

SPECIES OF WILD FLORA AS INDICATORS OF ENVIRONMENTAL GENOTOXICITY

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ABSTRACT

The present study evaluated the applicability of wild species in investigating environmental genotoxicity. The level of mutation in cells of the root meristem was examined in 8 wild plant species growing in environments affected by different types of pollution. Seeds collected in the disposal areas of a wolfram-molybdenum factory (Kabardino-Balkaria, Russia) were used to study the influence of heavy metal pollution. In the case of pollution with products of oil combustion and refining, the seeds were collected in areas adjacent to mini-factories for domestic oil refining (Chechen Republic, Russia). Pollution induced a 2.5-to-10.2-fold increase in mutation frequency in plant seedlings from contaminated zones compared to plants from "clean" zones. Thus, species of wild flora have proven as convenient and sensitive objects for estimating environmental genotoxicity.

Key words: environmental genotoxicity, heavy metals, oil pollution, wild plant species

SPECIE DI FLORA SELVATICA QUALI INDICATORI DI GENOTOSSICITÀ AMBIENTALE

SINTESI

Il presente studio ha valutato l'applicabilità delle specie selvatiche nello studio della genotossicità ambientale. Il livello di mutazione nelle cellule del meristema radicale è stato esaminato in 8 specie di piante selvatiche che crescono in ambienti affetti da diversi tipi di inquinamento. I semi raccolti nelle aree di smaltimento di una fabbrica di wolfram-molibdeno (Kabardino-Balkaria, Russia) sono stati utilizzati per studiare l'influenza dell'inquinamento da metalli pesanti. Nel caso dell'inquinamento da prodotti di combustione e raffinazione di petrolio, i semi sono stati raccolti in aree adiacenti alle mini-fabbriche per la raffinazione del petrolio (Repubblica Cecena, Russia). L'inquinamento ha indotto un aumento da 2,5 a 10,2 volte della frequenza di mutazione nelle piantine delle zone contaminate rispetto alle piante da zone "pulite". Le specie di flora selvatica si sono pertanto dimostrate utili e sensibili per la stima della genotossicità ambientale.

Parole chiave: genotossicità ambientale, metalli pesanti, inquinamento da petrolio, specie di piante selvatiche

INTRODUCTION

Understanding the influence of increasing environmental pollution on living organisms has been one of the most important problems to date. The progress of humankind is inseparably linked to the development of industry, which, in turn, inevitably leads to the contamination of the atmosphere, hydrosphere, lithosphere (soil) and biosphere. Water, air and soil pollution adversely affect living organisms, including humans.

A contaminated environment has both toxic and genetic effects on living organisms. While the toxic influences, as a rule, can easily be assessed, the impact of pollution on the genetic structures of an organism, including possible remote consequences, is difficult to determine.

There are a number of effective methods currently developed to estimate the quality of the environment. The classic methods include sampling of water, air and soil probes, and their laboratory analysis using physical-chemical procedures. This approach, however, does not allow the estimation of the hazardous effect of the detected pollutant on living organisms. Test systems designed to assess the genotoxic effects of pollution, on the other hand, can indicate the possible mutagenic, carcinogenic, teratogenic and other harmful consequences. An impressive number of newly synthesized chemicals and environmental components (soil, air, water and sediments) containing various types of pollutants have already been examined with this method throughout the world (Chen & White, 2004; Ohe et al., 2004; White & Claxton 2004). However, even this approach cannot provide complete information about how a genotoxic effect detected with a test system can influence plants, animals or their associations. Currently, another approach is being developed to estimate the quality of the environment: the assessment of the genotoxic effect of pollution *in situ*, directly on plants and animals living in a determinate area (Vardar et al., 2014; Watanabe et al., 2014). In this case, the whole complex of hazardous compounds will be taken into consideration, including their synergism and antagonism.

Since the 1970s, higher plants have been widely used to screen mutagens and monitor genotoxins in the environment (de Serres, 1978; Grant & Salamone, 1994; Sandhu et al., 1994; Ma et al., 2005; Sposito et al., 2015; Amato-Lourenco et al., 2017; Sposito et al., 2017). Most experiments *in situ* were performed using standard test systems with *Tradescantia*, *Allium cepa* and *Vicia faba* (White & Claxton, 2004). Plant test systems have proven to be highly sensitive to heavy metals (Knasmuller et al., 1998; Minissi et al., 1998; Majer et al., 2002; Correia et al., 2014), products of oil-processing industry (Minissi et al., 1998; Moraes Leme & Aparecida Marin-Morales, 2008) and other organic substances (Herrero et al., 2012; Mesi & Kopliku, 2013; Goujon et al., 2015; Rodriguez et al., 2015), as well as nanoparticles (Ghosh et al., 2016; Panda et al., 2016).

Nevertheless, plant test systems do have their limitations (Lazutka et al., 2003), so it is preferable to use naturally growing plant species for genetic monitoring (Gers'kin et al., 2005; Vardar et al., 2014; Watanabe et al., 2014). The aim of this work was to study the suitability of wild flora species to assess the genotoxicity of an environment.

MATERIAL AND METHODS

Characterization of the studied area and sampling

We used species of wild flora to test both inorganic (heavy metals) and organic (products of oil burning and refining) pollutants. The seeds collected at the tailing dump of a wolfram-molybdenum factory (Kabardino-Balkaria, Russia) were used to study the influence of heavy metal pollution. The factory was engaged in wolfram-molybdenum field exploitation and ore concentration until 2000, when it was closed down. The tailing dump we studied had been put into operation in 1967 and was located at an altitude of 1000–1200 meters above sea level. Surrounding areas similar to the studied areas in geo-climatic conditions (slope exposure and plants) were defined as the "clean zone" (background landscape).

In the case of pollution by the products of oil burning and refining, the seeds were collected in the Chechen Republic, in the surrounding areas of villages in which mini-factories for domestic oil refining were located. These mini-factories are usually built in the marginal areas of settlements or in woodland belts; their number can vary.

Several villages in which primary oil refining was practised for over 13 years were chosen as investigation sites. These were Dolynsk (Groznensky District), Alkhan-Yurt (Urus-Martanovsky District), Mesker-Yurt and Tsotsan-Yurt (Shalinsky Shalinskyi), and Geldegen (Kurchaloevsky District). They were selected for analysis because they are located in the same natural-climatic region and characterized by similar landscape features and vegetation. These villages were defined as the contaminated area. The village of Goity (Urus-Martanovsky District), which is known to be free of this type of industry and belongs to the same natural-climatic region was chosen as the "relatively clean" zone (termed as "relatively" because the benz(a)pyrene content in the soil was 0.03 mg/kg, which exceeds the legal limit of 0.02 mg/kg). The number of mini-factories in the studied polluted settlements ranged from 0 (Goity) to 62 (Dolynsk).

The plant collection proceeded as follows: a square territory of $10,000 \pm 200 \text{ m}^2$ was marked off virtually and divided along the diagonal into areas of approximately 50 m^2 . In each area, we selected 10 plants characterised by approximately the same behaviour at the stages of flowering and seed ripening, and then collected their seeds. Altogether, at each investigation site we collected seeds from at least 30 specimens of the same plants spe-

cies, and mixed them. Where only a few specimens of a plant species were available, the seeds were collected from all plants growing in that territory.

Test organisms

Plants of broadly distributed species were preferentially used for the investigation of genotoxicity of the environment in the Chechen Republic. These were waybread (*Plantago major* L.), medicinal dandelion (*Taraxacum officinale* Wigg. s.l.), Russian dock (*Rumex confertus* Willd.), and mayweed (*Matricaria recutita* L.). When studying the genotoxic effect of the waste from the wolfram-molybdenum factory, we used plants that grew in the surrounding regions and had ripe seeds at the moment of analysis (July-August). From the plants growing in dump areas we chose five species, specifically, cheatgrass (*Anisantha tectorum*); junegrass (*Koeleria cristata*); black henbane (*Hyoscyamus niger*); *Jurinea ciscaucasica* and medicinal dandelion (*Taraxacum officinale* Wigg. s.l.) to study the level of mutations; these species belong to distinct genera.

Tests procedure

The anaphase-telophase method was used to assess the level of chromosomal aberrations in plant seedlings. This method is based on the registration of chromosomal aberration at the anaphase and telophase stages. Owing to dormancy, the seeds only germinated in 1-3 months (or more) after harvesting, depending on plant species. Dandelion and grass seeds can germinate earlier, probably due to a shorter dormant period. It was important for the seeds of the same botanical species collected in "clean" and "contaminated" zone to have the same time of storage.

The seeds were germinated in glass Petri dishes on filter paper soaked with tap water in a thermostatic chamber at +26 °C. Germination times varied from 4 to 10 days, depending on the plant species. When necessary, the filter paper was additionally moisturized. The 5-10 mm long roots were fixed in a mix of ethanol and glacial acetic acid (3:1) for at least 2-3 hours. The material fixed this way can be stored up to 1-2 months in a refrigerator at 4 °C. The fixed seedlings were stained in acetocarmine (a 2% solution of carmine in 45% acetic acid) during a 10-12 min water bath. Temporary squash slides were prepared from the root meristem according to the standard method (Dubinina, 1978). At least 1000 anaphases were scored. Chromosome fragments, single and double "bridges" were registered. The percentage of abnormal anaphases against the total number of scored anaphases was calculated.

Chemical analysis

Soil samples were taken during dry summer days on the terraces of the dump area or in territories located ap-

proximately 100±20 m away from the oil-refinery factories, when the seeds of wild plants were also collected. Portions of soil were removed by probe from the upper 0-20 cm horizon in five repeats, then 5 kg of soil was mixed to prepare the sample for analysis (1 kg). The soil samples were put in tightly sealed polyethylene containers and marked with a tag indicating the place and time of sampling. For the purposes of chemical analysis, the soil was dried to obtain air-dry mass.

The level of heavy metals in the soils examined was determined using atomic-adsorption spectrophotometry (AAS). Analysis of soil samples for the presence of oil products was carried out in collaboration with the Laboratory of the Soil Sciences Department, Lomonosov Moscow State University by method of infrared spectroscopy (Orlov & Grishina, 1981). The method of low-temperature fluorescence spectroscopy developed by E. V. Shpolsky was used for the assessment of benz(a)pyrene content in soil (Shpolsky et al., 1968).

Statistical processing

To determine the reliability of the observed differences we used Fisher's conversion for the match against shares, and two-factor dispersion analysis.

RESULTS

Tables 1 and 2 summarize the obtained data on heavy metal content in the soils of the studied areas. The content of metals in the tailing dump of the tungsten-molybdenum factory (Kabardino-Balkaria, Russia) was significantly higher (Tab. 1). In the Chechen Republic, where the main pollutants are oil products, the content of heavy metals in the soil in the "relatively clean" village of Goity was not significantly different from other studied villages, in some cases it was even higher (Tab. 2). As for benz(a)pyrene and other petroleum products, their contents in the contaminated zone were 60–80 times higher than those in the "relatively clean" zone of Goity (Tab. 3). In Table 3 and all subsequent tables, the

Tab. 1: Heavy metal concentrations in the soils of the disposal areas of a wolfram-molybdenum factory (Kabardino-Balkaria, Russia).

Tab. 1: Koncentracije težkih kovin v prsti na odlagališčih tovarne volframa in molibdena (Kabardino-Balkaria, Rusija).

Sampling area	Heavy metal content (mg/kg)					
	Mo	Pb	Cu	Zn	Bi	Sn
Tailing dump	>40	33	37	30-60	6.5	4.5
Clean zone	<2	8-13.5	9.5-13	10-15	0	0

Tab. 2: Heavy metal concentration (mg/kg) in the soils of the studied villages of the Chechen Republic.**Tab. 2: Koncentracije težkih kovin (mg/kg) v prsti preiskanih naselij v Čečenski republiki.**

No	Site of soil sampling	Pb	Cd	Cr	Co	Mn
1	Goity	38.21	0.68	56.23	15.41	40.66
2	Tsotsan-Yurt	32.64	1.89	20.14	17.54	18.26
3	Geldegen	59.85	1.35	85.60	18.64	114.68
4	Mesker-Yurt	34.33	1.62	20.28	19.06	15.96
5	Alkhan-Yurt	54.70	1.22	72.44	17.80	136.78
6	Dolynsk	61.22	1.35	95.32	19.48	170.02

villages of the Chechen Republic are listed in order of increasing benz(a)pyrene content.

The anaphase-telophase method was used to assess the level of chromosomal aberrations in root meristem cells of plant seedlings. Figures 1a and 1b show a normal anaphase and telophase, respectively. Abnormal anaphases display acentric fragments and "bridges." Fragments of variable sizes emerge as a result of deletions (Fig. 1c) and chromosome lagging (Fig. 1d) during their movement to the poles. The joining of two centromere-containing fragments leads to the formation of a dicentric chromosome, which is affected by two mitotic centres and, being stretched between two daughter groups of anaphase or telophase chromosomes, forms a "bridge." Depending on the type of chromosome damage, different types of "bridges" may occur. The re-joining of two broken sister chromatids leads to the formation of a chromatid (usually single) "bridge" (Fig.

1e), whereas lateral re-joining of two broken chromosomes leads to the formation of a chromosomal (usually double) "bridge" (Fig. 1f).

Table 4 shows the results of the estimation of the levels of chromosomal aberrations (CA) in plants collected in the disposal areas of the wolfram-molybdenum factory (Kabardino-Balkaria, Russia) contaminated with heavy metals. The frequency of CA in the root meristem of plants collected in disposal areas was statistically significantly higher than the frequency of CA in plants from the "clean zone." Fragments proved to be the prevailing type of chromosomal aberration in all plant species we studied, with "bridges" found in minor quantities.

Wild flora species were also used for genetic monitoring of oil products pollution. Table 5 shows the results of the estimation of mutation level in seedlings obtained from seeds collected in the vicinity of the Chechen villages with mini-factories for domestic oil refining.

The study also revealed significantly increased levels of mutation in plant species collected in contaminated zones compared to the "relatively clean" zone of Goity. Of all types of induced alterations, the studied species displayed the highest increase in the frequency of fragments. The highest level of chromosomal rearrangement was recorded in plants from the village of Dolynsk, which is situated in the most contaminated zone.

Dispersion analysis has shown that in the case of contamination with oil products, mutation frequency was more dependent on the level of soil pollution than on the plant species used for testing. The influence of soil pollution was 67.1 %, the influence of species 22.5 %, their combined effect only 8.56 %.

DISCUSSION

In our studies we used 8 plant species, including common (*T. officinale*, *P. major*, *R. confertus*) and rare plants. All of them were quite suitable for genetic monitoring and fairly sensitive to various types of pollutants. All species from contaminated zones showed a significant increase in the level of anaphase/telophase abnormalities compared to the clean zone. In the case of the environmental pollution with heavy metals (dumps

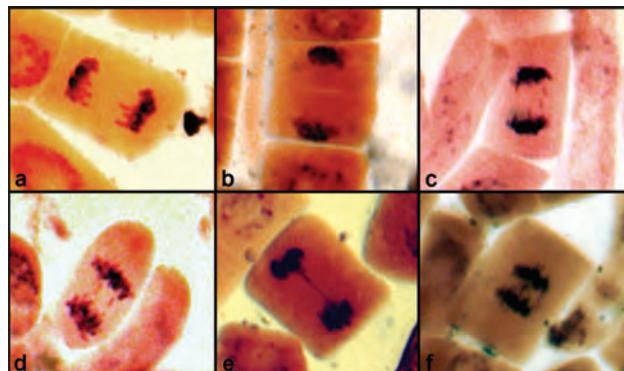


Fig. 1: Meristematic cells of *Taraxacum officinale* Wigg. s.l.: a – normal anaphase; b – normal telophase; c – anaphase with fragments; d – anaphase with lagging chromosomes; e – telophase with single "bridge"; f – anaphase with double "bridge".

Fig. 1: Meristemske celice regata *Taraxacum officinale* Wigg. s.l.: a – normalna anafaza; b – normalna telofaza; c – anafaza s fragmenti; d – anafaza z zamikom kromosomov; e – telofaza s posameznim "mostom"; f – anafaza z dvojnim "mostom".

Tab. 3: Oil products and benz(a)pyrene content in the soils of some villages of the Chechen Republic.**Tab. 3: Derivati naft in vsebnost benz(a)pirena v prsti nekaterih naselij v Čečenski republiki.**

No	Soil sample site	pH	benz(a)pyrene content (mg/kg)	Hydrocarbons (%)	Oil products (%)
1	Goity	8.30	0.03	0.03	0.02
2	Tsotsan-Yurt	8.42	0.06	0.86	0.73
3	Geldegen	7.79	0.14	0.63	0.52
4	Mesker-Yurt	8.63	0.15	1.17	1.10
5	Alkhan-Yurt	8.42	0.26	1.48	1.48
6	Dolynsk	7.86	1.83	1.72	1.56

of the tungsten-molybdenum factory), the frequency of CA was 1.5-5 times higher compared to the clean zone. In the case of oil and benz(a)pyrene pollution (Chechen Republic), the frequency of mutations increased 5-10 times. A comparison of results related to the two types of pollutants (heavy metals and oil products) revealed both similarities and differences in their actions. In the case of oil product influence, we observed an emergence of double "bridges" in seedlings, which was not detected in the case of contamination with heavy metals. In both cases, fragments were the predominant type of disturbance.

The results obtained using species of wild flora are in agreement with data obtained through standard plant test systems (Reutova, 2005, 2017). In these works the genotoxicity of the soil from the tailings of the same tungsten-molybdenum factory was studied using standard plant test systems, and the results indicated a 2-fold

increase in the frequency of mutations. The same studies were performed on soils of the villages in the Chechen Republic (Dzambetova & Reutova 2006). Soils contaminated with petroleum products caused a 5-fold increase in the frequency of mutations in plant test systems.

Virtually any plant species growing in a particular territory can be used for estimating genotoxic effects on the environment. It should be noted, however, that all species are not equally suitable objects for analysis. It is easier to work with species from the Poaceae and Asteraceae genera, as their seeds germinate easily and the roots are not very thick, which allows for good squash preparations. It is more difficult to work with seeds which have a dense, thick peel, as they germinate poorly and very slowly (e.g. the seeds of black henbane).

Mutagenic effects of HM, hydrocarbons, oil products and benz(a)pyrene have been studied for several dec-

Tab. 4: Level of chromosomal aberration in a wild plant from areas around a wolfram-molybdenum factory (Kabardino-Balkaria, Russia).**Tab. 4: Stopnja kromosomskih aberacij na divje rastocih rastlinah iz predelov okoli tovarne volframa in molibdena (Kabardino-Balkaria, Rusija).**

Species	No of cells (ana/telo)	cells with aberrations	Aberrations (%)	P
<i>Yurinea ciscaucasica</i> Clean zone dumps	976	17	1.76	<0,01
	1264	46	3.64	
<i>Anisantha tectorum</i> Clean zone dumps	1024	15	1.46	<0,05
	1004	51	3.09	
<i>Koeleria cristata</i> Clean zone dumps	1043	8	0.77	<0,001
	1187	41	3.45	
<i>Hyoscyamus niger</i> Clean zone dumps	270	3	1.10	<0,05
	322	9	2.79	
<i>Taraxacum officinale</i> Clean zone dumps	1033	41	3.97	<0,001
	1007	194	19.72	

ades, using various test systems and providing detailed reports of the results in the reviews (Chen & White, 2004; Ohe et al., 2004; White & Claxton, 2004). But there are very few works devoted to the study of environmental genotoxicity employing wild flora species growing in contaminated areas. In the past years, there have been some studies in which species of wild flora (mostly wood plants) were used for genetic monitoring and determining genotoxic effect on the environment. To this end, a micronuclei (MN) test was applied to the root meristem of pine (Geras'kin et al., 2005) and silver birch (Kalaev et al., 2006) seedlings. In all cases an increase of micronuclei frequencies was observed, even

when the types of pollutants were different: ionizing radiation and heavy metals.

Herbaceous plants – *Arabidopsis thaliana* and *Crepis tectorum* – have previously been used to study the genetic processes in permanently irradiated plant populations. These studies revealed that plants with rearranged karyotypes started to appear two years after the Chernobyl accident, their frequency correlated with the frequency of CA in the cells of the root meristem. Thirty-eight years after the Kyshtym nuclear accident no increase in the frequency of plants with rearranged karyotype has been detected, because such plants did not have any selective advantages and were subjected

Tab. 5: Level of chromosomal aberration in a wild plant from areas contaminated with oil refinery products (Chechen Republic, Russia).

Tab. 5: Stopnja kromosomskih aberacija na divje rastućih rastlinah iz predelov pod vplivom onesnaženja iz rafinerije (Čečenska republika, Rusija).

Plant species	Collection site	Number of cells (ana/telo)	Cells with aberrations	Aberrations (%)	P
<i>Taraxacum officinale</i> Wigg. s.l.	Goity	1056	24	2.27	
	Tsotsan-Yurt	1007	96	9.53	<0.001
	Geldegen	1050	114	10.85	<0.001
	Mesker-Yurt	1012	104	10.28	<0.001
	Alkhan-Yurt	1026	121	11.7	<0.001
	Dolynsk	1044	126	12.07	<0.001
<i>Matricaria recutita</i> L.	Goity	1164	18	1.55	
	Tsotsan-Yurt	1062	106	9.98	<0.001
	Geldegen	1018	98	9.63	<0.001
	Mesker-Yurt	1013	118	11.65	<0.001
	Alkhan-Yurt	1071	89	8.31	<0.001
	Dolynsk	1070	121	11.31	<0.001
<i>Rumex confertus</i> Wild.	Goity	1002	17	1.69	
	Tsotsan-Yurt	1027	89	8.67	<0.001
	Geldegen	1020	106	10.39	<0.001
	Mesker-Yurt	1006	96	9.54	<0.001
	Alkhan-Yurt	1082	87	8.04	<0.001
	Dolynsk	1051	113	10.75	<0.001
<i>Plantago major</i> L.	Goity	1018	9	0.89	
	Tsotsan-Yurt	1101	53	4.81	<0.001
	Geldegen	1059	61	5.76	<0.001
	Mesker-Yurt	1006	47	4.67	<0.001
	Alkhan-Yurt	1023	36	3.52	<0.001
	Dolynsk	1004	91	9.06	<0.001

to negative selection along with a decreasing irradiation dose rate (Abramov et al., 2006). The authors also noticed that the plant species they used in their studies could be recommended as test objects for the registration of genetic effects of low-dose ionizing radiation. Our study, too, has proved the suitability of wild flora species for identifying the genotoxicity of an environment when contaminated with inorganic (HM) and organic (hydrocarbons, oil products and benz(a)pyrene) substances.

CONCLUSIONS

Species of wild flora are very convenient objects for genetic monitoring, because they allow *in situ* analysis and an evaluation of the effects of all complex of environmental factors, they are highly sensitive to different

types of pollutions, and their testing does not require expensive facilities or highly qualified personnel.

To perform a comparative analysis of the results it is necessary to have an indicator species with a fairly broad distribution. From our point of view, medicinal dandelion (*Taraxacum officinale* Wigg. s.l.) is the best candidate. Dandelion proves to be a universal species, as its flowering or fruiting plants can be found throughout the summer period, with massive flowering typically occurring in May and early June. Dandelion seeds have a short dormant period; their germination rate is high and they germinate fast. The seedling roots are of an optimal size for making good squash preparations and the species is sensitive to various types of pollution (heavy metals, organic pollutants). Given all these factors we suggest that dandelion be used as a standard species for the examination of genotoxic effects on the environment.

RASTLINSKE VRSTE KOT INDIKATORJI OKOLJSKE GENOTOKSIČNOSTI

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POVZETEK

Pričajoča raziskava opredeljuje uporabnost divje rastočih rastlinskih vrst za ugotavljanje okoljske genotoksičnosti. Nivo mutacij v celicah v koreninskem meristemuh je bil preiskan na 8 rastlinskih vrstah, ki uspevajo v okolju pod vplivom različnih okoljskih dejavnikov. Za ugotavljanje vpliva onesnaženja s težkimi kovinami so uporabili semena, nabранa na odlagalniščih tovarne wolframa in molibdena (Kabardino-Balkaria, Rusija). V primeru onesnaževanja z produkti izgorevanja in rafiniranja nafte so bila semena pobrana v predelih z mini-tovarnami za predelavo nafte (Čečenska republika, Rusija). Onesnaženje je povzročilo 2,5 do 10,2 – kratno povečanje frekvence mutacij na sadikah iz onesnaženega okolja v primerjavi s sadikami v "čistem" okolju. S tega vidika so divje rastoče rastlinske vrste uporabne kot občutljivi objekti za ugotavljanje okoljske genotoksičnosti.

Ključne besede: okoljska genotoksičnost, težke kovine, onesnaženje z nafto, divje rastoče rastlinske vrste

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