displayed that RAPD may potentially form the basis of novel biomarker assay to detect DNA damage and mutational events in cells of bacteria, plants, invertebrate and vertebrateanimals (27, 30). Although there are several studies on the genotoxic effects of heavy metals on various organisms, studies about lichens about genotoxicity have started in our labratory in recent years and few reports have been published (22-24, 31-35). Genotoxicity studies with lichen species demonstrated the possible ecotoxicological impacts of such contamination by providing valuable information on environmental pollution and improving the process of risk assessment through RAPD analyses (22-24, 31-35).

The cement industry produced cement dust which contains metals such as Cd, Cr, Cu, Ni and Pb (36). Although cement factories are generally established far from city centers, local areas are affected negatively. Schuhmacher et al. (36), demonstrated that cement dust and associated chemicals can spread over a large area through wind and rain, accumulate in lichens, plants, animals and soils, downwind from the cement plant (36). Different types of contamination originating from industrial and agricultural activities may have harmful impact on organisms (37).

The aim of the current study was to evaluate the in situ DNA integrity and protein profile in four lichen species (*Pseudevernia furfuracea, Lobaria pulmonaria, Cetraria islandica* and *Usnea longissima*) by using the molecular technique. For this purpose, four lichen species were collected from Giresun which were not exposed to any kind of contamination. Four lichen species exposed to pollutants in Aşkale cement factory affected heavily by industries. Biomarkers are used to evaluate the effects of exposure to chemical contaminants and detect responses to environmental stress in lichen species.

MATERIALS AND METHODS

Study area

The cement factory areais located in eastern Turkey (N 39° 55′ 31″, E 40° 40’ 12″). The area has a terrestial climate characterized by hot and dry summers and cold, snowy winters. The major type of plant cover is steppe. Forests are located in the higher parts of mountains in the north and northeast. Forests include *Pinus sylvestris, Picea orientalis, Fagus orientalis, Quercus petrea, Juniperus oxycedrus, Abies nordmanniana, Ulmus minor* and *Fraxinus excelsior* species and conifers, mostly at altitudes of 700-2500 m. The Aşkale cement plant is located 55 km west of Erzurum city. The cement plant has been operating in the area since 1974.

Lichen material

Pseudevernia furfuracea, Lobaria pulmonaria, Cetraria islandica and *Usnea longissima* lichen species were collected from three different locations

in Dereli-Giresun, Eastern Anatolia, Turkey, in July 2008. The samples collected from the Dereli-Giresun which were supposedly not exposed to any kind of contamination were used as control in experiments. Three different control samples for four different lichen species collected randomly from different substrates of their own were used in this study. The samples were exposed to air at different sites (Site 1, 2 and 3), according to the far from the cement factory by using bag technique for 4, 8 and 12 months in 2008. Lichen species were transplanted on trees placed 50 (site 1), 100 (site 2) or 200 m (site 3) downwind from the combustion unit of the plant, as three replicas. Lichens samples were collected after four, eight and twelve months of transplantion. Among three sites, Site 1 (50 m) is closer to the cement factory in Aşkale, Erzurum. On the other hand, Site 2 (100 m) and 3 (200 m) are close to the cement factory.

Total Soluble Protein Level

Four different lichen thallus were homogenized (1:1, w/v) with 0,2 M phosphate buffer (pH 7.0) with a cold mortar and pestle. The homogenate was centrifuged at 27.000 x *g* for 20 min. The supernatant was used for assays of total soluble protein content. The total soluble protein content of the lichen extracts was determined according to Bradford method (38), with bovine serum albumin (BSA) as a standard. Experiments were repeated three times.

Genomic DNA isolation and RAPD procedures

Genomic DNA extraction was performed according to the protocol defined by Aras and Cansaran (39). Concentration and purity of DNA were measured at 260 nm and by 260 nm/280 nm absorbance ratios with nanodrop (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific, Wilmington, USA).

Standard 10-base primers supplied by Operon Technologies Inc. (Alameda, CA, USA) were used to screen RAPD variation. Fourteen oligonucleotide primers [CGCCCGCAGT (B389), TTCGAGCCAG (OPC01), GTGAGGCGTC (OPC02), GGGGGTCTTT (OPC03), CCGCATCTAC (OPC04), TGTCTGGGTG (OPC10), CTGTTGCTAC (OPO03), CAGCACTGAC (OPO07), GGTGCACGTT (OPO19), CGGATCGACA (P437), CAGGCCCTTC (TubeA01), TGCCGAGCTG (TubeA02), AGTCAGCCAC (TubeA03), AGTCAGCCAC (TubeA03)] were screened and among them five primers [OPC01, OPC02, OPC03, OPC04, OPC10] were amplified clear and reproducible bands. PCR was performed in a reaction volume of 25 µl containing 200 ng genomic DNA, 2.5 µl 10 x reaction buffer, 2.5 mM MgCl2, 20 µM dNTPs, 0.2 µM of primer and 0.5 unit of Taq polymerase (Promega, Madison, USA) and ddH2O was added to the standard volume. The PCR programme consisted of the following steps: initial denaturation at 94°C for 30 sec, annealing at 36°C for 1 min at 35 cycles, extension at 72°C for 45 sec and a final extension at 72°C for 8 min. Amplified samples were loaded on %1.2 agarose gels (mixture of %50 agarose and %50 Nu Sieve GTG agarose, FMC Corporation, Wokingham, Berkshire, United Kingdom), and run at 100 V for 4 h. For detection of any other kinds of DNA contaminants, a negative control of PCR mix without any template DNA was also used. To test the reproducibility of the RAPD-PCR, experiments were repeated at least twice for each primer and faint bands were ignored and only reproducible bands obtained in repeated experiments were taken into account.

Statistical analyses

The SPSS (Statistical package software v.15.0 for Windows) was used to analyze the changes in total soluble protein content. Data were tested by analysis with variance analysis (ANOVA). Least significat difference test at 0.01 significance levels was performed. In the analysis of RAPD profiles; bands which appeared and disappeared in the control sample were considered as the criterion of the judgment (Table 1). Polymorphisms observed in RAPD profiles included disappearance of a control band and appearance of a new band (36) (Table 2). Genomic

Table 1. Changes of total bands in control, and of polymorphic bands and varied bands after 4, 8 and 12 months exposure in four lichen species, Aşkale, Erzurum, 2015

Pseudevernia furfuracea		Site 1 (50m)						Site 2 (100m)						Site 3 (200m)						
Primer	C TB	S1		S2		S3		S4		S5		S6		S7		S8		S9		
		a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	
OPC 01	12	3	1	3	0	0	1	2	0	3	1	2	1	1	1	0	2	1	1	A
OPC 02	17	2	2	1	1	2	1	3	1	1	3	2	0	2	0	3	0	2	0	
OPC 03	13	2	2	2	4	3	1	1	0	2	0	1	1	0	2	1	1	1	1	
OPC 04	22	4	1	5	2	2	2	4	1	0	1	1	2	1	0	2	0	1	1	
OPC 10	19	2	6	1	4	1	3	1	0	1	2	1	0	1	1	1	2	0	3	
	83	13	12	12	11	8	8	11	2	7	7	7	4	5	4	7	5	5	6	
	a+b	25		23		16		13		14		11		9		12		11		
Lobaria pulmonaria		Site 1 (50m)						Site 2 (100m)						Site 3 (200m)						
Primer	C TB	S1		S2		S3		S4		S5		S6		S7		S8		S9		
		a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	
OPC 01	11	2	0	3	1	0	0	3	0	2	1	1	0	0	0	0	1	0	1	B
OPC 02	18	1	2	0	2	2	1	1	0	4	0	0	0	0	1	2	2	1	1	
OPC 03	14	3	2	3	1	0	2	3	1	2	0	0	1	2	1	0	0	1	0	
OPC 04	15	5	2	4	2	3	2	4	1	0	4	1	4	1	2	3	1	2	1	
OPC 10	17	3	1	1	0	4	1	1	0	0	1	0	4	1	2	2	1	2	1	
	75	14	7	11	6	9	6	12	2	8	6	2	9	4	6	7	5	6	4	
	a+b	21		17		15		14		14		11		10		12		10		
Cetraliaislandica		Site 1 (50m)						Site 2 (100m)						Site 3 (200m)						
Primer	C TB	S1		S2		S3		S4		S5		S6		S7		S8		S9		
		a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	
OPC 01	10	0	2	1	1	1	0	0	0	0	2	0	2	2	0	0	0	0	0	C
OPC 02	16	1	2	2	1	2	1	1	1	0	2	3	1	0	2	0	1	1	0	
OPC 03	11	2	1	2	2	0	1	3	0	1	0	1	0	3	0	0	0	1	0	
OPC 04	17	4	3	2	2	4	1	3	3	2	0	0	4	2	0	3	2	2	1	
OPC 10	15	2	2	4	1	3	1	0	2	1	3	2	0	1	1	0	3	1	3	
	69	9	10	11	7	10	4	7	6	4	7	6	7	8	3	3	6	5	4	
	a+b	19		18		14		13		11		13		11		9		9		
Usnea longissima		Site 1 (50m)						Site 2 (100m)						Site 3 (200m)						
Primer	C TB	S1		S2		S3		S4		S5		S6		S7		S8		S9		
		a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	
OPC 01	12	1	0	0	2	1	0	0	1	0	2	1	1	1	2	1	0	0	0	D
OPC 02	13	0	3	1	2	0	3	1	0	0	1	0	2	0	1	0	1	0	3	
OPC 03	11	0	2	3	0	0	1	1	1	0	1	0	1	1	1	1	0	1	0	
OPC 04	15	3	3	1	4	3	1	1	4	2	3	2	2	0	2	1	1	1	0	
OPC 10	15	5	1	1	2	2	2	3	1	1	1	1	1	2	0	1	2	0	2	
	66	9	9	6	10	6	7	6	7	3	8	4	7	4	6	4	4	2	5	
	a+b	18		16		13		13		11		11		10		8		7		

S1, S4, S7: 4 month S2, S5, S8: 8 month, S3, S6, S9: 12 month

S: Sample, C: Control sample, a: Appearance of new bands, b: Disapperance of control bands, a+b: Indicates polymorphic bands, TB: Total bands.

template stability (%GTS) was calculated as where 'a' indicates the RAPD polymorphic profiles the total number of polymorphic bands obtained for the five primers) in each sample exposed to environmental pollution around the cement factory, Aşkale-Erzurum, and 'n' is the number of total bands in the control (40). Changes in the RAPD patterns were expressed as decreases in GTS which is related to the change to the number of RAPD profiles generated by the lichen samples exposed to the polluted areas, in

Table 2. The polymorphism ratios of the primers

Primers	TB	PB	Ratio (%)
Pseudevernia furfuracea			
OPC01	12	6	50.0
OPC02	17	9	52.9
OPC03	13	7	53.8
OPC04	22	18	81.8
OPC10	19	14	73.6
Lobaria pulmonaria			
OPC01	11	5	45.4
OPC02	18	13	72.2
OPC03	14	11	78.5
OPC04	15	13	86.6
OPC10	17	14	82.3
Cetraria islandica			
OPC01	10	7	70.0
OPC02	16	11	68.7
OPC03	11	8	72.7
OPC04	17	14	82.3
OPC10	15	11	73.3
Usnea longissima			
OPC01	12	7	58.3
OPC02	13	7	53.8
OPC03	11	8	72.7
OPC04	15	13	86.6
OPC10	15	12	80.0

Table 3. Changes of GTS for all primers in study

Sites	Samples	GTS ratio (%)			
		Pseudevernia furfuracea	*Lobaria pulmonaria*	*Cetraria islandica*	*Usnea longissima*
Site 1 (50m)	S1	69.87	72.00	72.46	72.72
	S2	72.28	77.33	73.91	75.75
	S3	80.72	80.00	79.71	80.30
Site 2 (100m)	S4	84.33	81.33	81.15	80.30
	S5	83.13	81.30	84.05	83.33
	S6	86.74	85.33	81.15	83.33
Site 3 (200m)	S7	89.15	86.66	84.05	84.84
	S8	85.55	84.00	86.95	87.87
	S9	86.74	86.60	86.95	89.39

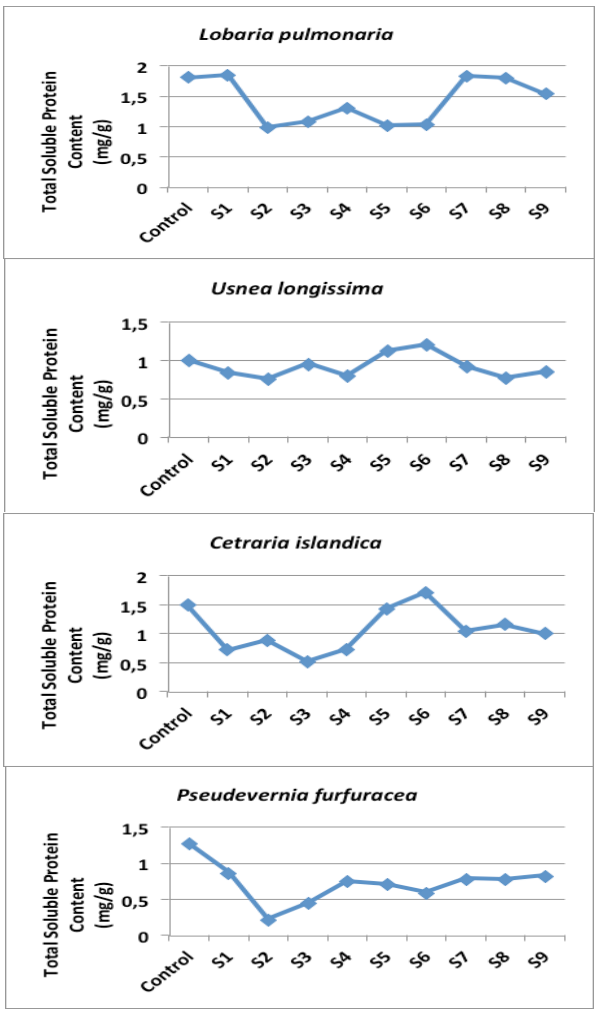
S1,S4, S7: 4 month, S2,S5, S8: 8 month, S3, S6, S9: 12 month

relation to profiles obtained from the control lichen samples (Table 3).

RESULTS

Effect of pollutants on total soluble protein level

The results showing the total soluble protein levels in four lichen species are presented in Fig. 1. Total soluble protein content profile of the control samples was similar to four examined lichen species. Total soluble protein content was significantly ($P<0.01$) changed with the exposure time to the pollutants ($P<0.01$) (4, 8 and 12 months). It was shown that protein content changed with four lichen species after 8 month of exposure. When 50 m far from cement factory in Aşkale, protein content significantly decreased. At the other sites far from cement factory (100 and 200 m) were observed little changes compared with control *L. pulmonaria* lichen species (Fig 1). Compared with the four lichen species and protein levels of pollutants exposure, *P. furfuracea* was determination to the maximum change of protein contents in all sites and exposure



S1: *Lobaria pulmonaria*, 4 month, 50 m
S2: *L. pulmonaria*, 8 month, 50 m
S3: *L. pulmonaria*, 12 month, 50 m
S4: *L. pulmonaria*, 4 month, 100 m
S5: *L. pulmonaria*, 8 month, 100 m
S6: *L. pulmonaria*, 12 month, 100 m
S7: *L. pulmonaria*, 4 month, 200 m

S1: *Usnea longissima*,4 month,50m
S2: *U. longissima*, 8 month, 50 m
S3: *U. longissima*, 12 month, 50 m
S4: *U. longissima*, 4 month, 100 m
S5: *U. longissima*, 8 month, 100 m
S6: *U. longissima*, 12 month, 100 m
S7: *U. longissima*, 4 month, 200 m

S1: *Cetraria islandica*, 4 month, 50 m
S2: *C. islandica*, 8 month, 50 m
S3: *C. islandica*, 12 month, 50 m
S4: *C. islandica*, 4 month, 100 m
S5: *C. islandica*, 8 month, 100 m
S6: *C. islandica*, 12 month, 100 m
S7: *C. islandica*, 4 month, 200 m

S1: *Pseudevernia furfuracea*, 4 month, 50 m
S2: *P. furfuracea*, 8 month, 50 m
S3: *P. furfuracea*, 12 month, 50 m
S4: *P. furfuracea*, 4 month, 100 m
S5: *P. furfuracea*, 8 month, 100 m
S6: *P. furfuracea*, 12 month, 100 m
S7: *P. furfuracea*, 4 month, 200 m

Figure 1. *Lobaria pulmonaria, Usnea longissima, Cetraria islandica, Pseudevernia furfuracea* species of Total Soluble Protein Content (mg/g)

times.

The RAPD-PCR profiles of the control and exposed samples in cement plant (Aşkale-Erzurum)

In the current study the genotoxic effects of various environmental pollutants were studied with the *Pseudevernia furfuracea, Lobaria pulmonaria, Cetralia islandica* and *Usnea longissima* that were exposed to various pollutants around the cement factory in Aşkale-Erzurum. Control sites are located far away from the allocation units. Table 1 represents the summation of all polymorphic bands in RAPD profile and Figure 2 presents all RAPD bands of selected primer. The DNA concentrations measured for the samples were in the range of 887 ng/µl to 4149 ng/µl, and the 260 nm/280 nm ratios ranged from 1.81 to 1.99. The total number of bands obtained in control samples by RAPD analyses were 83, 75, 69 and 66 for four lichen species (*P. furfuracea, L. pulmonaria, C. islandica* and *U. longissima*) used in the study, respectively. According to the results, each primer generated 10-22 bands with an average of 16.6, 15.0, 13.8 and 13.2 bands per primer for four lichen species, respectively..

RAPD profiles of the control and exposed samples around the cement factory showed significant differences (Table 1). In this regard, the main observation or changes in the RAPD patterns included appearance of new bands and/or disappearance of normal bands compared with the control samples. Furthermore, all primers resulted in alteration of a few amplification products gave complicated patterns of gains or losses. Although *P. furfuracea* species showed the highest levels of disappearence of new bands in the polluted samples, *U. longissima* displayed the lowest levels of band changes. Total number of bands were more in *P. furfuracea* species (83 in control, site 1: 64 (a+b); site 2:38 (a+b); site 3: 32 (a+b) as compared to other lichen species. The lowest number of bands that appeared was observed in the *U. longissima* samples

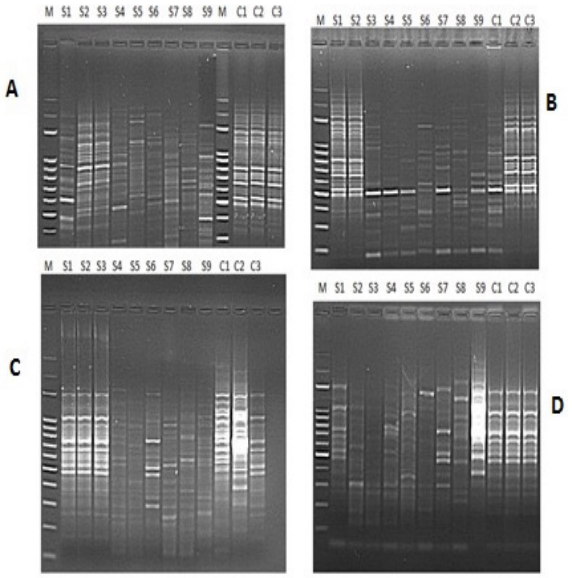


Figure 2. RADP profiles generated by OPCO2 primer from Peltigera praetextat and Usnea longissima exposed to polluted areas in Aşkale-Erzurum.
Lane M, Moleculer weight marker (100 bp. ladder)
M: Marker, N: Negative Control, C1-C2-C3: Control, S1-S4-S7-S10: 4 months, S2-S5-S8-S11: 8 months, S3-S6-S9-S12: 12 months, Site 1: 10 m, Site 2: 50 m, Site 3: 100 m, Site 4: 200 m.

(66 in control, site 1: 47 (a+b); site 2:35 (a+b); site 3: 25 (a+b) (Table 1). After four month of exposure, 13, 14, 9 and 9 extra bands appeared at site 1 (50 m far from the cement factory) in *P. furfuracea, L. pulmonaria, C. islandica* and *U. longissima*, respectively. Compared with the examined four lichen species, totally 19, 45 and 51 unexposed RAPD bands were appeared in P. furfuracea from sites 1, 2 and 3 (50, 100 and 200 m far from cement plant, respectively) after exposed to pollutants in cement plant in Aşkale-Erzurum (Table 1). Totally 19, 31 and 41 normal RAPD bands appeared in control sample of *U. longissima* from the sites 1 (50, 100 and 200 m far from cement plant) (Table 1). In addition to this, 31, 13 and 15 new bands disappeared in the *P. furfuracea* samples from sites 1, 2 and 3 of exposed pollutants. The polymorphisms were occurred as disappearance or appearance of the

bands in exposed samples compared to the control. The polymorphism ratios of the primers were given in Table 2. The polymorphic bands obtained by RAPD primers showed variability for each site for four lichen species. The highest polymorphism value obtained is P% = 86.6% in *U. longissima* and *L. pulmonaria* by the primer OPC04, and the lowest polymorphism value observed is P% = 45.4% in *L. pulmonaria* by the primer OPC01. The genomic template stability (GTS) which is a quantitative measurement reflects changes in RAPD patterns was calculated for each five primers and presented at Table 3. It was observed that average GTS values were decreased obviously with an nearest a site to cement plant. An obvious decrease in GTS values was observed as the sample collection site gets closer to the cement plant. The results were interpreted for genotoxic effect, considering the sampling points where the lichens were exposed to pollutants aroundthe cement factory in Aşkale-Erzurum.

DISCUSSION

With fast economic development and industrialization, a vast range of genotoxic chemicals were produced, and spread to the environment. These chemicals adversely affect living organisms, and often lead to serious diseases in human beings. Atmospheric pollution is composed of mixed pollutants and the inherent complexity of the composition and subsequent reaction products make it very difficult to estimate the ambient genotoxicity risk of air by traditional pollution measurements. The best way to determine environmental genotoxicity could be the direct quantification of the genotoxic effect (i.e., DNA damage) (38) of the pollutant to a living organism. Due to highly conserved structure of the genetic material, it is possible to use a broad variety of species including bacteria, yeasts, lichens, animals and plants in genotoxicity tests (30, 31, 41). In this study, we suggest that molecular and biological assays examined in *Pseudevernia furfuracea, Lobaria pulmonaria, Cetraria islandica* and *Usnea longissima* species could be used together as reliable and powerful biomarkers to determine genotoxic effects of pollutants in ecotoxicology.

Developing an understanding of the mechanisms of heavy metal tolerance in organisms at a biochemical and molecular level is the focus of today's ongoing research efforts. Toxicant induced population genetic effects may arise from the direct action of the toxicant at the DNA level (mutagenic effects) (35) or may indirectly result from population mediated process that are related to the toxicant exposure (36). Initially protein markers (i.e. allozymes) were used to infer the population genetic effects of toxicant exposure (38), but currently a wide variety of DNA markers/techniques are available. These techniques can be applied to infer all routes through which toxicants may affect the genetic structure of exposed organisms. After proper optimisation condition, the RAPD is a reliable, sensitive and reproducible assay, and therefore can be applied to genotoxicity studies. Toxic chemicals induce several cellular stress responses and damage different cellular components such as membranes, proteins and DNA (26, 42, 43). Mohd-Anwar et al. (2012) reported that, when 14 days old rice seedlings were treated with different As (III) concentrations for different time periods, protein content was significantly decreased at a higher concentration (300 µM) and duration (96 h), however at a lower concentration (50 µM) less changes were observed (44). Similar effect on protein content was observed in the current study. In this studies revealed that total soluble protein content considerably changes to pollutants exposure in four lichen species in cement factory after the different time arrivals.

In recent years, lichens have begun to be used as good bioindicators of genetic toxicity of environmental pollutants (22-24, 31-35). Genotoxicity as a result of metal toxicity is also described to play a major role in DNA-damage induction (33, 45). In this study, probable DNA damages induced by various environmental pollutants, were reflected by changes in RAPD profiles:

disappearance of normal RAPD bands and appearance of new PCR products occurred in the profiles. In the study three controls collected randomly from different substrates of their own was used. In all experiments three control species revealed the same band pattern. The profiles of informative primer OPC04 in examined four lichen speciesare shown in Fig. 2. The highest number of band changes (25) was detected in *P. furfuracea* lichen species from site 1 (far from 50 m in cement factory) after four months exposure of pollutants.

The objectives of this study were to determine the impact of distance from the combustor of the cement plant (predominant wind direction) and the effect of duration of exposure on bioaccumulation in the four lichens species. Previous studies indicated that nickel, Cd, Cr, Cu and Pb accumulated in lichen thalli with the greatest accumulation within 50 m far from the plant and exposure time, while the concentrations of Al was not consistently impacted by distance from the plant and duration of exposure (21). When four lichen species were compared according to their heavy metal accumulation, *P. furfuracea* was found to be the highest accumulator of pollution sources (21). *P. furfuracea* was observed as the most effective indicator of cement dust pollution. As the results of the current studies revealed the highest level of band variation, in other words genotoxic effect, *P. furfuracea* might be considered as a good candidate for genotoxicity indicator. In this study and previous study were parallel results of *P. furfuracea* lichen species which have the most heavy metal capacity and reflects of genotoxic effect. Our results show that a heterogenous mixture of pollutants might have contributed to the changes in the DNA-band patterns revealed by RAPD analysis, reflecting the induction of DNA damage in *P. furfuracea*.

The appearance of a new DNA band could occur because some oligonucleotide priming sites could become accessible to oligonucleotide primers after structural change or because some changes in DNA sequence have occurred due to mutations, large deletions, and/or homologous recombination (46). Appearance of new bands may also be the result of genomic template stability related to the level of DNA damage, the efficiency of DNA repair and replication (47). The results indicate that GTS level in *P. furfuracea* was the most affected lichen species by the pollution around cement factory (Aşkale, Erzurum).

In a previous study conducted in parallel with the same samples, some of the heavy metal concentrations were determined and the lowest levels of heavy metals were found in *U. longissima* (21). RAPD patterns generated for four different lichen species from polluted sites are clearly different from the control group and exhibit a distinct change with increasing concentrations of pollutants. Our results indicate that site 1 which is the nearest to the cement factory has high pollutants levels (21) and therefore may lead to a high level of genotoxic effect in the four lichen species as the samples from this location revealed the lowest GTS. The highest GTS values which may be considered as lowest genotoxic effect were detected in the samples from site 3 which is distinguished from other sampling locations by pollutants in cement factory.

However, to our knowledge, little information is available on lichens about their potential genotoxicity indicator capacity against pollutants. DNA alterations in the exposed *P. furfuracea* samples and *Evernia prunastri* samples were aimed to be described by RAPD analysis, in order to reveal the pattern of genetic variation influenced by the various environmental pollutants (23, 32, 34). Thus, the findings in the current study confirmed the idea that environmental pollutants, mainly heavy metals cause DNA damages in organisms and demonstrate the potential of RAPD analyses to monitor the level of genotoxicity in lichens. According to a previous study by Cansaran-Duman et al. (2011), the highest number of band changes in *E. prunastri* were found at (sites 8 and 10) close to iron steel factory in Karabük (34). In this study, *P. furfuracea* appears to

reveal higher genotoxic effects than *L. pulmonaria, C. islandica* and *U. longissima* the samples from around cement factory, Aşkale-Erzurum. Heavy metals are a major component of air pollution and many studies have shown that concentrations of absorbed heavy metal elements in lichen samples rise as they get closer to polluted sites like busy motorways and steel mills and also depending on the exposure time. In this study, at site 1, which is the nearest to the cement factory, genotoxicity ratios in the samples were much higher than the values obtained for other sites. In this study, lichen samples exposed close to a pollution source were compared in order to provide genotoxicity information of mixed pollutants found in the air. High number of polymorphic bands was observed in the *P. furfuracea* samples taken from areas close to the cement factory (site 1).

The present study shows the suitability of the lichen samples for the detection of genotoxicity and also provides information about the level of potential genotoxic agents around a cement factory. To our knowledge, there is no single study as yet on genotoxicity assessments in lichen species in cement factory. Our findings confirm that lichen species *P. furfuracea, L. pulmonaria, Cetraria islandica and U. longissima* can be used to monitor genotoxicity. Particularly P. furfuracea is revealead as a good indicator of genotoxicity in this respect. As lichens grow very slowly and are rarely encountered in polluted areas, lichen transplantation seems to be a promising method for monitoring pollutants effects and genotoxicity testing. Biomarkers can provide valuable information on exposure of pollutants and be used to measure a wide range of risk assessment of pollutants on organisms at the molecular level. This study reveal to allow visual integration of a set of early warning responses measured with biomarkers and lichen species provide valuable data for taking preventive measures.

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