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Original Research

Genotoxicity of Soil Pollutants Extracted with Different Solvents

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Abstract

The aim of our study was to compare the efficacy of extraction of genotoxic pollutants from soils performed by means of several solvents. Soil pollutants were extracted in the Soxhlet apparatus. Extract genotoxicity was assessed using the Ames Test. All the examined soils (12) contained genotoxic pollutants. Extraction with hexane allowed detection of pollution genotoxicity in 11 soils, whereas with methanol and dichloromethane in 10 soils each. The other solvents allowed detection of genotoxicity in fewer soil samples. Methanol extract had the highest genotoxicity in the majority of soils (7 out of 12). Results obtained in the present study and literature data allow recommendation of methanol for extraction of genotoxic pollutants from soils. Methanol is more versatile, efficient, common, and less harmful for laboratory workers than dichloromethane and hexane.

Keywords: mutagenicity, soil pollutant, Ames Test, *Salmonella typhimurium*, organic solvents

Introduction

Soil coming from various parts of the world becomes polluted as a result of industrial, mining, communication, agricultural, and military activities [1]. Some pollutants enter the soil directly, others appear as a deposit of pollutants in the atmospheric air [2-3]. As a result, liquid fuels, pesticides and fertilizers, chloro-derivatives of polycyclic aromatic hydrocarbons, and heavy metals enter the soil [3-7]. Many of them demonstrate genotoxicity. Mutagens polluting the soil pose a great health hazard. They could accumulate in crop plants tissues and in the animals that feed on them. It leads to the contamination of food eaten by people. Some of these pollutants could migrate into both subsoil [8] and surface waters used by people, also as potable water. Therefore, monitoring soil contamination caused by genotoxic pollutants is necessary, thus the literature of this subject is abundant [1]. The authors of these works used genotoxicity studies to evaluate the bioremadiation process of polluted soil [2, 5-7, 9].

These authors were using different extraction methods and different solvents [10, 11]. The most common method assessing genotoxicity of soil pollutants was the Ames Test. It employs different strains of *Salmonella typhimurium* [10, 12, 13] and different test variants: a traditional one with Petri dishes without preincubation [11] and with preincubation [14], or with microtitre plates [15]. It makes the results difficult to compare. Studies conducted so far aiming at optimizing extraction methods and proper solvent selection [8, 10, 14-17] have not led to a uniform methodology that is widely recognized and used.

Therefore, the aim of our study was to determine the usefulness of 5 organic solvents in extraction of genotoxic pollutants from soils on the basis of the comparison of their versatility and efficacy. The criteria for solvent selection included their common use for genotoxic pollutant extraction from soils and other environmental samples, as well as differentiation of boiling points and polarity. The obtained results also have allowed determination of the benefit and

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Table 1. Characteristics of the tested soils.

No.	Soil type	Type of utilization and pollution
1	sandy loam	newly contaminated with gas oil
2	sandy loam	contaminated with gas oil after bioremediation
3	silty	contaminated with explosives (mainly DNT) after self-purification
4	silty	soil from a military area contaminated with explosives (mainly DNT), after 30 days of bioremediation in aerobic conditions (pot experiment)
5	silty	soil from a military area contaminated with explosives (mainly DNT), after 30 days of bioremediation in anaerobic conditions (pot experiment)
6	silty	contaminated with explosives (mainly TNT)
7	silty	soil from a military area contaminated with explosives (mainly TNT), after 30 days of bioremediation in aerobic conditions (pot experiment)
8	silty	soil from a military area contaminated with explosives (mainly TNT), after 30 days of bioremediation in anaerobic conditions (pot experiment)
9	loam	non-agricultural soil contaminated with aviation fuel, after self-purification
10	sandy	non-agricultural soil contaminated with chloroorganic compounds, after self-purification
11	sandy loam	agricultural soil, 1 km from the road (suburbs of Wrocław - SW Poland)
12	sandy loam	agricultural soil, 50 m from the road (Nadolice Wielkie near Wrocław – SW Poland)

cost ratio related to the use of one or more solvents. The use of the results in practice will contribute to a more rational planning of studies on genotoxicity of soil pollutants.

Material and Methods

Test Soils

The genotoxicity of 12 soil samples was tested (Table 1). The samples represented different genetic types of soils: sandy, sandy loam, loam, and silty. The soils differed with respect to the type of utilization (non-agricultural, agricultural, military) and the type of pollution (remains of liquid fuels, explosives, chloroorganic compounds).

Methods of Sampling and Sample Preparation

1-2 kg soil samples were collected in and near Wrocław with a shovel at a depth of about 20 cm. They were placed in bags made from the polyvinyl chloride (which do not demonstrate genotoxicity) then transported to the laboratory. In the laboratory they were dried for 2-3 days at room temperature. 50 g of each sample was extracted in the Soxhlet apparatus for 8 hours. The following Sigma solvents were used: methanol (polar proton, BP = 64.7ºC), hexane (apolar, BP = 34.6ºC), dichloromethane (apolar, BP = 40.7ºC), diethyl ether (apolar, BP = 68.7ºC), and a mixture of diethyl ether and hexane 1:1 by volume. The solvent was evaporated to dryness and then dissolved in 10 cm^3 dimethyl sulphoxide (DMSO). The extract dissolved in DMSO (Sigma) was sterilized by filtration (0.2 µm pore diameter). The extracts in DMSO were stored at -70ºC.

Methods of Genotoxicity Testing

The tests were based on the strain *Salmonella typhimurium* TA 98 obtained from the Ames Laboratory Department of Biochemistry at the University of California. It has the following set of genetic markers: his^-, rfa, ΔuvrB, and +R.

The procedure described by Maron and Ames [18] was used. All the trials consisted of 4-5 replicates. 0.5 cm^3 of phosphate buffer or S-9 mix fraction was poured into a sterile test tube. Then the following components were added: 0.1 cm^3 test sample, 0.1 cm^3 overnight broth culture of the test strain, TOP-agar at 45ºC containing 0.2 cm^3 0.5 mM histidine and biotin. The content of the test tube was mixed and within 20 s poured on the Petri dish with Vogel-Bonner minimal medium. The dishes were incubated for 48 hours at 37ºC. After that, the revertants growing on the dishes were counted. The mean numbers of spontaneously occurring revertants were similar to those given by Maron and Ames [18]. Mutagenic effects of the samples were tested with and without metabolic activation with S-9 mix.

1 cm^3 of S-9 mix fraction prepared according to the procedure recommended by Maron and Ames [18] contained: 8 mM $MgCl_2$, 33 mM KCl, 4 mM NADP, 5 mM glucose 6-phosphate, 100 mM sodium phosphate, and 0.05 cm^3 homogenate of rat liver with 40 g/cm^3 protein content activated by Aroclor 1254.

Spontaneous reversion amounted to 30-50 colonies per dish. In order to test sensitivity of the strains, a positive control was performed by subjecting bacteria to a control mutagen (without metabolic activation with S-9 fraction: 0.2 µg 2,4,7-trinitro-9-fluorenone per dish; with metabolic activation with S-9 fraction: 10 µg 2-aminofluorene per dish).

Table 2. The net number of revertants per 1 g soil (spontaneous reversion = 40).

Soil sample	Metabolic activation with S-9 mix fraction	Solvent used for extraction				
		Methanol	Dichloromethane	Hexane	Hexane + diethyl ether	Diethyl ether
1	no	713.4	919.3	**1423.5**	0	0
	yes	0	0		0	0
2	no	0	**829.1**	828.8	0	0
	yes	0	0	0	0	0
3	no	0	0	0	0	0
	yes	**922002.8**	1412.8	12368.0	961.8	1585.2
4	no	**10152.0**	0	3264.0	0	2901.0
	yes	**4258.0**	1513.6	1534.6	0	0
5	no	0	0	**650.3**	0	0
	yes	1109.7	665.6	**3277.7**	0	0
6	no	7231.5	**11380.1**	1198.3	4976.3	28977.5
	yes	**5014.2**	4865.3	1065.4	2315.7	2675.4
7	no	**17345.3**	0	14423.7	0	14163.6
	yes	**83401.35**	2801.7	6628.2	0	3123.8
8	no	**41786.4**	0	27244.6	4662.7	15960.9
	yes	29721.3	5367.9	**38888.9**	2120.2	2067.9
9	no	0	0	0	0	0
	yes	1588.4	0	**6920.0**	0	0
10	no	0	0	0	0	0
	yes	0	0	0	0	**1986.9**
11	no	**120.8**	0	119.5	0	0
	yes	0	0	0	0	0
12	no	**218.0**	0	183.9	0	0
	yes	**1616.5**	111.6	51.8	0	0

The highest numbers of revertants for particular soil samples are indicated in bold.

Also, mutagenic activity of the applied solvents was tested. According to the procedure, the trials with mutagenicity ratio MR≥2 and with a linear dose-response relationship were considered mutagenic [18, 19]. Specific genotoxicity of the test soils was calculated on the basis of the dose-response function and was expressed as the net number of revertants/1g soil. For extracts with no genotoxicity, the value of net 0 revertants/1g soil was assumed.

Results

Genotoxicity of Test Soils

All the test soils were polluted with mutagenic chemical compounds (Table 2). At least one extract in each test soil sample caused development of mutation in the strain *Salmonella typhimurium* TA 98. Particular soils differed with respect to the number of solvents required to detect genotoxicity with the Ames Test, as well as with respect to the intensity of the mutagenic effect. Extracts of half of the tested soils (soils 4-8 and 12) revealed genotoxicity in the Ames test with and without metabolic activation with S-9 mix. This means that the pollutants extracted from those soils contained both direct and indirect mutagens. 5 soils were contaminated with trinitrotoluene (TNT) and 1 soil was agricultural. Extracts of the other soils revealed genotoxicity only with or only without activation with S-9 mix fraction. This means that they contained only direct or only indirect mutagens.

The results reveal different solubility levels of genotoxic pollutants in particular soils in different solvents. They do

Table 3. The probability of mutagenicity detection by means of one and two solvents out of 5 used in the present study [%].

Solvent 1	Solvent 2					Only solvent 1
	Methanol	Dichloromethane	Hexane	Hexane + dibutyl ether	Dibutyl ether	
Methanol	x	92	92	83	92	83
Dichloromethane	92	x	92	83	92	83
Hexane	92	92	x	92	100	92
Hexane + diethyl ether	83	83	92	x	58	33
Dibutyl ether	92	92	100	58	x	58

not give rise, however, to the assumption that particular solvents have different efficacy in genotoxic pollutant extraction for soils representing different genetic types or differing in the type of pollutant. The only conclusion that could be drawn was that specific genotoxicity of soils polluted with the remains of explosives was higher than in soils contaminated with other pollutants. The indicator of specific genotoxicity of soil was the net number of revertants per 1 g soil caused in the test by an extract of a particular soil revealing the highest genotoxicity. The mean number of revertants for 6 soils polluted with the remains of trinitrotoluene (TNT) was 81 times higher than for the other 6 soils (177606 and 2166). This indicates significant TNT genotoxicity as compared to other soil pollutants.

Selection of the Best Solvent

The usefulness of the tested solvents for extraction of genotoxic pollutants from soils was assessed on the basis of two criteria:

- versatility (the most versatile solvent was the one whose extracts allowed detection of genotoxicity in the largest number of soil samples)
- efficacy (the most efficient solvent was the one whose extracts from a particular soil was more genotoxic than extracts of the same soil obtained by other solvents)

Pollutant extraction with hexane allowed detection of genotoxic pollutants in 11 samples, whereas with methanol and dichloromethane in 10 samples. Extraction with diethyl ether and the mixture of hexane with diethyl ether allowed detection of mutagenic activity in pollutants extracted from fewer soil samples (7 samples for diethyl ether and 4 samples for the mixture of hexane and diethyl ether). It was revealed that the most versatile solvents of genotoxic soil pollutants were hexane, methanol and dichloromethane.

Extraction with dichloromethane allowed detection of activity of direct mutagens in a larger number of soil samples compared to indirect mutagens. Extraction with methanol and hexane allowed detection of the activity of direct and indirect mutagens in a comparable number of tested soil samples. Versatility of the other solvents toward direct and indirect mutagens was not compared due to few soil samples whose extracts obtained with those solvents resulted in a mutagenic effect in the Ames Test.

Methanol extract revealed the highest mutagenicity in 7 out of 12 soils, the hexane extract in 3 soils, and the dichloromethane extract in 1 soil. The extract of soil 10 obtained with diethyl ether was the only extract of this soil that revealed genotoxicity in the Ames Test. Extracts obtained with the mixture of hexane and diethyl ether did not reveal the highest mutagenic activity in any tested soils. In some cases, pollutants extracted with the mixture of hexane and diethyl ether revealed lower genotoxicity than pollutants extracted with one of these solvents (soils 6 and 8 without S-9 activation and soil 3 with S-9 activation). Thus, the solvent revealing the highest efficacy of extracting genotoxic pollutants from the tested soils was methanol.

The solvent with the highest probability of genotoxicity detection in a given soil sample was hexane. The solvent with the highest probability of full assessment of real mutagenicity in a given sample was methanol. The use of this solvent allowed detection of genotoxicity in almost the same number of samples as with hexane.

Benefits and Costs of Extraction with More than One Solvent

Knowledge about applying the extraction of pollutants with many solvents, significantly increasing the probability of genotoxicity detection of samples, could contribute to taking less time and using fewer materials in the laboratory. Depending on the aim of the study, it may be more rational to test a larger number of soil samples with a higher probability of non-detection of genotoxicity in some samples, or to test a smaller number of samples with a higher probability of non-detection of genotoxicity in some samples. Compilation of the solvents into pairs revealed that in 70% of cases (14 out of 20 pairs) the use of the second solvent increased the number of soil samples whose genotoxicity was detected with the Ames test (Table 3). In cases where solvent 1 was methanol or dichloromethane or hexane (solvents resulting in detection of genotoxicity in 9-11 samples, i.e. 75-92%), an increase in genotoxicity detection was 1-2 samples, i.e. up to 17%. A higher increase in genotoxicity detection was observed only with diethyl ether and the mixture of hexane with diethyl ether, i.e. the solvents that individually caused detection of genotoxicity in 42% or 58% of samples. While making a decision about employing

Table 4. The probability of mutagenicity detection by means of two and three solvents out of 5 used in the present study [%].

Solvent 1	Solvent 2	Solvent 3					Only solvent 1 and 2
		Methanol	Dichloromethane	Hexane	Hexane + diethyl ether	Diethyl ether	
Methanol	dichloromethane	x	x	92	92	100	92
	hexane	x	92	x	92	100	92
	hexane + diethyl ether	x	92	92	x	92	83
	diethyl ether	x	100	100	92	x	92
Dichloromethane	methanol	x	x	92	92	100	92
	hexane	92	x	x	92	100	92
	hexane + diethyl ether	92	x	92	x	92	83
	diethyl ether	100	x	100	92	x	92
Hexane	methanol	x	92	x	92	100	92
	dichloromethane	92	x	x	92	100	92
	hexane + diethyl ether	92	92	x	x	100	92
	diethyl ether	100	100	x	100	x	100
Hexane + diethyl ether	methanol	x	92	92	x	92	83
	dichloromethane	92	x	92	x	92	83
	hexane	92	100	x	x	100	92
	diethyl ether	92	92	100	x	x	58
Diethyl ether	methanol	x	100	100	92	x	92
	dichloromethane	100	x	100	92	x	92
	hexane	100	100	x	100	x	100
	hexane + diethyl ether	92	92	100	x	x	58

the second solvent, one must remember that it would double the workload and the use of materials for extraction and biotest.

Adding the third solvent to those pairs increased the number of soil samples whose genotoxicity was detected with the Ames test for 26 out of 28 groups of three solvents (Table 4). However, in 22 cases the number increased by only 1 sample (about 8%). A higher increase in genotoxicity detection concerned only those solvent sets in which the two first solvents were diethyl ether and the mixture of hexane and diethyl ether. The use of extraction with a subsequent solvent resulted in an increase in workload and the use of materials that was unproportionally high to the increase in genotoxicity detection for particular samples.

Discussion

All the soil samples tested in this study contained pollutants revealing genotoxicity in the Ames test. The situation was similar when the soil samples were assessed with the Ames Test by other authors. Many of those samples revealed genotoxicity toward *Salmonella typhimurium* with or without metabolic activation with fraction S-9, and less frequently only with or only without the said activation [9, 17, 20]. This means that the tested samples contained both direct and indirect mutagens or only one of these two types. There were also some samples revealing no genotoxicity [9]. The results obtained in the present study (as well as literature data) revealed that soils containing genotoxic pollutants represented different genetic types [8] and have been used for different purposes. The soils came from forest [17], agricultural [17], urban [15], industrial [9], communication [13], and military areas [4, 5]. In many cases, soils containing the remains of petroleum products [3, 7], explosives [4, 5], and pesticides were studied [6]. Authors of some papers used the study on genotoxicity of soil pollutants for monitoring the progress of bioremediation of contaminated soils [2, 5].

All the samples tested in the present study contained pollutants causing frameshift mutations that might be detected by *Salmonella typhimurium* TA 98. Studies performed by other authors gave similar results for many soil samples [12, 20, 21]. Soil samples tested by other authors

were also causing base pair substitution mutations that could be detected by the strain *Salmonella typhimurium* TA 100 [12, 20], as well as other mutations, including those occurring in eukaryotic cells [22].

The majority of solvents used for extraction of pollutants from the soils tested in the present study were already used by other authors: methanol [10, 12, 15, 17, 21], dichloromethane [2, 9, 11, 21], hexane [10, 15], diethyl ether [8]. The only solvent that has not been used before in the available literature is the mixture of hexane and diethyl ether. The majority of authors used only one solvent for extraction of soil pollutants. Therefore, results obtained by them could not be used for drawing conclusions about different efficiencies of particular solvents used for extraction of genotoxic pollutants. White and Claxton [23] analyzed literature data concerning genotoxicity studies conducted on 1633 soil samples and found out that more than 50% of the samples were extracted with methanol, 24% with the mixture of hexane and acetone, 14% with dichloromethane, and 11% with other solvents. Common use of the same solvents makes it easier to compare the results, but it should not be the only criterion for solvent selection. The additional criteria should be established as follows: the versatility of solvent (which gives the higher probability of genotoxicity detection of samples, if they contain genotoxic pollutants) and extraction effectiveness (which gives the higher probability that genotoxic effects will not be lowered as a result of extraction, only a part of pollutants from the genotoxic sample).

The highest versatility in extraction of genotoxic pollutants from the soils tested in the present study was revealed by hexane, then methanol and dichloromethane. The highest efficiency was revealed by methanol. Donelly et al. [9, 21] tested 40 soil samples, of which 27 revealed genotoxicity in dichloromethane and 25 in methanol. The use of two solvents allowed detection of genotoxicity in 29 soil samples. In 21 samples, higher genotoxicity was revealed in the extract obtained with methanol and in 12 samples in the extract obtained with dichloromethane. The usefulness of methanol for extraction of genotoxic pollutants from soils was also indicated by authors studying individual soil samples [10, 16, 24]. However, this was not always so. Courty et al. [14] studied mutagenicity on 4 soil samples extracted with 8 different solvents, including methanol. They concluded that dichloromethane, acetone, and acetonitrile were the most efficient in extracting mutagenic pollutants from soils. In some cases, other solvents turned out to be more efficient than methanol or dichloromethane, i.e. acetonitrile [17] and diethyl ether [8]. These were, however, results from studies on individual soil samples. Monarca et al. [20] claimed that acetone extracted twice as much pollution than cyclohexane (mean for 10 soil samples). Still, they did not compare mutagenic activity of these two extracts. The common feature of methanol, acetone, and acetonitrile is polarity and relatively high boiling points. On the other hand, dichloromethane and diethyl ether are non-polar solvents of relatively low boiling points. The results obtained in the present study as well as other literature data indicate that the most efficient solvents in extracting genotoxic pollutants from soils are polar solvents of high boiling points and dichloromethane.

Some authors emphasise that selection of an appropriate solvent should also take into account the characteristics of the tested soils, such as the type of contamination and genetic type of the soil. Statistical analysis of genotoxicity toward *Salmonella typhimurium* of soil samples extracted with methanol, dichloromethane, and the mixture hexane + acetone showed a dependence of extraction efficiency not only on the solvent used but also on the type of pollutants present in soils (industrial, urban, and agricultural). It was not possible to point to one versatile solvent that would be the most efficient in extraction of all soil samples [23]. A similar result was obtained after comparison of extraction efficacy while using different solvents for soils representing different genetic types. Extraction with diethyl ether was the most efficient with garden, peaty, and podsolic soil, while for the sandy soil there was not one extract that would show the highest mutagenic activity toward different *Salmonella* strains with or without metabolic activation [8]. Current knowledge of the correlation between the efficacy of genotoxic pollutant extraction by means of different solvents and the genetic type of the tested soil and the type of pollutant does not allow recommendation of different solvents for extraction of pollutants from soils differing with respect to those features.

The results obtained in the present study and other literature data indicate that the use of two or more solvents only to a small extent increases the probability of non-detection of pollutant genotoxicity in one soil sample, but it largely increases the workload and materials required to perform the tests. The use of more than one solvent for extraction of soil pollutants is justified only when the trust to negative genotoxicity test results is much more important than the number of the tested samples. The choice of solvents should be preceded by analysis of their physical and chemical properties.

The obtained results and literature data allow recommendation of methanol for extraction of genotoxic pollutants from soils. Methanol revealed the highest efficiency in extracting genotoxic pollutants from soils and a significant versatility that makes it highly probable to detect genotoxicity of pollutants in the tested soils. The argument for methanol is also related to protection of health of workers performing extraction of soil pollutants. The use of more volatile dichloromethane exposes the workers to the risk of neoplasms due to limited evidence of its carcinogenic effect, and the use of hexane exposes workers to the risk of fertility impairment. Genotoxicity studies performed on extracts of the same soil samples obtained with different solvents result in an increased workload and material usage that is unproportionally high to the increase in detection of genotoxicity of the tested samples. Therefore, they should only be used when it is necessary to avoid negative false results caused by insufficient solubility of genotoxic pollutants that are present in some of the tested soil samples.

Conclusion

All the examined soils contained genotoxic pollutants. The number of extracts of particular soils revealing genotoxicity was different. Extraction with hexane allowed detection of pollution genotoxicity in 11 soils, and with methanol and dichloromethane in 10 soils each. The other solvents allowed detection of genotoxicity in fewer soil samples. Methanol extract had the highest genotoxicity in the majority of soils (7 out of 12). When extracts of particular soils obtained by means of different solvents were compiled in groups of two or three, it was revealed that testing more than one extract from each soil sample only slightly increased the probability of detection of genotoxic pollutants, while it significantly increased the workload and materials needed to conduct the biotests. Depending on the aim of a study, it may be better to assess fewer or more soil samples, but with a higher probability of avoiding false negative results. Results obtained in the present study and literature data allow recommendation of methanol for extraction of genotoxic pollutants from soils. Methanol is more versatile, efficient, common, and less harmful for laboratory workers than dichloromethane and hexane.

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