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Original Research

Soil Pollution Screening Using Physico-Chemical and Cytogenetic Approaches: a Case Study of a Bulgarian Suburban Nature Park

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Abstract

Increasing attention has been paid recently to soil pollution. The aim of this study was to evaluate the ecological risk of soils collected from Nature Park Shumen Plateau (Bulgaria) (NP) using physico-chemical and cytogenetic approaches (pH analysis, analysis of the content of heavy metals included in Bulgarian soil pollution standards, and *Allium cepa*-test). Soil samples from NP were collected from seven sites. A sample from a heavily anthropogenic-influenced urban site was also collected. As signs for environmental risk used pH values, we found total concentration of heavy metals and mitotic abnormalities in *Allium cepa* root meristems included in Bulgarian legislation. Only two samples from NP were found to be slightly acidic. The other samples have slightly alkaline pH. The highest pH value was obtained in the urban sample. Heavy metal concentrations did not exceeded national standards for soil pollution. Several NP samples and urban samples exerted cytotoxic and genotoxic effects in *Allium* root meristems. Our data on soil pH and heavy metal concentrations did not indicate potential ecological risk, but cytogenetic endpoints showed the presence of harmful compounds in studied areas. In conclusion, the ecological risk for the investigated suburban area may be assessed as moderate.

Keywords: suburban recreational area, soil pollution, *Allium cepa*-test, pH, heavy metals

Introduction

Environmental problems resulting from anthropogenic activities have become matters of great concern today. Industrialization and urbanization have both economic and environmental effects. They cause environmental pollution and alter biogeochemical cycles. Causes and effects of air and water pollution are widely studied. On the other hand, until recently soil pollution has remained in the background [1]. The contaminants in soil could influence the ecosystem and humans directly, contaminating ground water and the food chain [2]. In the last few years increasing attention has been paid to contaminants of urban soils [3-7]. Soils in towns and their vicinity are scarcely used for food production, but people have direct contact – especially using recreational areas [8]. The present study focused on a suburban recreational area in Bulgaria: Shumen Plateau Nature Park, which was declared a protected area in 1980. According to Nelson and Chomitz [9], protected areas are "a clearly defined geographical space, recognized, dedicated and managed,

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through legal or other effective means, to achieve the long-term conservation of nature with associated ecosystem services and cultural values." NP is a heavily visited place used for outdoor sport and recreation on its hiking trails, bicycle routes, sites for rock climbing and paragliding, hiking shelters, and plenty of places to picnic.

Studies on soil pollution are very important for providing information about the intensity of anthropogenic activity. These data could contribute to a municipal policy on environmental management [10]. A "site by site" approach is recommended in such studies, since every site has unique characteristics [1]. In the case of NP, on its periphery are located the town of Shumen and several villages. Shumen is an important transport hub with well-developed light industry. Djingova et al. [11-12], using *Populus nigra* 'Italica' as a biomonitor, revealed an absence of detectable acute pollution in the region of Shumen. Two decades later, investigations of elemental composition of some medicinal plants of NP have shown the selective accumulation of some heavy metals and rare earth elements [13-15]. In addition to ecological hazards, these observations also revealed health risks, since herbs can transfer contaminants from the environment to humans through the food chain [16]. NP is a source of more than 300 species of medicinal plants [17], some of which are collected by visitors for personal use.

Soil is a complex mixture of inorganic and organic compounds. There is no simple conventional method for evaluating soils for their potential negative health effects [18-19]. Physico-chemical and biological approaches are complementary for assessing environmental risk [20]. Determining pH and elemental composition are common ways to test soil quality. Soil pollution is mainly associated with heavy metals concentrations. A lot of studies have been provided worldwide in order to measure and compare elemental content to acceptable standards [21-22]. Among the soil contaminants, special attention should be paid to soil mutagens due to their potentially harmful effects on living organisms [23]. Higher plant bioassays are excellent genetic models for testing the toxicity of various environmental contaminants [24-25]. These tests are simple compared to animal bioassays [23, 26], and can serve as the first alert for the presence of environmental hazards [27]. Among them, the *Allium cepa*-test has been widely used as a bioindicator of environmental pollution, showing high sensitivity and good correlation with other test systems [28-30]. Changes in mitotic activity and distribution of different stages of mitotic division are accepted as indicators of cytotoxic influence [31]. The presence of mitotic abnormalities and the induction of micronuclei in interphase cells indicate the genotoxic effect [32].

The aim of this study was to evaluate the ecological risk of soils collected from the Shumen Plateau Nature Park using physico-chemical and cytogenetic approaches (pH analysis, analysis of the content of heavy metals included in Bulgarian soil pollution standards, and the *Allium cepa*-test).

Materials and Methods

Studied Areas and Soil Sampling

The study area in the present investigation is Shumen Plateau Nature Park (NP) in northeastern Bulgaria. Soil samples from NP (SNP) were collected from seven sites. A sample of soil from an urban site (SU) was collected from a grassy area along the Simeon Veliki Avenue in Shumen city.

Soil samples were collected during August 2015 under dry weather conditions. Sampling was provided by digging for soil to a depth of 0-10 cm (soil horizon A, in accordance with [33]). For each site, 10 topsoil sub-samples were taken and bulked in order to give a composite sample. The samples were air-dried at room temperature in a laboratory to constant weight, pulverized, and then sieved to < 1 mm particle size.

Some features of sampling sites are listed below (location; soil horizon A: soil type according to [34]; human impact):

– SNP-1: Acacia forest (Acacia); N43°16'24.12" E26°51'28.94", 481 m a.s.l.; leached chernozems; there are no roads, paths, or human activity in the location.
– SNP-2: Oriental Hornbeam forest (*Carpinus orientalis*); N43°14'23.36" E26°52'41.61", 436 m a.s.l.; grey forest soils; there are no roads, paths, or human activity in the location.
– SNP-3: Bukaka Forest (Strict Nature Reserve) with dominant plant species *Fagus sylvatica* ssp. *moesiaca;* N43°15'26.53" E26°51'54.56", 506 m a.s.l.; lithosols; there are no roads, paths, or human activity in the location.
– SNP-4: mixed deciduous forests; N43°15'42.03" E26°52'42.45", 488 m a.s.l.; lithosols; a third-class road passes nearby.
– SNP-5: Open meadow near the Monument to 1300 Years of Bulgaria; N43°15'37.35" E26°55'21.14", 440 m a.s.l.; carbonate chernozem; the monument is a highlight for many visitors and tourists and can be reached by a processional concrete stairway from Shumen or by road; a large parking lot, restaurant, playgrounds, and shelters with fireplaces are nearby.
– SNP-6: Hornbeam forest (Carpinus); N43°14'51.94" E26°54'14.33", 475 m a.s.l.; grey forest soils; there is a big shelter with fireplaces, which is a starting point for many trails and is heavily visited; a road between the Monument to 1300 Years of Bulgaria and Shumen Fortress passes nearby.
– SNP-7: Open meadow near Shumen Fortress; N43°15'45.41" E26°53'17.24", 442 m a.s.l.; rendzina; the many shelters with fireplaces here are used by various public events.
– SU: Grassy area along the Simeon Veliki Avenue in Shumen city; N43°16'29.77" E26°56'6.01", 233 m a.s.l.; chernozem; a heavily used road in an urbanized space with railway traffic lies nearby.

Physico-Chemical Analyses

pH Analysis

Soil samples were suspended in deionized water at a ratio of 1:2 (w/v). pH of filtrate was measured using a Hanna handheld HI-8314 meter system with a gel-filled Hanna HI-1217D pH electrode (Hanna Instruments, Bedfordshire, U.K.).

Total Elemental Analysis

The content of heavy metals included in Bulgarian soil pollution standards [33] was determined. Direct quantification of total element concentration in soils using energy dispersive x-ray fluorescence (EDXRF) was performed at the Institute for Nuclear Research and Nuclear Energy, Bulgarian Academy of Sciences. We used a spectrometric system with Ketek, PGT, and DSP detectors, and software for PFA. Excitation sources were Fe^{55}, Pu^{238}, and Am^{241}. Samples were triplicate excited for 2,000 s, 2,500 s and 3,000 s.

Cytogenetic Analysis

Allium cepa L.-Test.

The onion bulbs were purchased from a biofarm certified to the BCS Öko Garantie, GLOBAL G.A.P, and IFS Food. The outer scales of the bulbs and the old dry roots were carefully removed without destroying the root primordia. The bulbs were kept for root germination in deionized water for 24 h. Bulbs with new roots and length of 1.5 cm were placed on soil:water suspension (25:1) and allowed to root for 24 h at 25 ±1ºC. Deionized water was used as a negative control and methyl methanesulfonate (11 mg/l, for 24 h) was used as a positive control. Then the root tips were washed thoroughly with distilled water, fixed in a Clarke's fixative (95% ethanol:acetic acid glacial, 3:1) for 90 minutes and hydrolyzed in 1N HCl for 8 min and in 45% acetic acid for 60 min at room temperature. Then they were stained for 90 min in 1% aceto-orcein and the terminal root tips (1-2 mm) were excised and squashed in 45% CH_3COOH. Each soil sample and the control group consisted of nine meristems from three bulbs. At least 1,000 cells of each root meristem were analyzed. The following cytogenetic endpoints were scored: mitotic index, index of each phase of mitotic division, mitotic cells with chromosome bridges, fragments, laggard chromosomes, multipolar anaphase/telophase, diagonal spindle, C-mitosis, and interphase cells with micronuclei or two nuclei.

Statistical Analyses

Mean data of pH in soil samples were calculated. Results from elemental analysis were expressed as mean value. Student's t-test was used to assess the significant differences between heavy metals in NP soil samples and in urban soil sample. Pearson Correlation test was used in order to estimate the relationships between soil pH and the content of selected chemical elements. This was provided only in the case of the presence of particular elements in all sites. Depending on the values of the Pearson's coefficient (r), the following types of correlation were differentiated: $0<r<0.3$ weak correlation, $0.3< r<0.5$ moderate correlation, $0.5<r<0.7$ significant correlation, $0.7< r <0.9$ strong correlation, $0.9< r <1.0$ very strong correlation (significance level at $P\leq0.05$). Results from *Allium*-test were expressed as the mean ±standard deviation (SD). Student's t-test was performed with $P\leq0.05$ taken as a significance level.

Results

Physico-Chemical Characteristics of Soil Samples

Studies on soils usually involve pH measurements, since pH is a very important soil property [7]. Data on pH values of soil samples tested are presented in Table 1. Only two samples from the Nature Park (SNP-2 and SNP-3) were found to be slightly acidic. The other samples (SNP-1, SNP-4, SNP-5, SNP-6 and SNP-7) have slightly alkaline pH. The highest pH value (7.90) was obtained in an urban soil sample (SU).

Table 1 shows concentrations of heavy metals in the soil samples, as well as the maximum permissible

Table 1. Total heavy metal concentrations in soil samples and relationships between soil pH and elements content.

		Elements (mg/kg)			
	pH	Pb	Zn	Cu	Cd
SNP-1	7.38	44	57*	nd	nd
SNP-2	6.52	47	83*	nd	3
SNP-3	5.89	52*	68	nd	nd
SNP-4	7.20	23	93	nd	3
SNP-5	7.82	21	113*	nd	nd
SNP-6	7.64	44	78	20	2
SNP-7	7.49	42	92	28	2
SU	7.90	26	108	24	3
MCL		200	400	300	8
r		-0.66 (NS)	0.55 (NS)	-	-

SNP – water suspension of soil from *Nature Park Shumen Plateau;* SU – water suspension of soil from an urban site (Simeon Veliki Avenue in Shumen); nd – not-detectable; MCL – maximum concentration limits for living places, parks, playgrounds (Regulation No. 3/01.08.2008); r – Pearson's contingency coefficient (relationships between soil pH and elements); *– significant differences between heavy metals in SNP and SU; P ≤ 0.05; NS – not significant

Table 2. Mitotic index and phase indices in root meristematic cells of *Allium cepa* L. exposed to soil-water suspensions for 24 h.

	Mitotic index		Prophase index		Metaphase index		Anaphase index		Telophase index	
Sample	%	SD	%	SD	%	SD	%	SD	%	SD
NC	5.51	±0.76	34.31	±14.48	20.86	±6.42	11.89	±8.15	32.94	±9.65
SNP-1	5.33	±1.79	40.45	±17.00	29.77	±12.19	9.86	±6.68	19.92*	±5.88
SNP-2	4.26*	±1.05	42.93	±11.66	23.39	±7.72	16.20	±6.59	17.48*	±9.90
SNP-3	3.85*	±1.22	39.20	±9.37	27.84*	±7.96	18.18	±6.38	14.77*	±4.21
SNP-4	1.74*	±0.72	49.37*	±13.69	25.32	±12.85	10.76	±6.76	14.56*	±8.24
SNP-5	4.74	±1.61	38.76	±8.10	25.00	±8.61	14.91	±4.85	21.33*	±9.29
SNP-6	1.58*	±0.71	50.34	±20.04	20.69	±12.41	15.17	±9.90	13.79*	±13.46
SNP-7	5.29	±0.85	36.10	±7.37	26.97*	±6.61	14.73	±3.30	22.20*	±4.53
SU	4.08*	±0.78	33.78	±9.98	25.20	±7.28	21.98*	±6.22	19.03*	±6.21
PC	3.43*	±1.25	53.99*	±13.12	20.77	±8.11	7.67	±4.42	17.57*	±5.22

NC: negative control (deionized water); SNP – water suspension of soil from *Nature Park Shumen Plateau;* SU – water suspension of soil from an urban site (Simeon Veliki Avenue in Shumen); PC: positive control (methyl methanesulfonate, 11 mg/l); n – number. Data are means ± SD (standard deviation), *P≤0.05.

concentrations according to Bulgarian legislation [33]. Four heavy metals included in the above-mentioned document (Cu, Zn, Cd, and Pb) were detected using the EDXRF technique. Among them, Zn and Pb were relatively abundant in all areas we investigated. Cu and Cd were found in several sites. All of the measured values were below the regulatory limits (see Table 1).

Relationships between soil pH and elemental content have often been reported in similar studies [35-38]. Taking this into account, correlation coefficients between soil pH and content of heavy metals were calculated (Table 1). A positive correlation with Zn implies that the concentration of this element becomes higher with the increase in soil pH. The negative correlation between soil pH and Pb implies that lead concentration is higher at a lower soil pH.

Allium cepa-Test

We used the *Allium cepa*-test to detect cytotoxicity and genotoxicity of soil samples. Effects of soil suspensions on mitotic index and mitotic phase distribution in root meristematic cells are summarized in Table 2. Mitotic activity was extremely decreased in comparison with the

Table 3. Mitotic abnormalities in root tips of *Allium cepa* L. exposed to soil-water suspensions for 24 h.

Sample	Dividing cells (n)	B (%)	L (%)	F (%)	MP (%)	CM (%)	DS (%)	Total abnormalities	
								%	SD
NC	513	0.58	2.73	0.00	0.00	0.00	2.73	6.04	±4.71
SNP-1	487	1.44	0.21	0.00	0.00	0.21	9.45	11.29*	±5.94
SNP-2	389	0.00	1.02	0.00	0.00	0.00	6.17	7.20	±4.05
SNP-3	352	0.28	2.84	0.00	0.00	0.00	10.80	13.92*	±10.30
SNP-4	158	0.00	1.27	0.00	0.00	1.27	9.49	12.03	±10.49
SNP-5	436	1.15	2.98	0.00	0.23	0.23	12.16	16.74*	±5.06
SNP-6	145	1.38	5.52	0.00	0.00	1.38	17.24	25.52*	±22.00
SNP-7	482	0.83	2.70	0.00	0.00	0.21	13.49	17.22*	±6.43
SU	373	1.61	5.36	0.27	0.00	0.80	14.48	22.52*	±11.24
PC	313	0.00	3.84	0.32	0.00	0.96	5.75	10.86	±7.47

NC: negative control (deionized water); SNP – water suspension of soil from *Nature Park Shumen Plateau;* SU – water suspension of soil from an urban site (Simeon Veliki Avenue in Shumen); PC: positive control (methyl methanesulfonate, 11 mg/l); n – number; B – bridges; L – laggard chromosomes; F – fragments; MP – multipolar anaphase/telophase; CM – C-mitosis; DS – diagonal spindle. Data are means ± SD (standard deviation), *P≤0.05.

Table 4. Interphase cells with micronuclei and two nuclei in root tips of *Allium cepa* L. exposed to soil water suspensions for 24 h.

Sample	Interphase cells (n)	Micronuclei			Two nuclei		
		n	%	SD	n	%	SD
NC	8,792	1	0.01	±0.04	22	0.25	±0.15
SNP-1	8,645	2	0.02	±0.05	36	0.42	±0.20
SNP-2	8,740	4	0.05	±0.09	34	0.39	±0.28
SNP-3	8,794	2	0.02	±0.04	32	0.36	±0.39
SNP-4	8,941	2	0.02	±0.07	12	0.13	±0.14
SNP-5	8,759	3	0.03	±0.07	52	0.59	±0.64
SNP-6	9,048	0	0.00	±0.00	13	0.14	±0.21
SNP-7	8,624	0	0.00	±0.00	35	0.41	±0.56
SU	8,759	0	0.00	±0.00	9	0.10	±0.24
PC	8,821	16	0.18*	±0.20	19	0.22	±0.20

NC: negative control (deionized water); SNP – water suspension of soil from *Nature Park Shumen Plateau;* SU – water suspension of soil from an urban site (Simeon Veliki Avenue in Shumen); PC: positive control (methyl methanesulfonate, 11 mg/l); n – number. Data are means ± SD (standard deviation), *P≤0.05.

negative control after treatment with two soil samples from NP (SNP-4 and SNP-6). Significant negative influence exerted two other samples from NP (SNP-2 and SNP-3) and an urban sample. The treatment also changed the mitotic phase distribution. The characteristic effect caused by all soil samples was a decrease of telophase index. Notable accumulation of prophase cells was observed after treatment with SNP-4 and SNP-6.

The treatment increased by ~4-fold the percent of chromosome aberrations in urban samples in comparison to negative control (Table 3). A similar effect caused one sample from NP (SNP-6). The number of abnormal mitoses detected was ~3-fold higher in meristems treated with two other samples from NP (SNP-5 and SNP-7). Aberrant cells frequency after treatment with other samples (SNP-1, SNP-3 and SNP-4) was raised ~2-fold. Diagonal metaphases and anaphases were detected in all samples. Their existence was notable in an urban sample and in three of the samples from NP (SNP-5, SNP-6 and SNP-7). Laggard chromosomes were also frequently observed abnormalities in the treated cells. Bridges in ana-telophase and C-mitoses were also scored. Fragments and multipolar anaphases were found only in two samples.

Additionally, we scored abnormalities in interphase cells (Table 4). The percentage of micronuclei in treated groups was not influenced as compared to those exposed to water only. On the other hand, five soil samples from NP (SNP-1, SNP-2, SNP-3, SNP-5 and SNP-7) induced an increase in the number of binucleated cells.

Discussion

We used pH values, heavy metal concentrations, and the cytotoxic and genotoxic potentials of the investigated soils as indicators for environmental risk. Different soil compounds of natural or anthropogenic origin were able to influence these parameters. In nature, soil pH is determined both by the type of predominant plants in the ecosystem and the parent material. Soil pH of native forests usually is acidic [25, 39]. In the plateau, soil samples SNP-1, SNP-2, SNP-3, SNP-4, and SNP-6 were collected from forest areas, but only two of them were found to be acidic. A possible explanation could be the type of the main foundation rock in the Shumen Plateau: limestone [40]. According to Chahal et al. [25], the limestone-derived soils in nature are usually alkaline (pH 7.5 to 8). So, the slightly alkaline pH detected could be explained by the parent material. A higher value of pH in SU sample in comparison with other samples could be due to proximity to the road. It is known that alkalinity of streetside soil is a consequence of the release of calcium from the weathering of cementing materials in a road surface [41-42].

As mentioned above, studies on soil contamination are mainly focused on heavy metals [43-44]. Some heavy metals (Cu and Zn) are required in minute quantities by organisms, but excessive amounts of them exert negative effects. Other heavy metals (Pb, Cd, Hg, and As) are harmful to both plants and animals [45]. Bulgarian legislation [33] focuses on the monitoring of the following heavy metals: Cr, Ni, Cu, Zn, As, Cd, Hg, and Pb. Relatively abundant amongst them in SNP are Zn and Pb. Zinc content in studied soils varied in the range 57-113 mg/kg (Table 1). Similar values were obtained by Klimek [46] in unpolluted soil: 141 mg/kg. The main source of Zn pollution is traffic emissions [47]. Lead poisoning, a global environmental hazard, also is associated mainly with roadside soils [48-49]. Interestingly, the levels of lead in an urban soil sample is about two-fold lower than NP`samples (Table 1). Similar low levels of Pb in roadside soils in an urban area (range 26-81 mg/kg) were reported in other studies [50]. It should be noted that Pb content is influenced by pH of the soil. Watmough et al. [51] reported that Pb concentrations in the forest floor increased with decreasing soil pH (range ~3-80 mg/kg). This is in accordance with our data – the pH value of urban soil sample was higher in comparison with NP`samples. On the basis of the distribution pattern of Cu and Cd in the tested soils we might suppose that their presence is not anthropogenic related, since they were found in the urban soil and in some sites on the plateau.

In the present study, soil pH and measured heavy metal concentrations didn't reveal the existence of ecological risk. On the other hand, cytogenetic endpoints indicated the presence of harmful compounds in studied areas. The decline of mitotic activity and changes in the proportion of mitotic phases after treatment (Table 2) indicate the occurrence of a cytotoxic effect [52]. The tested soil samples were more genotoxic as compared with negative control. The most genotoxic sample from NP was collected from SNP-6. Data in Table 3 indicated that SNP-6 possess a genotoxic potential comparable to urban soils. A possible explanation for the toxicity is that SNP-6 is located near the road and is extensively used for picnics as a site where people build fires for cooking. Other sites with detected high genotoxicity were the two most-visited locations: SNP-5 and SNP-7. Increased soil genotoxic potential could be related to the presence of a large monument. Samples from a preserve (SNP-3) and nearby (SNP-4) also were more genotoxic as compared with negative control, but the percentage of chromosome aberrations was about 1.7 times lower in comparison with urban soil. On the basis of the observed genotoxicity of the soils in NP we could conclude that there is a moderate ecological risk, since only one site was shown to be comparable with urban soil negative influence on genetic material.

Elevated cytotoxic and genotoxic levels in the soils from NP in comparison with negative control could be partly explained by the location of the park near the town of Shumen. The plateau is in close proximity to the town, where there are highways and a huge, heavily visited memorial. Additionally, a road connects Shumen Pplateau with the town and Shumen Fortress (a place of intense tourist activity). Public events, including rallies, are provided on the park [53]. So traffic is not excluded as a source of pollution.

There are also some agricultural areas in the park vicinity. As described by Firbas [54], contamination of relatively distant areas is possible due to pesticide spraying techniques. Moreover, some chemicals such as antimite insecticides are regularly used in the territory of the park. Various ingredients of pesticides can damage genetic material [55]. Another harmful impact associated with winds is the wide use of wood and coal heating during winter in the region, and the park includes picnic areas with fireplaces. The impact of cooking fires used in the operation of picnic grounds is not extensively studied, but fuel is also a source of pollution.

Due to the significance of the soil as part of the ecosystem and because of its impact on human health, it is necessary to find proper bioindicators and biomarkers for ecotoxicological analyses. Data presented in this paper suggest that the influence of soil samples on onion root growth is a complex phenomenon. We used mitotic index and chromosome aberrations as cytogenetic endpoints. Some samples influenced both of them, while others affected only mitotic activity or frequency of aberrant cells. It should be noted that our data on soil pH and heavy metal concentrations, included in Bulgarian legislation, did not indicate potential ecological risk, but cytogenetic endpoints showed the presence of harmful compounds in studied areas. So this study confirmed that the *Alium cepa*-test can be used for reliably evaluating the effects of potential cytotoxic and genotoxic compounds in complex mixtures like soils [28-30, 32]. Moreover, the *Allium*-test shows interactions between different compounds (additive, antagonistic, or synergistic) [54, 56].

Conclusions

The physico-chemical approaches used in the present study are not sufficient as tools for environmental screening: the limiting heavy metal concentrations did not exceed national standards for soil pollution, and the range of pH values found in soils studied was not extreme. However, bioassays could provide basic information about hazards of complex mixtures like soil. Established cytotoxicity and genotoxicity of NP soils revealed a moderate ecological risk. Further analyses of soils in NP should be provided, because positive results from the *Allium*-test serve as an alarm for ecological degradation of the area.

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