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Pressurized liquid extraction with water as a tool for chemical and toxicological screening of soil samples at army live-fire training ranges

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Abstract

Significant discrepancies in the results of risk assessments based on chemical and toxicity analyses of soils may arise through differences in the efficiency of the extraction or leaching methods used. A rapid technique that may be used in the screening phase of live-fire training ranges and suitable for extracting explosive residues is pressurized liquid extraction (PLE) with water. Therefore, PLE and the commonly used batch leaching method EN-124 57-2 were compared for their utility to extract specific residues from soil samples collected from the Canadian Forces Base (CFB) Petawawa, Ontario. After extraction the cytotoxicity of the samples were assessed in the L-929 growth inhibition assay. The PLE method yielded extracts suitable for direct use in the toxicity assay within 20 min as compared to 24 h for the batch leaching method. Analysis of the extracts showed that the PLE water extracts tended to give higher recoveries of explosive residues and the resulting exposure concentrations were confirmed by higher cytotoxicities. Furthermore, gas chromatography–mass spectrometry analyses showed that the samples contained significant amounts of several munition-related stabilizers and plasticizers of toxicological significance in addition to the analysed explosive residues. In conclusion, PLE using water is a promising extraction technique for both chemical and toxicological screening of soil samples from areas that may be contaminated with explosive residues.
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1. Introduction

Due to a program of demilitarization, nearly all of the military sites of military training in Sweden will be closed and in some cases the land will be re-used for activities such as recreation and building. However, elevated levels of explosive residues have been found in soil at live-firing shooting and training ranges in the USA and Canada [1], and investigations will be required at Swedish sites following national environmental risk assessment procedures [2]. Environmental contaminants at military training sites originate from live-firing exercises, low-order detonations and unexploded ordnance, and include a large number of substances such as nitroaromatics (trinitrotoluene, TNT; 2,4-dinitrotoluene, 2,4-DNT; trinitrobenzene, TNB, etc.); cyclic nitroamines (1,3,5,7-tetranitro-1,3,5,7-tetrazacyclooctane, HMX; 1,3,5-trinitro-1,3,5-triazacyclohexan, RDX, etc.) and nitrate esters (nitroglycerine, NG; nitrocellulose, NC). Live-firing training at these military sites may also give rise to significant contamination by heavy metals, petroleum and combustion residues [3,4]. In addition, the distribution of explosive residues is particularly heterogenous and thus requires appropriate choices of sample number and sub-sampling techniques [5,6], making chemical characterization of contaminants at live-fire training areas (such as hand grenade, anti-tank rocket and mortar ranges and areas for detonation and open burning) especially challenging.

To address the problems associated with complex contamination, several authors have pointed out the need for toxicological analysis of contaminated soils [7–9]. Soil water extracts are commonly used for toxicity measurements of contaminated soils, and although there is no standard technique, shaking soil in water in a liquid/solid-ratio (L/S-ratio) of 10:1 seems to be the most frequently used [10]. For compounds with low water solubility, extracts prepared in organic solvents are used instead;

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for example, a soil extraction procedure for ecotoxicological and chemical characterization of explosive residues based on ultrasonication with acetonitrile has been described by Sunahara et al. [11]. Since most organic solvents are incompatible with toxicological tests evaporation to dryness and dilution with e.g. DMSO/water is necessary. Nevertheless, according to the cited authors, under ideal conditions this procedure yields extracts reflecting the worst case scenario in terms of organic contaminant concentrations and thus avoids any possible underestimation of the potential extent of contamination. However, this procedure does not give representative samples for cases of mixed contamination of inorganic and organic analytes.

For chemical analyses of explosive residues in soil samples, US EPA Method 8330 recommends to perform the extraction step by shaking or ultrasonicating in acetonitrile. However, as a consequence of the different techniques used to obtain extracts for chemical and toxicological evaluation of polluted sites, discrepancies in the results may arise. It is thus imperative, when doing both chemical and toxicological analysis, to obtain extracts suitable for both types of analysis. Other available extraction techniques include supercritical fluid extraction (SFE) with CO2 [12], microwave-assisted extraction (MAE) [13] and pressurized liquid extraction (PLE) with methanol–acetonitrile [14]. These techniques have all proven to be either comparable, or more effective, than the conventional extraction techniques. Performing extractions with pressurized water has been extensively studied as the dielectric constant of water can easily be suppressed by increasing the temperature, thereby enabling the extraction of more apolar substances [15–17].

Since environmental monitoring and evaluation of live-fire training ranges involves several hundreds of samples and thus a high cost a screening phase would benefit by a technique to aid important steps such as hot-spot identification (high risk zones), area reduction (sample exclusion) and as a tool to alarm for the need of more in-depth chemical and/or toxicological characterization.

In this study, the utility of PLE, with water as the extraction solvent, was investigated for rapidly producing extracts of samples contaminated by explosive residues for subsequent chemical and toxicological screening. The PLE method was compared to the commonly used batch leaching method EN-124 57-2 [18] with respect to its utility for producing extracts for subsequent chemical and toxicological screening, in terms of speed and ability of indicating toxicity. Additionally, conventional chemical analysis for metals and explosives, and screening by gas chromatography–mass spectrometry (GC–MS) for other organic compounds was executed to further characterize the samples.

2. Materials and methods

2.1. Samples

Sub-surface (0–2 cm depth) composite soil samples (50 increments) were collected according to a judgmental random design to obtain representative samples from two live-fire training ranges at CFB Petawawa in Ontario, Canada [19]. Two samples (S1 and S2) were collected from the impact area of an anti-tank range, around a tank used as a target. S1 was taken within 2 m of the tank and S2 2–4 m from the tank. A third sample (S3) was collected at the firing point of another range heavily used by artillery and mortars, in front of the main firing pad. Samples from these sites should thus reflect residues and deposits from various activities associated with live firing training. Composite soil samples (1–2 kg) were homogenized using an acetone slurry technique [4] and then dried for several days before aliquots for chemical and toxicological analysis were withdrawn.

2.2. Sample preparation for toxicological analysis

Two methods were used for water extraction prior to toxicological analysis. For PLE, 8 g soil samples were mixed with acid-washed sand (Fontainebleue, Prolabo, France), placed in a 11 ml extraction cell, and extracted with ~15 ml of Milli-Q®-water at the following settings: 150 °C, 10 MPa, preheating 5 min, static 10 min, purge 60 s (N_2) and 1 cycle using a PLE instrument (ASE 200, Dionex Corp., Sunnyvale, USA). A built-in rinsing step was performed between each extraction. The L/S-ratio was 1.5–2 during the extractions and the total extraction duration was less than 20 min, including 5 min equilibration and 10 min in static extraction mode. For the second method, soil was leached using standard method EN-124 57-2 [18] by adding 80 ml of Milli-Q®-water to 8 g of sample (L/S-ratio 10) and agitating the mixture on a laboratory shaker for 24 h. Before assaying toxicity, extracts produced using the two methods were passed through 0.45 μm PTFE-syringe filter (Supelco, Bellefonte, Pennsylvania, USA). The remaining water extracts were cleaned up and concentrated to 2 ml for LC–UV analysis using solid phase extraction (SPE) columns (Supelco, Lichrolut EN 200 mg). The SPE columns were conditioned sequentially with 3 ml of MeOH, AcN and water before the water samples were applied and finally eluted with 2 ml of AcN.

2.3. Sample preparation for chemical analysis

The extraction method for chemical analysis was essentially following Campbell et al. [14]. In short, 10 g samples were mixed with acid-washed sand (Fontainebleue, Prolabo, France) and extracted with ~15 ml of acetonitrile at 150 °C and 10 MPa using the same PLE instrument and settings as above. Filtered extracts were concentrated to 1 ml in a rotary evaporator and further adjusted by a gentle stream of nitrogen (Buchi, Germany). The vials were finally weighed to a correct volume based on solvent density.

2.4. Chemical analysis

Samples (20 μl) were analysed using a liquid chromatograph (Waters 2695), equipped with a 4.6 mm × 150 mm, 5 μm particle size Purospher C18 column (Merck, Darmstadt, Germany), coupled to a ultraviolet (LC–UV) photo diode array detector (Waters; 2996 PDA) monitoring the eluate at 254 nm, and an isocratic liquid phase of MeOH/H_2O, 50:50 (flow rate, 1 ml min^{-1}). Integrated peak areas were compared with those

Table 1
Concentrations of analytes found (by HPLC–UV) in the soil samples after extraction by pressurized liquid extraction and a batch leaching method (EN 12457-2)

	PLE/AcN (μg/g)				Ref. lab using EPA 8330 (μg/g)[a]		
	S1	S2	S3	Blank	S1	S2	S3
HMX	210	81	2.1	<0.03	170	91	0.061
RDX	1.4	0.74	<0.02	<0.02	0.42	0.30	<0.001
1,3,5-NB	<0.02	<0.02	<0.02	<0.01	<0.001	<0.001	<0.001
1,3-DNB	<0.01	<0.01	<0.01	<0.01	<0.001	<0.001	<0.001
Tetryl	<0.02	<0.01	<0.01	<0.01	<0.001	0.20	<0.001
TNT	0.49	0.32	0.081	<0.01	0.38	0.29	<0.001
NB	<0.01	<0.01	<0.01	<0.01	<0.001	<0.001	<0.001
4-A-DNT	<0.02	<0.02	<0.02	<0.02	<0.001	<0.001	<0.001
2-A-DNT	0.31	0.23	0.17	<0.01	0.045	0.049	<0.001
2,6-DNT	<0.02	<0.02	<0.02	<0.02	<0.001	<0.001	0.049
2,4-DNT	<0.01	<0.01	1.3	<0.01	<0.001[b]	<0.001[b]	<0.001[b]

	PLE/H_2O (μg/g)				EN 124 57-2/H_2O (μg/g)			
	S1	S2	S3	Blank	S1	S2	S3	Blank
HMX	100	98	1.6	<0.04	42	35	0.40	<0.06
RDX	3.1	<0.04	<0.04	<0.04	<0.05	<0.05	<0.07	<0.05
1,3,5-NB	<0.02	<0.02	<0.02	<0.02	<0.03	<0.03	<0.04	0.03
1,3-DNB	<0.01	<0.01	<0.01	<0.01	<0.02	<0.02	<0.03	<0.02
Tetryl	<0.03	<0.03	<0.03	<0.03	<0.04	<0.04	<0.06	<0.04
TNT	0.28	0.41	<0.02	<0.02	<0.03	<0.03	<0.04	<0.03
NB	0.27	<0.02	<0.02	<0.02	<0.02	<0.02	<0.03	<0.02
4-A-DNT	<0.02	<0.02	<0.02	<0.02	<0.03	<0.03	<0.04	<0.03
2-A-DNT	<0.01	<0.01	<0.01	<0.01	<0.02	<0.02	<0.03	<0.02
2,6-DNT	<0.03	<0.03	<0.03	<0.03	<0.04	<0.04	<0.06	<0.04
2,4-DNT	<0.01	<0.01	1.7	<0.01	<0.02	<0.02	0.82	<0.02

	PLE/H_2O[c] (μg/ml)			EN 124 57-2/H_2O[c] (μg/ml)		
	3[d] (μg/ml)	3[d] (μg/ml)	3[d] (μg/ml)	12.5[d] (μg/ml)	12.5[d] (μg/ml)	12.5[d] (μg/ml)
HMX	35	33	0.50	4.2	3.5	0.040
RDX	1.0	<0.01	<0.01	<0.005	<0.005	<0.005
TNT	0.1	0.1	<0.01	<0.003	<0.003	<0.003
NB	0.090	<0.01	<0.01	<0.002	<0.002	<0.002
2,4-DNT	<0.003	<0.003	0.60	<0.002	<0.002	0.082
Total	36	33	1.1	4.2	3.5	0.1

The water extracts were cleaned up/concentrated on solid phase extraction before analysis and results are reported both based on the weight soil and exposure concentration in the water extract.
[a] TNO-Prins Maurits Laboratory Holland (Research Group: Pyrotechnics and Energetic Materials).
[b] 2,4-DNT and NG coeluted under the gradient program used.
[c] Exposure concentration.
[d] L/S ratio.

of standards prepared from a certified calibration standard (USEPA 8330 Mix A and B, Supelco). A 1 μl of the PLE-AcN extracts prepared as described above were also analyzed by GC–MS (HP-GC-5890, HP-MSD-5972, with a 30 m long, 0.25 mm i.d., film thickness 0.25 μm, DB5MS column from J&W Scientific, Folsom, CA, USA) in fullscan mode to screen for the possible presence of compounds not covered by the US EPA 8330 protocol [20] (see Table 1). The GC conditions were as follows: injector temperature 200 °C, column temperature program: initially 2 min at 60 °C, increasing to 280 °C at 10 °C min^{-1} and finally held at 280 °C for 5 min, with helium as carrier gas at a flow rate of 1 ml min^{-1}. The ion source was kept at 280 °C for the whole run and electron impact spectra (70 eV) were recorded (50–550 *m*/*z*) at unit resolution. The criterion for a positive identification was a library match quality greater than 90% (NIST 98), unless standards were available, in which case retention times of ±2 s were used for verification. A reference laboratory, the TNO-Prins Maurits Laboratory in Holland, (Research Group: Pyrotechnics and Energetic Materials) was asked to analyze the same three samples according to US-EPA 8330. This method is based on ultrasonication extraction with acetonitrile followed by analysis with LC–UV.

Samples were also analyzed for metals by inductively coupled plasma mass spectrometry (ICP-MS) following method Ont SOP 0624, based on method EPA 6020 [21]. Typically, 0.5 g of each soil sample was digested in a mixture of concentrated nitric and hydrochloric acids. Samples were then scanned for the presence of aluminum, antimony, arsenic, barium, beryllium,

bismuth, boron, cadmium, calcium, chromium, cobalt, copper, iron, lead, lithium, magnesium, manganese, mercury, molybdenum, nickel, potassium, selenium, silver, sodium, strontium, tellurium, tin, uranium, vanadium and zinc. Background samples (28) were also analyzed to establish the non-anthropogenic or natural level of metals in soils within the same geological formation as the training area. The results reported in this study are those which exceeded background concentrations.

2.5. *Toxicity measurements—the L-929 growth inhibition assay*

Cells were counted in a hematocytometer and 3000 cells/well were seeded in 96-well microplates (Falcon) and preincubated for 24 h in a humidified 37 °C atmosphere containing 5% CO_2. Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum (HyClone, FRA), 1% non-essential amino acids (Gibco, UK), 1% L-glutamine (Gibco, UK) and 0.5% gentamicin (Gibco, UK) were used as growth medium. Exposure (72 h) started when medium were removed and replaced by fresh EMEM containing water based soil extracts corresponding to liquid/solid (L/S) ratios of 3, 6 and 12 for PLE-extracts and the lowest obtainable L/S = 12.5 for the EN-12457-3 extracts. In all readings, acryl amide, EMEM and PLE extracts of acid washed sand were positive and negative controls and blanks, respectively. Cytotoxicity was measured by the neutral red incorporation assay originally described by Borenfreund and Puerner [22] and later adapted to L-929 cells [23]. After exposure, cell cultures were incubated with neutral red dye solution (50 μg/ml) for 3 h and quickly fixed in 4% formaldehyde/1% $CaCl_2$. After neutral extraction in 1%/50% acetic acid/ethanol-solution, cell viability were measured spectrophotometrically at a specific wavelength of 540 nm. Low cell viability, hence low neutral red uptake results in a correspondingly low optical density.

3. Results and discussion

Soil concentrations of explosives and related compounds are reported in Table 1. Regardless of the differences observed between each extraction technique, it can be seen that HMX (40–200 μg/g) was the most abundant explosive found in the samples from the anti-tank rocket range target area (S1 and S2). This is in agreement with results from samples collected at a similar range at CFB Valcartier, Quebec, Canada [5]. The composition of most of the explosives used at these sites was Octol, (70–75% HMX, 30–25% TNT). The relatively low levels of TNT (0.3–0.5 μg/g) found in the Petawawa samples as compared to the theoretical ratio is probably due to the fact that TNT is known to rapidly disappear in clayish and/or organic rich soils due to sorption of TNT and its degradation products [24,25].

HMX (<2.1 μg/g) and 2,4-DNT (0.8–1.7 μg/g) were the most abundant analytes in sample S3. The presence of DNT in S3, which is a firing position, was expected because of its use as a propellant stabilizer. However, the reported concentrations of HMX in S3, using LC–UV might be a false positive since HMX is part of the detonation charge and should not be found at the firing position. It is further an early eluting compound

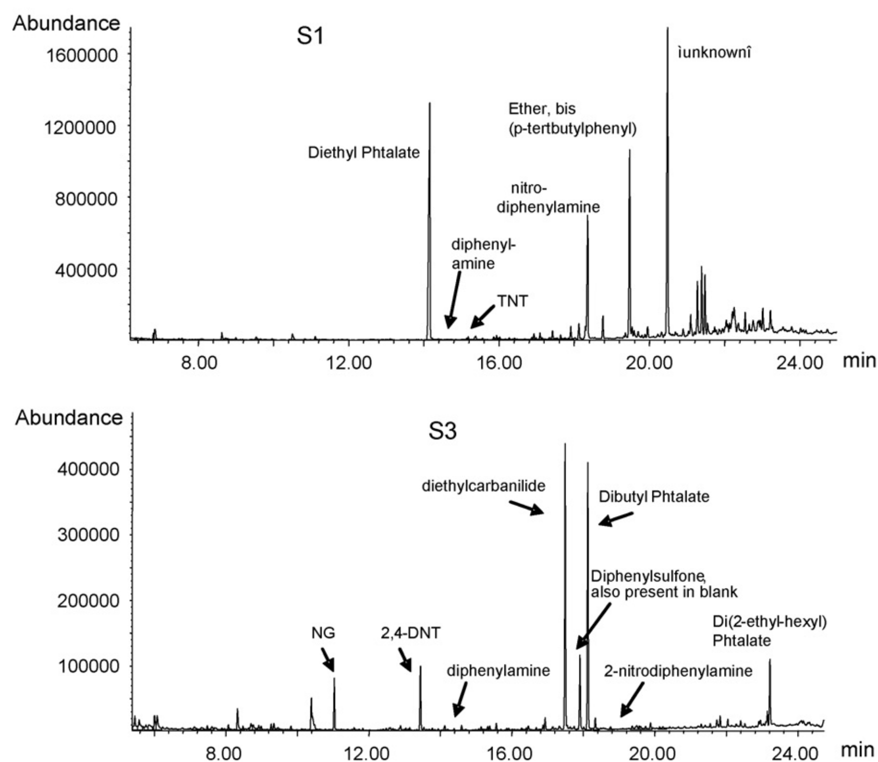

Fig. 1. Total ion chromatogram obtained by GC–MS from samples collected at the detonation area (PS1) and firing position (PS3). NG: nitroglycerine; 2,4-DNT: 2,4-dinitrotoluene.

where interfering sample constituents usually appears in the chromatogram. This possibility could have been checked by using a second, confirmation column or mass spectrometry. Nevertheless, essentially the same compounds were identified by the reference laboratory (see Table 1) with the exception that tetryl and 2,6-DNT were detected at low concentrations in sample S2 and S3, respectively. Although more replicates would be needed to draw statistical conclusions, it can be noted that samples extracted with water at room temperature (EN 124 57-2) tend to give lower concentrations of HMX, RDX and TNT than the other extraction methods. This result is not surprising considering the low water solubility of many explosive residues. However the higher solvent temperature in PLE, which increases the solubility of the analytes, and the combination of static and dynamic modes, can probably overcome the problematic low water solubility of most of these energetic residues, if not all of them.

The total ion chromatograms (TICs) generated by GC–MS for samples S1 and S3 (Fig. 1) revealed significant levels of several munition-related substances (additives) such as the stabilizers diphenylamine (DPA) and diethylcarbanilide (also known as centralite I), and plasticizers such as dibutyl-, di(2-ethylhexyl)- and diethyl-phthalate. Unlike the explosives, additives are not designed to completely combust during detonation and/or deflagration, but to form stable secondary compounds during deflagration by binding decomposition products or to enhance storage or casting. Residues of these additives are therefore likely to be found at sites where explosives have been used. In addition, nitroglycerine, which is a major component in double base-propellants but is not covered by the US EPA 8330 protocol, was also found (as expected) in the GC–MS analyses of sample S3. The metal concentrations, shown in Table 2, show elevated levels of principally lead, cupper and antimony which are consistent with other reports from shooting ranges [3].

The difference of the water extracts were even more obvious while studying the total exposure concentrations (see Table 1) where the batch leachates were roughly 10-fold lower (e.g. 4.2 μg/ml versus 36 μg/ml and 3.5 μg/ml versus 33 μg/ml for S1 and S2, respectively) based on the total concentrations of explosives according to the LC–UV analysis. The difference in total concentrations of explosive residues obtained by the two extraction methods was also apparent in the toxicological measurements. As can be seen in Fig. 2, no significant acute cytotoxicity was indicated on exposure to EN-124 57-2 extracts at the L/S-ratio of 12.5 (which was the lowest possible) while the higher L/S-ratios corresponding to 3 and 6 of PLE-extracts of all three samples showed acute cytotoxicity. These results highlight the advantage of PLE water extraction over EN-124 57-2

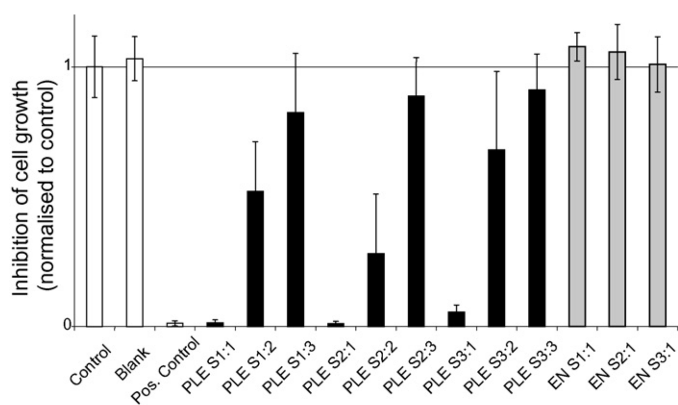

Fig. 2. Measurements of relative toxicity of water extracts in L-929 cell cultures. Values are from six replicates with 95% confidence intervals. PLE: pressurized liquid extraction; EN: batch leaching method EN-124 57-2. For PLE the L/S-ratios 3, 6 and 12 corresponds to 1, 2 and 3 and for EN an L/S-ratio of 12.5.

for producing samples with contaminant concentrations high enough for toxicity assays. The PLE-extracts are further produced within 20 min as compared to 24 h for the batch leaching method.

The positive toxicity response to sample S3 (firing position) may seem anomalous as the concentration of explosives and metals was relatively low in it and we speculate that the additives might be responsible for the outcome. In Table 3, identified explosive residues and their related compounds are listed alongside published toxicological data and their respective water solubilities. In summary, the toxicological data in Table 3 indicate that the explosives are more toxic than the identified additives in various cytotoxicity and rat LD50 assays, but the toxicity of the explosives and additives are in the same range in aquatic assays with the water flea (*Daphnia magna*). The reported water solubilities vary among the substances, but they are essentially in the same range, indicating that additives and explosives should be extracted by the PLE-water method. Obviously, it is difficult to establish a causal relationship between quantified exposure levels and toxicity but the significant amounts of additives detected in the samples (especially sample S3) as shown in Fig. 1 were quite convincing.

The investigated extraction technique is primarily intended to increase the speed of the screening phase of soil samples from live-fire training ranges in order to identify high risk zones and to focus the analytical cost on relevant samples. Extracts obtained by the PLE-water method could also be used in other water-based toxicity assays and a number of biological tests representing different trophic levels would enhance the relevance to account for different sensitivities between species (as shown by

Table 2
Metal concentrations (mg/kg) exceeding background concentrations in the Petawawa area

	Cu	Pb	Sb	Mb	Cd	Ni	Sr	Sn	Zn	Ag
Background	31	44	2.5	0.9	0.6	24	27	ND	82	1.7
S1	330	660	6	1					120	
S2	835	14600	314	2.2	1	30	104	5.41	250	8.3
S3	434	100	3.5							

ND: not detected.

Table 3
Toxicological data for explosives and identified additives

Substance	Toxicity data			
	Water solubility (mg/L)	LD_{50} rat (mg/kg b.w.)	EC_{50} *D. magna* (mg/L)	Cytotoxicity, IC_{50} on mammalian cell culture (μg/L)
HMX	6.6[a]	6490	32	>22[b]
RDX	38.4[a]	100	15	>180[b]
TNT	130[a]	607	11.9	33[c], 22–197[b], 4–24[d]
2,4-DNT	300	268	35	439[c], 98–460[e]
NG	1305	105	32	nd
DPA	40	1165–2000	7.0	nd
2-Nitro-DPA	27.7	nd	nd	nd
Diethylcarbanilide	4.8	2750	nd	nd
Diethyl phthalate	1080	8600	52	>1000
Di(*p*-tertbutyl-phenyl) ether	nd	nd	nd	nd
Di(2-ethylhexyl) phtalate	0.34	3000	9.4	>1280[f], 700[g], ≥1000[h], 25[i]

All other toxicological data from Chemical substances database 11.0, Prevent AB [36]. nd: no data.
[a] Talmage et al. [35].
[b] Lachance et al. [27].
[c] Wellington and Mitchell [28].
[d] Honeycutt et al. [29].
[e] Perchermeier et al. [30].
[f] Gyu Seek Rhee et al. [31].
[g] Phillips et al. [32].
[h] Dees et al. [33].
[i] Rothenbacher et al. [34].

Robidoux et al. [26]). However, we recognise that validation of the PLE-screening technique, preferably by running it parallel to traditional methods in an environmental risk assessment of a live-fire training range is needed to draw a clearer conclusion of its full potential utility.

4. Conclusions

The present study has demonstrated the potential utility of pressurized liquid extraction with water as a rapid technique to produce extracts that are suitable for both direct assaying of toxicity and chemical analysis to speed up the screening of soil samples from live-fire training ranges. The extraction efficiency and the possibility of yielding extracts with a low L/S-ratio should be beneficial in obtaining results that may reflect worst case scenarios in terms of ecotoxicological impact, even though validation in a real environmental risk assessment is needed. Furthermore, a number of substances related to explosives, not usually covered in the assessment of these sites, were also identified. The complexity of these samples thus highlights the need to combine toxicological and chemical analyses when screening large live-fire training ranges.

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