VALIDATION OF A TWO-PHASE BIOASSAY FOR RISK ASSESSMENT OF CONTAMINATED SOILS

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This paper summarizes a 45 credits Master's degree project in Environmental Chemistry conducted during the period November 2010 – September 2011 at the Environmental Chemistry laboratory at Umeå University, Department of Chemistry, and the environmental consultant company "Pelagia Miljökonsult AB" in Umeå.

Abstract

A modification of the acute toxicity test with *Daphnia magna* has previously been developed at the environmental consultancy company Pelagia Miljökonsult in collaboration with researchers at Department of chemistry, Umeå University. In this test, a two-phase system is used instead of the aqueous leachate used in the standard *Daphnia magna* acute toxicity test. In the present study, the two-phase test is further evaluated in order to figure out its advantages and disadvantages compared to the standard test. The study includes a thorough follow up of several soil samples from a former wood preservation site in a chemical and ecotoxicological evaluation. Multivariate methods are used to correlate the toxic response (EC50) in the two-phase test with the pollutants analyzed (PAHs, Oxy-PAHs, azaarenes, and metals). The results indicate a moderate correlation between the total amount of PAHs and Oxy-PAHs, and the toxic response (i.e. a negative correlation with the EC50-value), but no correlation of the EC50 with the total amount of metals. Comparing the standard test against the two-phase test, the results show that the toxicity is mainly associated with the particle bound contaminants, as no response is observed when the water leachates of the soil are tested. However, after extraction of the lipophilic contaminants from the soil using cyclohexane at moderate temperature (50°C), the toxicity is removed from the soil, indicating that the toxicity is not due to the particles themselves. After reapplication of the extracted contaminants, the toxicity was completely recuperated; although the particle bound toxicity was greatly affected by the content of organic matter (OM) in the soil, limiting the availability of PAHs and oxy-PAHs. Thus, we believe that the modified ecotox test gives a better estimate of the soil toxicity and should replace the standard test performed on water leachates of the soil.

Content

AIM

The aim of this study was to evaluate a two-phase version of the *Daphnia magna* immobilization test[1] on soil samples collected at a former wood-preservation site, which was heavily contaminated with PAHs, in particular, but also with metals. In addition, the underlying cause of the toxic response on *Daphnia magna* in the two-phase test was to be elucidated.

More specifically, the following issues were addressed:

- How does the toxicological response (EC50) in the two-phase test correlate with the contaminant contents (content of PAHs and other contaminants)?
- Are there any other factors, such as the availability of the contaminants that influence the response?
- Will the two-phase test give a different response compared to a standard *Daphnia magna* test performed on the aqueous leachate of the soil? (Is the toxicity of the soil particle bound or is it water soluble?)
- Will the particles after extraction with cyclohexane cause toxic response, or is the toxicity due to lipophilic contaminants adsorbed to them?

INTRODUCTION

Risk assessment of contaminated sites: chemical analysis versus bioassays

Risk assessment of contaminated sites is usually performed through chemical analyses of contaminants that are expected to be present at the site, and by comparing these values to concentrations that are assumed to cause no adverse effects, under the no observed effect level (NOEL)[2].

While chemical analyses are faster and give a concentration value more precise than biological testing, they have been unable to resemble environmental availability or bioavailability[2]. Chemical analysis are also limited to a pre-specified number of compounds that can be extracted and quantified with the chosen analytical methods, and leave out all other potentially toxic compounds present[2]. Furthermore, effects caused by mixtures of compounds cannot be estimated in a compound by compound chemical risk assessment, and potential contributions to the overall effect from individual contaminants below the NOEL or below the analytical detection limits are not taken into account[2].

Consequently, bioassays are recommended as a complement to chemical analysis in risk assessments. The advantage of biological methods is that they estimate and show the effect of the direct exposure upon a biological system, giving indications of the combined toxicity of all compounds present as well as of their bioavailability. On the other hand, bioassays are limited by the test organism's sensitivity, meaning that a battery of bioassays (including different endpoints and different organisms representing different trophic levels) would need to be performed since not one biological test can predict the hazards for all other species in the environment[3].

Therefore the recommended risk assessment must include both, bioassays and chemical analysis at least in the screening phase and during determination of the pollutant's bioavailability.

Bioassays for soil toxicity: advantages and limitations

For the assessment of soil samples, biological testing have most often been performed on water leachates of the soils, assuming that only dissolved contaminants are available for uptake in organisms and that sorbed contaminants are too immobile to reach surface waters and groundwater, and therefore the human food chain[4]. Additionally there are very few standardized tests for soils available, while there are numerous water based tests. Consequently, some aquatic bioassays have been adapted to be used for soils as well, using soil leachates. This was done for simplicity, but also because bioassays on the whole soil matrix may be more expensive, as well as time and space consuming[5]. Leachates are also used in an attempt to simulate environmentally available pollutants, although various studies have suggested that leaching and extraction steps preceding the biological test can both over and under estimate the availability of the contaminants in the soil. A water leachate can underestimate the bioavailable fraction, while an extract using an organic solvent can overestimate it, or even lead to a toxic response due to the organic solvent itself[6].

Tests of particle/soil suspensions could be a useful compromise in the adaptation of standardized aquatic bioassays. As an example it has been observed that when testing algal growth inhibition, the toxic response caused by soil suspensions was much higher than that of water based leachates[6].

In order for an ecotoxicological test to be relevant and widely used in risk assessment, a few considerations must be kept in mind such as: to use ecological relevant species with high sensitivity, that the biology is well known, that the organism is available year around and is easily bred, and that the test is cost effective and standardized. The *Daphnia magna* acute toxicity test fulfills all these requirements and it has so far been the most frequently used aquatic bioassay.

The Daphnia magna acute toxicity test

As it was mentioned previously the *Daphnia magna* acute toxicity test has been used as a standard method for risk assessment of soils by testing the aqueous leachate.

Daphnia magna is a freshwater crustacean with an adult size of 2-5 mm and an average life span of 40 days[7,8]. *Daphnia magna* is a very sensitive species, as an example it reproduces asexually through parthenogenesis under optimal environmental conditions, while stressful conditions cause sexual reproduction and production of ephippia (resting stage eggs)[9].

The optimal growth conditions for daphnids is within a pH range of 7- 8.6, a temperature between 20-25ºC, dissolved oxygen (DO) >6 mg/l, and water hardness 160-180 mg $CaCO_3$/l [7].

The *Daphnia magna* acute toxicity test follows the procedures of the international standard ISO 6341[1]. The test is applicable to chemical substances, industrial or sewage effluents, waste waters, aqueous extracts and leachates, fresh water, eluates of fresh water sediments and pore water[1]. The soil aqueous leachates are used to characterize the water soluble pollutants[5]. The endpoint of this test is mobility inhibition after 24 hours, which is measured as the concentration of the soil, in a specified volume of dilution medium, which aqueous phase alone immobilizes 50% of the exposed daphnids (EC50)[1].

Two-phase toxicity test with Daphnia magna

Although *Daphnia magna* is a water dwelling species, it can be useful for soil testing as well, at least if it is performed in a two-phase system (soil suspension). An important characteristic of *Daphnia magna* is that it feeds unselectively on suspended particles in the water by filtering large volumes of water and retaining any particle over 0.45 μm up to 50 μm [8,10]. Also when lacking of food they can stir the sediments to feed on the resuspended particles[10]. Daphnia does not digest the sediments but keeps them in its gut for a long time unless new food is provided[10]. This is fundamental for the developed two-phase variant of the *Daphnia magna* acute toxicity test, considering that the possible exposure routes are the contact with the dissolved phase, and dietary uptake through the ingestion of water and particles[11]. There are also advantages of using a well investigated and standardized organism like *Daphnia magna*, and additionally the *Daphnia magna* acute toxicity test is also a relatively cost effective method.

Some studies have shown that biological testing of aqueous leachates of soils contaminated with PAHs has low relevance. Bispo et al.[5] observed that no toxic response was observed in *Daphnia magna* when testing aqueous leachates of a former coke oven soil, and even though the other tested organisms in that study (*Thamnocephalus platyurus* and *Vibrio fischeri*) showed higher response to the aqueous leachate compared to *Daphnia magna*, the overall response of all these organisms to aqueous leachates was significantly lower than the response to methanol leachates (methanol leachates were used in that study to evaluate the less soluble and soil-bound pollutants)[5]. The lack of response in *Daphnia magna* to water leachates strongly discourages using the standard method for risk assessment of such soils. However, using a soil suspension in a two-phase test would probably enhance the sensitivity and the relevance of the *Daphnia magna* test.

In the two-phase acute toxicity test with *Daphnia magna* the soil particles are suspended in the water based medium, and daphnids are exposed to the suspension rather than to the aqueous leachate alone. Besides the sample preparation as a suspension instead of a water leachate, the following steps proceed as it is described in the standard *Daphnia magna* acute toxicity test[1].

In previous studies at Pelagia and Umeå University, the two-phase test has shown good correlation between the PAH-content of the soil and the toxic response[12].

The soils to be used for validation of the test

- **Wood preservation site**

In this study, various soil samples from a former wood preservation site in Holmsund, Västerbotten County, Sweden, were used to evaluate the two-phase test. The contaminants at the site mainly consists of PAHs and various metals, such as copper (Cu), chromium (Cr) and arsenic (As), all originating from wood preservation agents used at the site, i.e. creosote and CCA salts respectively. Impregnation at this site was done with CCA salt since the beginning of operations in 1944 until 1981, and with creosote since 1953 until 1976 [WSP-rapport 2007].

- **PAHs, Oxy-PAHs and azaarenes**

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds composed of two or more fused benzene rings, of which those with up to 7-rings are environmentally most relevant. Several PAHs have

been identified as acutely toxic, carcinogenic, mutagenic, and teratogenic[2]. PAHs occur naturally in oil, coal, and tar deposits, and are formed as byproducts during incomplete combustion of fuel (whether fossil fuel or biomass)[2]. Creosote, a coal tar distillate, is used in the impregnation of wood and has high quantities of PAHs, phenolic compounds, and N-, O- , and S-heterocyclics, which represent an environmental hazard[13].

Alkylated PAHs, heterocyclic PAHs (N, S, O), and oxidation products of PAHs (oxy-PAHs) are referred as polycyclic aromatic compounds (PACs), and are usually found associated with PAHs as they share the same sources [2].

In the environment PAHs are generally found adsorbed to particles and humic matter (organic matter), due to their lipophilic character, so increasing levels of organic matter result in decreased bioavailability[14]. Low molecular weight (LMW) PAHs (2-3 rings) are somewhat more water soluble and volatile, and hence more available than high molecular weight (HMW) PAHs that are more strongly bonded to particles[2]. The higher availability of LMW PAHs makes them more susceptible to environmental transformation processes such as biodegradation and photooxidation[2].

PAHs with octanol-water partition coefficients (Kow) up to 5.2 have shown acute effects on soil dwelling organisms such as *Folsomia fimetaria*, but this behavior was not observed for PAHs with higher Kow[15]. A possible suggested explanation was the limited water solubility in pore-water (as Kow is positively correlated with hydrophobicity) that reduced the bioavailability of compounds[15]. Another study by Lors et al.[16] on aqueous soil leachates using *Daphnia magna* also suggests that the acute ecotoxicity is directly associated with the content of 2- and 3- ring PAHs[16].

Oxy-PAHs are defined as PAHs with one or more carbonylic oxygen attached to the aromatic ring structure. They may be emitted from the same sources as PAHs, but may also be formed through transformation of PAHs in the environment[17]. Oxy-PAHs are generally more mobile in the environment than PAHs, but similar to PAHs; lighter oxy-PAHs are more mobile than heavier ones[17]. It has been observed that many oxy-PAHs accumulate during degradation of PAHs in soil (biological or photo-oxidation), posing another environmental threat[17].

Oxy-PAHs have shown to be acutely toxic to the bacteria *Vibrio fischeri* and to *Daphnia magna* [17,18]. As an example 9,10-phenanthrenequinone has shown greater toxicity than its parent compound in invertebrate assays, and it can be easily formed under sunlight[18,19]. Oxy-PAHs have also shown mutagenic effects, although they are usually less potent than unsubstituted PAHs[17].

Azaarenes or N-PAHs are a family of heterocyclic PAHs that contain one or more nitrogen atoms in the aromatic rings. They are usually present at somewhat lower concentrations than the PAHs but they have higher water solubility and are therefore more mobile and bioavailable. Some N-PAHs have been found to be mutagenic, carcinogenic, teratogenic and genotoxic, but so far the ecotoxicological studies have focused on the low molecular weight azaarenes, leaving a lot more to be investigated regarding their toxic potential [20].

The molecular structure of the organic contaminants analyzed during this study is presented in Figure 1 (PAHs and alkylated PAHs), Figure 2 (oxy-PAHs), and Figure 3 (azaarenes).

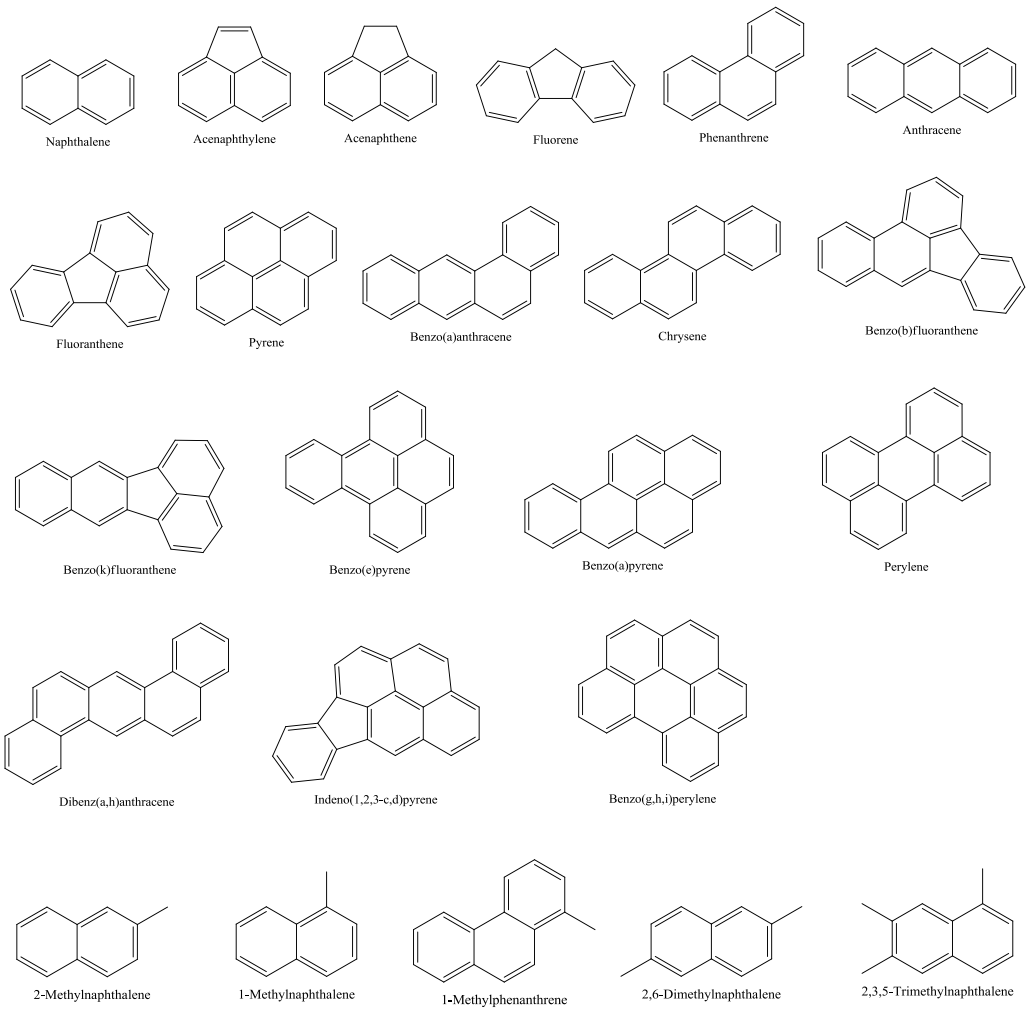

Figure 1: PAHs and alkylated PAHs measured and evaluated in the validation of the two-phase ecotox test with *Daphnia magna*

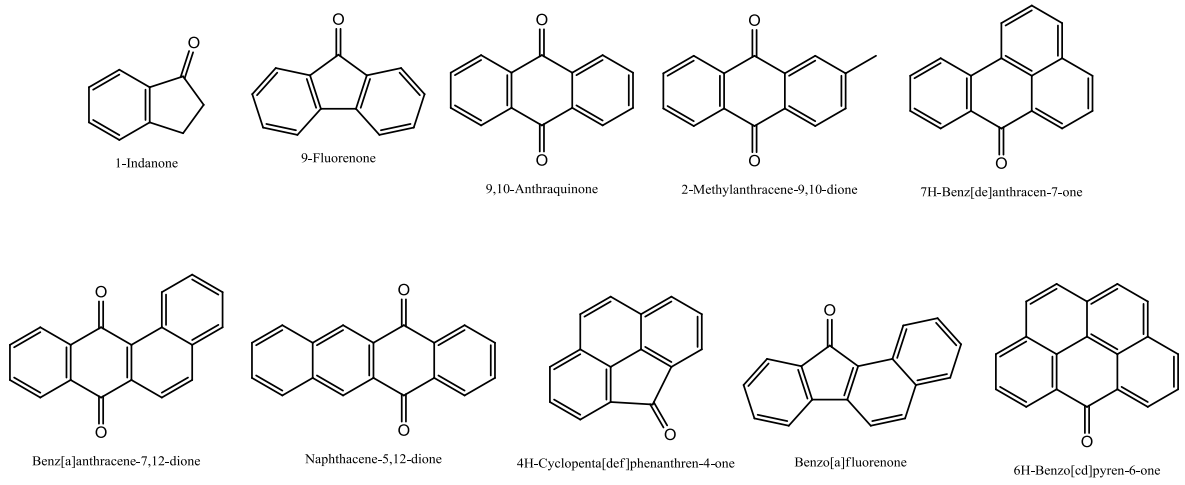

Figure 2: 10 Oxy-PAHs measured and evaluated in the validation of the two-phase ecotox test with *Daphnia magna*

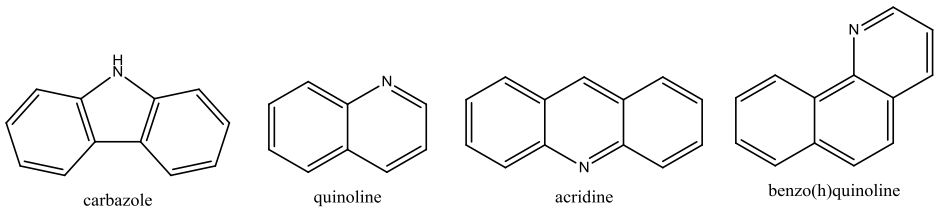

Figure 3: Azaarenes measured and evaluated in the validation of the two-phase ecotox test with *Daphnia magna*

- **CCA/metals**

Chromated copper arsenate (CCA) is a wood preserving agent that commonly contains Cu (II), Cr (VI) and As (V), as the major active components[21]. All these metals may be of environmental concern, but particularly chromium and arsenic may cause negative effects for humans and the environment. For instance, arsenic and chromium have proven to be carcinogenic to humans and when released in soil As and Cr have higher mobility than Cu[3,21]. Even though Cu has shown to be much less toxic, synergistic and additive effects have been observed between Cu and 1,2-dihydroxianthraquinone, Cu and 9,10-phenanthrenequinone, and Cu and phenanthrene, in previous studies[19].

Metals availability in soils is dependent on several factors such as content of clay-size mineral particles, organic matter (OM) content and pH[3]. Clay minerals and OM offer surface areas with negative charges that bind to metals limiting its availability[3]. Usually when pH decreases metal solubility increases, but As mobility is increased as pH increases[3].

MATERIALS AND METHODS

Six soils samples from the wood preservation site in Holmsund were used for the validation of the method. The criteria for the selection of soil samples were to cover a wide range of PAHs content and to use surface soils only (see Table 1). Soil 4 was obtained by mixing two other different soil samples from the preservation site, in order to obtain an intermediate concentration within the range of PAHs.

Table 1 : List of soil samples and some of its characteristics used in the validation of the two-phase ecotox test

Soil sample code	Sample depth	Dry content %	Org matter % d.w.	Sum 16 US EPA PAHs (mg/Kg)*
1	10-30 cm	93	2.9	1.9
2	10-20 cm	96	3.8	5.4
3	20-60 cm	93	21	54
4	Mix (20-30 cm and 0-1 m)	94	5.1	710
5	10-20 cm	95	7.9	1100
6	20-30 cm	99	4.8	3400

*results from measurements performed in this study as described in the Results and Discussion section

The experimental procedures performed in this study are briefly described in the following paragraphs and a schematic flow chart is shown in Figure 4.

Firstly, for the soil sample preparation, soils were sieved and the fine fraction was used for all analysis and tests in this study. Organic matter and dry content were also measured in this step.

Secondly, a chemical characterization was performed measuring several organic and inorganic contaminants. Among the organic contaminants PAHs, oxy-PAHs and azaarenes were measured, both as total contents and as the chemically estimated bioavailable fraction. Among the inorganic compounds, a number of metals of environmental significance were measured.

Thirdly, ecotoxicological testing was performed using three groups of tests. This started with the developed Two-phase test with *Daphnia magna*, where the results (EC50) were correlated with the content of contaminants, using multivariate analysis.

Afterwards, a comparison of toxic responses between the standard testing of aqueous leachates and the Two-phase test with Daphnia magna was performed. This was followed by a resuspension of the leached particles and repeated testing using the two-phase approach in order to see if the toxicity was kept with the particles or drawn with the aqueous leachate.

Finally, the organic contaminants were extracted from the soil with cyclohexane, after which the two-phase test method was applied on the extracted soil. This was done to investigate if the toxicity was due to the lipophilic contaminants in the soil or to the particles themselves. The lipophilic contaminants in the cyclohexane extract were then reapplied on the extracted soil, which was tested again with the two-phase ecotox method to see if the toxicity was coming back with the reapplied lipophilic contaminants.

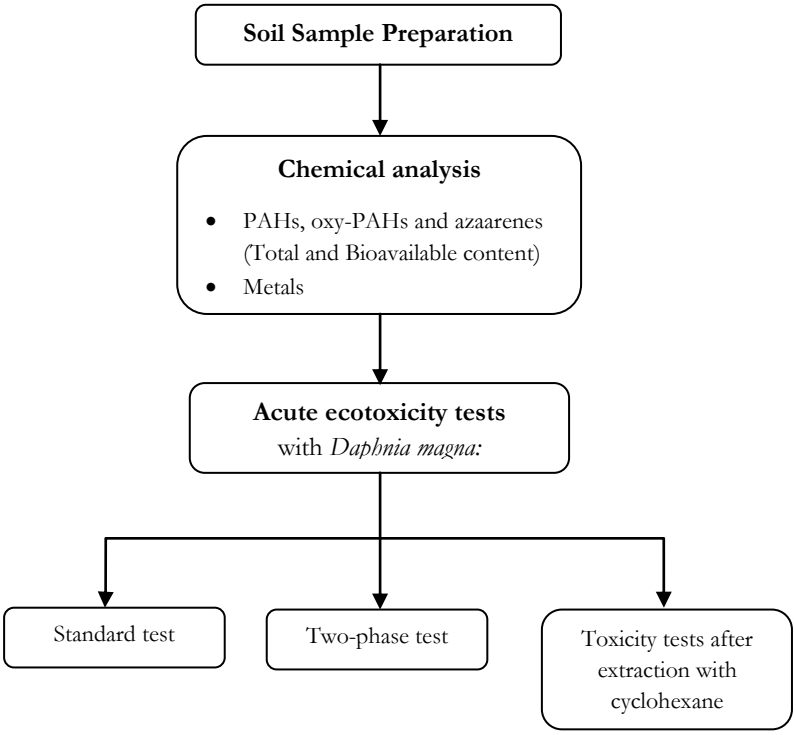

Figure 4: Flow chart of experimental procedures performed in this study for the validation of the Two-phase ecotox test with *Daphnia magna*

Soil sample preparation

As a first step for the sample preparation, soils were sieved using a 0.5 mm mesh prior to any test or analysis. The fine fraction was used to ensure a homogenous subsampling for the chemical analysis and ecotoxicological tests, and a reproducible dilution of the slurry in the Two-phase ecotox test. Also, as PAHs accumulate on smaller particles, the small size is also beneficial for the diffusion of the solvent[22].

The dry weight of the soils was determined by treating sub-samples of them at 105 ºC for 24 hr after which the weight loss was measured[23]. To estimate the organic matter content the method of Loss-on-ignition (LOI) was used[23]. The soil sample was heated at 550ºC for 3 hours (organic matter is oxidized at 500–550 °C to carbon dioxide and ash) and the weight loss was measured and presented as percentage mass loss[23]. LOI at 550 ºC is considered a simple method to estimate OM but other reactions may take place at this temperature in the sample, causing an overestimation of OM[24]. Nevertheless LOI is still suitable to study differences in OM, and in this case, where soil samples were obtained from the same site and sieved at the same particle size before LOI procedure, we will assume LOI at 550 ºC as OM content.

Chemical Analysis of PAHs, Oxy-PAHs and Azaarenes

The chemical analysis was performed according to Figure 5. It describes at the same time two extraction processes, one for the determination of total contents and one for the chemical estimation of the bioavailable fraction. Both extraction processes follow the same fractionation steps for clean-up and separation of the PAHs, from the oxy-PAHs and azaarenes, before instrumental analysis.

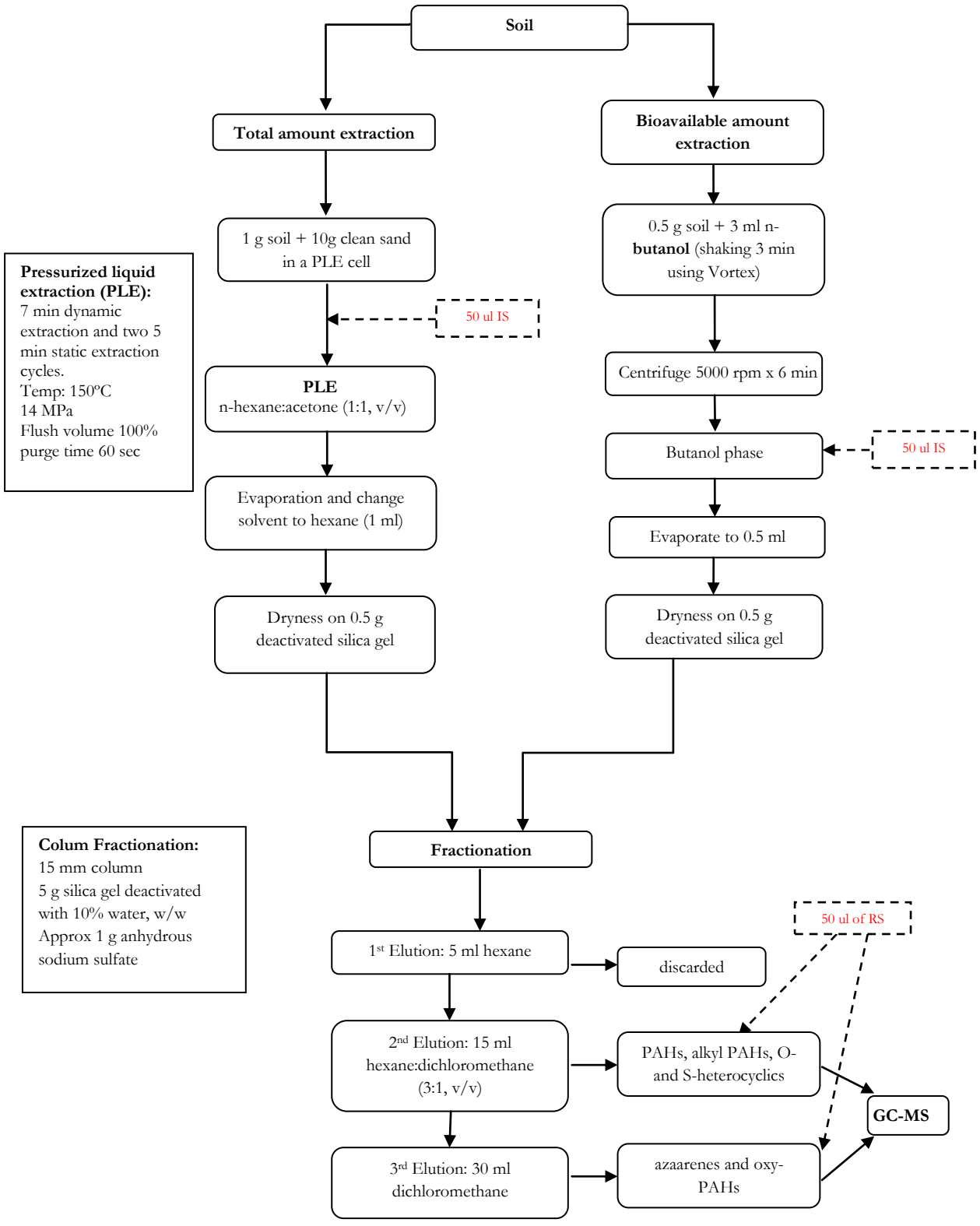

Figure 5: Schematic of the chemical analysis of PAHs, Oxy-PAHs, and azaarenes. The two extraction processes used for measurement of Total amounts and the Bioavailable fractions, respectively, are described in parallel to each other. The following fractionation scheme and the instrumental analysis is identical for both procedures.

For the determination of total content, Pressurized Liquid Extraction (PLE) with a binary solvent (n-hexane:acetone 1:1) was used[25]. This technique ensured efficient release of all PAHs, oxy-PAHs and azaarenes from the soil matrix[17, 25]. The solvent was evaporated using a rotavapor to approximately 1-ml, making sure that all acetone is evaporated by adding more hexane[26]. Before transferring the extract to the fractionation column, the extract was carefully added to a small amount of deactivated silica gel (0.5 g), letting it evaporate one drop at the time. The impregnated silica gel was then placed on top of a previously prepared fractionation column.

Concomitantly, a mild extraction method with butanol was used to extract the lightly adsorbed pollutants of the soil, as an estimate of the bioavailable fraction[27]. The butanol extraction was done at room temperature, and for duration of only 3 min under vortex treatment before centrifugation. In this case the internal standard (IS) was added after the extraction. Evaporation and transfer of the extract to the fractionation column was done in the same way described for the determination of the total content of the target compounds.

A cleanup step followed the extraction, using open-column chromatography with silica gel as the chromatographic material, and solvents of increasing polarity to elute PAHs, oxy-PAHs and azaarenes in different fractions[26, 28]. The fractions were analyzed by gas chromatography-mass spectrometry (GC-MS) [26].

Internal Standards (IS) were used to be able to compensate for the loss of target compounds during the sample preparation. A mixture of perdeurated PAHs was used as IS for the PAHs quantification: [$^2H_8$]naphthalene, [$^2H_8$]acenaphtylene, [$^2H_{10}$]acenaphthene, [$^2H_{12}$]chrysene, [$^2H_{10}$]fluorene, [$^2H_{10}$]pyrene, [$^2H_{10}$]anthracene, [$^2H_{12}$]benzo[k]fluoranthene, [$^2H_{12}$]benzo[ghi]perylene. For the quantification of oxy-PAHs the native 2,3-dimethylanthraquinone was used as IS for the measurements of total amounts, and [$^2H_8$]anthracenedione for the measurement of the bioavailable amounts. 2,3-dimethylanthraquinone was replaced as IS by [$^2H_8$]anthracene dione since the former was found to be present, in low amounts, naturally in the soils, and because isotopically labelled compound are generally better suited as IS[26].

A recovery standard (RS) is used to calculate the amount of IS lost during the analytical procedure[2]. For both Total and Bioavailable content determination the RS used was [$^2H_{10}$] fluoranthene.

Quantification was performed using certified reference standards, comparing the retention time and peak areas per each analyte[26]. Mixtures of reference standards were prepared separately for PAHs, oxy-PAHs and azaarenes.

The PAHs analyzed in the Bioavailable fraction were the 16 PAHs designated as priority pollutants by US-EPA[17]. The analysis for Total content included some more PAHs as well as some alkylated PAHs (see Figure 1). Ten oxy-PAHs (see Figure 2) and four azaarenes (Figure 3) were analyzed in both Total and Bioavailable measurements.

Trace Metal Analysis

The method used for the analysis of metals was method 3051A "Microwave assisted acid digestion of soils" US EPA[29], in which the sample is not completely decomposed (no usage of HF). Rather than measuring total concentrations obtained through a total decomposition method, for environmental purposes it is more useful to measure total recoverable amounts as obtained with the method 3051A[30].

In this study, after microwave digestion the supernatant was reduced carefully in an open vessel on the furnace at very low temperature to avoid loss of As. For the experimental procedure see Figure 6.

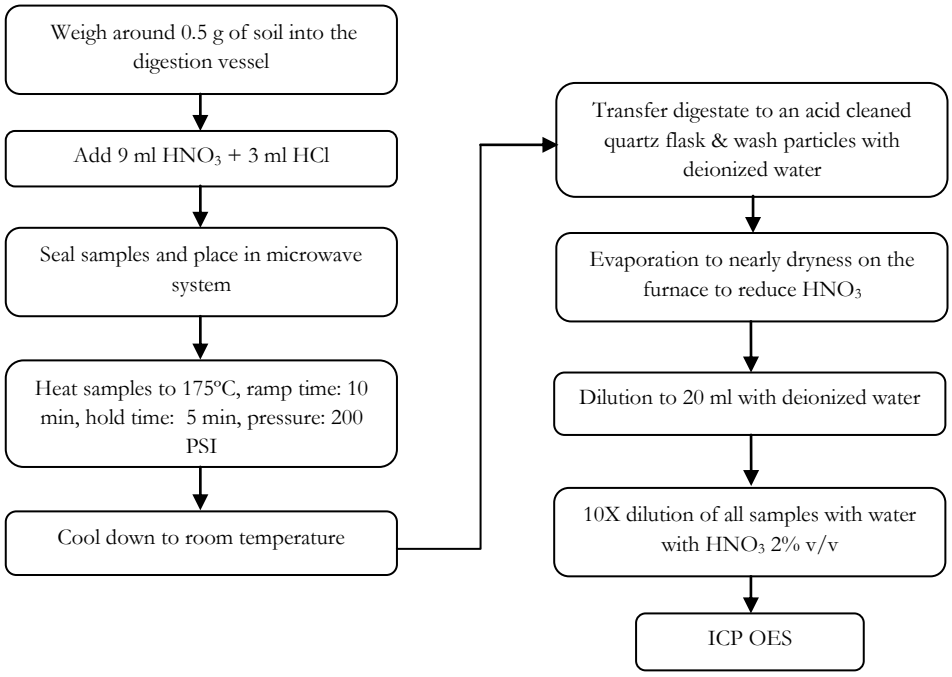

Figure 6 : Procedure for the trace metal analysis of the soil samples following method 3051A USEPA.

Two certified reference materials (MESS-3 and PACS-2) from the National Research Council Canada were used as quality control.

The metals measured in the soil samples were Zn, Cd, Cu, As, Pb, Cr, Hg. Certified reference solutions for those metals were used to prepare standards solutions of different concentrations for the calibration curve.

Two-Phase Ecotoxicological Test

The two-phase test is a variant of the standard acute toxicity test with *Daphnia magna*[1], which was developed in collaboration between Umeå University and Pelagia Miljökonsult AB, Umeå. The main steps in the procedure of this test are presented in Figure 7.

Dilution medium was fixed according to the formula: 0.33 g of sea salt + 2, 3 ml of 1.1 M $CaCl_2$ + 2,2 ml of 0,3M $NaHCO_3$ + 0,1 ml of 0,1M SeO_2 in 1 liter of distilled water[31]. This medium is not the standard recommended by ISO 6341, but it has given better results than the ISO medium in survival and reproduction tests with *Daphnia magna*[31].

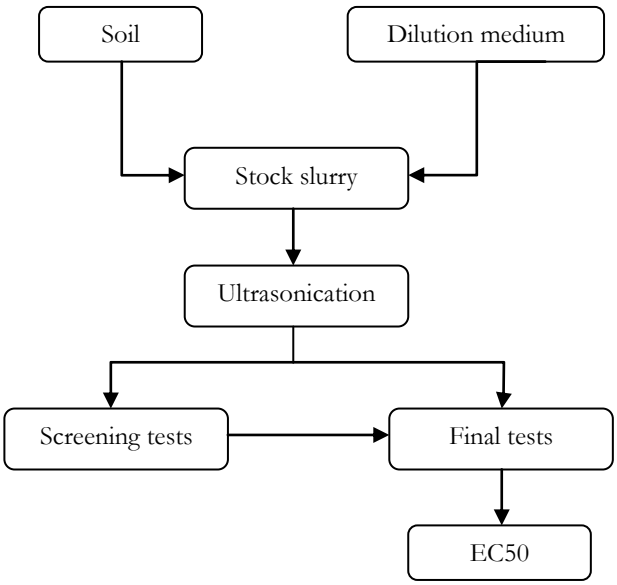

Figure 7: Schematic of main steps in the Two-phase ecotox method. Daphnids are added in the sample vessels of the Screening and Final tests for 24 hours exposure as described in the ISO 6341.

Stock slurries (medium/soil suspension) for the screening tests were prepared, containing 6g of dry soil and 36 ml of dilution medium. After mixing, the stock slurries were placed in an ultrasonic bath for 30 min to disperse soil aggregates and to homogenize the samples.

The screening or preliminary tests are used to determine the range of concentrations to test in the final test[1]. Samples for the screening test were prepared by diluting the stock slurry stepwise in a geometric progression (6X), at least in five levels. For each level, 30 ml suspension was used to expose 5 daphnids. No replicates were performed during the screening.

The final tests enable the determination of the EC50[1]. The screening tests give an estimate of the range of concentrations for the final tests, which should include an immobilization degree between 10% and 90% at least[1]. The stock slurry for the final test was prepared at the highest concentration to be tested according to the screening tests. The dilution of the stock slurry for the final tests was done in an arithmetic progression in this study. At least 5 different concentration levels were tested to construct the dose response curve, each level with 5 replicates consisting of 20 ml of the suspension for 10 daphnids.

All sample vessels were left to settle for 12 hours in darkness to stabilize the pH before exposure of daphnids. The temperature in the incubator was set to 20-22 ºC[1].

For all the ecotoxicological tests in this study, dormant eggs (ephippia) of *Daphnia magna* were used, since there is evidence that test organisms obtained from ephippia have similar sensitivity and precision to laboratory cultures[32]. Ephippia was obtained from MicroBioTests Inc.

Hatching of ephippia was carried out in a special chamber to keep constant illumination of around 6000 lux and temperature of 20-22 ºC [33]. Daphnids were collected after hatching and kept in beakers for 12 hours to guarantee an age between 12-24 hours before using them in the screening or final tests[1].

Young daphnids were exposed to the soil suspensions during 24 hours without feeding and in complete darkness[1]. After this period the daphnids were collected from the sample vessels into a watch glass for the observation of immobilization. Percentage of immobilization was determined after observing the number of daphnids that were not able to move within 15 seconds after gentle agitation, even if they were moving their antennas only[1].

A dose-response curve was constructed for each ecotox test, in which the X axis described the logarithm of the concentration, while the Y axis described the response as % mobile daphnids. The curves were generated by non linear regression analysis, sigmoidal curve with variable slope. The GraphPad Prism 5.0 software was used to plot the data and to calculate EC50 and its 95% confidence interval (CI).

When a screening test resulted in no mobility inhibition of daphnids at the highest concentration tested in this study (0.1667 g/ml), the final test was performed only at this high concentration to confirm the lack of toxic response.

Standard Daphnia magna Ecotoxicological Tests

The standard test consists of testing the toxicity of the supernatant of the soil slurry (aqueous leachate)[1]. The objective of this test was to compare the response of *Daphnia magna* using the Standard test and the Two-phase test, and to see if the toxicity was drawn with the aqueous leachate or stayed particle bound.

Samples 3 and 6 were chosen as representative samples because of their higher toxic response obtained in the Two-phase tests, and those were the only samples tested at this stage. Only one concentration level was used for all tests in this section, this was of 0.06 g/ml for soil 3 and 0.005 g/ml for soil 6.

The sample preparation procedure for the Standard test is shown in Figure 8. A concentrated soil suspension was fixed first and the supernatant was diluted in order to acquire the desired concentration level for soils 3 and 6 mentioned previously.

Additionally, the residual, washed out, particles from the Standard test were resuspended in dilution medium and tested with *Daphnia magna* in a two-phase system. The slurry was prepared and diluted to obtain the desired concentration level in each sample vessel (0.06 g/ml for soil 3 and 0.005 g/ml for soil 6). Figure 8 also shows the procedure to obtain the samples with resuspended particles.

In summary, three experimental set ups were tested alongside in this section:

- Two-phase test at the single concentration level (with untreated soil to compare toxic response values)
- Standard test (aqueous leachate)
- Test of the resuspended residual particles in a two-phase system

For each experimental set up in this section 10 replicates were used with 15 ml of solution or suspension for 5 daphnids.

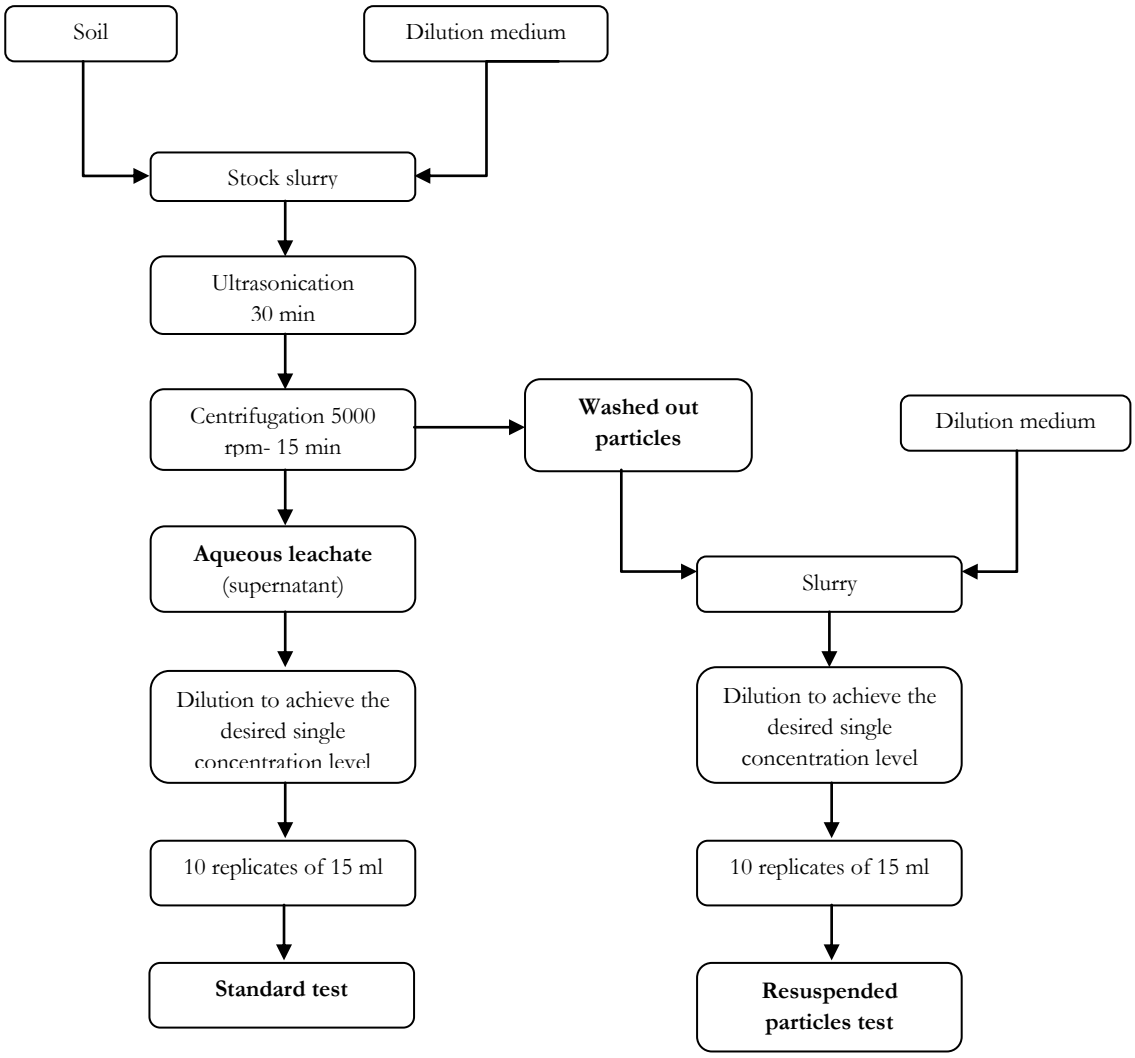

Figure 8: Procedure for sample preparation of the Standard test is shown in main column (left). Washed out particles were also resuspended to be further tested in a two-phase system, this sample preparation procedure is shown to the right of the standard test procedure.

Toxicity tests after extraction with cyclohexane

The objective of this test was to determine whether the toxic response observed in the Two-phase test was due to lipophilic compounds in the soil or to the particles themselves. Lipophilic compounds were removed from the particles through extraction with cyclohexane, according to the procedure described in Figure 9.

Sample 3 and 6 were chosen again as representative samples, and those were the only samples used in the experimental set ups of this section.

A soft extraction with cyclohexane at 50°C was performed on the soil sample. Initially, the extracted soil was tested for toxicity at a single concentration level in a two-phase system, soil 3 at the concentration of 0.038 g/ml and soil 6 at 0.06 g/ml. The toxic response (% immobilized daphnids) of the extracted soil was compared to that previously obtained in the Two-phase tests section.

Later on, the extract containing the lipophilic compounds was reapplied to the extracted soil, and the toxicity of the reapplied soil was tested following the procedure described for the Two-phase test to obtain a reapplied soil EC50. The steps in the sample preparation of the extracted soil and reapplied soil are described in Figure 9.

Spiking of the soil sample was done through a high volume of solvent method that results in a good distribution of the added compounds due to solvent permeation[34]. The ratio of solvent carrier volume to soil weight was 1:2 (ml:g). Mixing after addition of the solvent was done manually with a glass rod. The extract was added until slightly flooding the soil sample, letting the solvent evaporate for approximately two hours in order to add carefully the remaining of the extract. The spiked soil samples were left to dry under the hood for 72 hours.

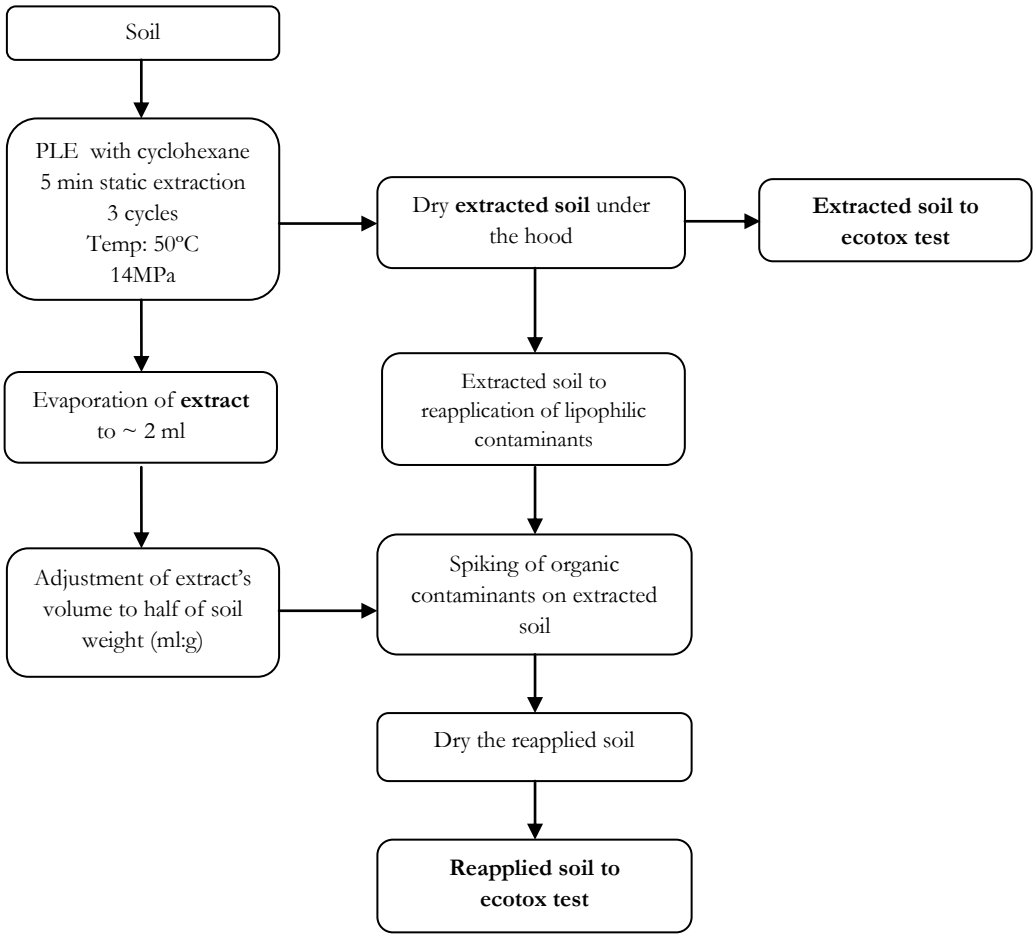

Figure 9: Schematic procedure for the sample preparation in the toxicity tests after extraction with cyclohexane. Two sets of samples are obtained: Extracted soil and Reapplied soil.

In this set of tests, 10 replicates with at least 10 ml of suspension for 5 daphnids were used at each concentration level tested. At least 5 concentration levels were tested to construct the dose-response curve of the reapplied soil.

Multivariate Analysis

Multivariate analysis has several advantages, it can handle many variables and many observations, it copes with multicollinearity (when variables are approximately linearly related as is the case with PAHs), it copes with missing data, it separates regularities from noise, and it provides diagnostic and graphical tools [35].

For this study the software SIMCA-P+ Version 12 was used. Data from the chemical characterization (Total and Bioavailable contents), along with the OM content, and EC50 of the Two-phase tests were compiled in an excel database, which was used as the work set for multivariate data analysis. All values under the limit of detection were considered as missing. All variables (measurements) were mean centered and scaled to unit variance (UV scaling option).

One of the tools used to evaluate the results of this study was principal component analysis (PCA) which is a statistic explorative tool used to identify clusters, trends and outliers within the observations and variables[35]. Principal components are latent structures that describe the variance of the data, the first principal component is a line that best describes the greatest variance in the data using the least squares method, the second principal component describes the second greatest variance in the data and is orthogonal to the first component, the following components are estimated in the same way[35]. R^2X is the variation in the matrix of variables (X-matrix) that is explained by each modeled component, this tells us how important is the component in describing the data.

Observations in this study are the soil samples (1 to 6), while variables are the measurements performed in the samples such as PAHs, Oxy-PAHs, azaarenes, metals, organic matter content and EC50. PCA was used to identify the predominant variables that characterized the soil samples. The results can be visualized with two plots; the loading plot and the score plot. The loadings plot shows the correlation between variables (measurements), while the score plot shows the correlations between observations (samples). Both plots are evaluated at the same time to elucidate how the observations and variables are connected. As an example, in a PCA with two principal components, observations positioned in a given quadrant of the score plot are positively influenced by the variables positioned in the same quadrant in the loading plot[35].

Loadings Bi Plots in PCA display the loadings and the scores expressed as correlation coefficients in the same plot. The interpretation of the relationship between variables and observations is easier in this plot as the scores and loadings are presented together superimposed. Observations situated near variables in the plot have high contents of these variables, while variables situated opposite to the observation in the plot are present in low amounts in the observation. Loadings Bi Plots were used for the characterization of the soils samples regarding their content of total PAHs, Oxy-PAHs, azaarenes, and metals.

PLS (projections to latent structures by means of partial least squares) is a method that relates two blocks of variables X and Y by a linear multivariate model [35]. The objective of PLS is to build a model that predicts Y (the response) based on X (process measurements). In this study the modeled response Y is the toxicity expressed as EC50, and all the measurements characterizing the samples constitute the X matrix.

OPLS (orthogonal PLS) is a variant of PLS that separates the systematic variation in X in two model parts, one that models the linear correlation between X and Y (predictive components), and one part that models the variation that is orthogonal or unrelated to Y (orthogonal components)[35]. OPLS is used

in a very similar way as PLS but gives only one predictive component for one response Y modeled, as in this study, which makes the evaluation much easier[35]. OPLS was used to correlate EC50 (Y) to the measurements of PAHs, Oxy-PAHs, azaarenes, metals and OM content.

RESULTS AND DISCUSSION

Chemical Analysis of PAHs, Oxy-PAHs and azaarenes

Chemical analysis of the soil samples confirmed that they represented a wide range of PAHs pollution, see Table 2. It should be noted that sample 4 was a mixed sample as it is mentioned in the Materials and Methods section, and its content of PAHs fits as an intermediate step in the range of concentrations. The overall results for dry content, organic matter content, PAHs, oxy-PAHs and azaarenes are listed in Table 2. The results for Total and Bioavailable concentrations of individual PAHs, oxy-PAHs and azaarenes in the soil samples are listed in Annex 1.

Table 2: Summary of overall results of the chemical analysis of PAHs, oxy-PAHs and azaarenes, along with the determination of dry weight content and organic matter content for the six samples evaluated in this study.

Sample	Dry content %	Org matter % d.w.	Sum 16 US EPA PAHs (mg/Kg)	Sum oxy-PAHs (mg/Kg)	Sum N PAHs (mg/Kg)
1	93	2.9	1.9	0.28	0.06
2	96	3.8	5.4	1.2	0.08
3	93	21	54	9.7	1.09
4	94	5.1	710	45	5.4
5	95	7.9	1100	98	1.9
6	99	4.8	3400	280	2.9

An observation that was noted was the very dark color of soil 3, which also had the largest content of organic matter. It is possible that these components of the soil affected the extractability as well as the mobility and bioavailability of the target analytes by strong sorption[36,37].

For characterization of the samples regarding PAHs content, a PCA Loadings Bi Plot is shown in Figure 10, where PAHs grouped according to the number of fused rings are correlated to the observations. The same procedure was performed to observe the predominance of oxy-PAHs and azaarenes in the soil samples as illustrated in Figure 11.

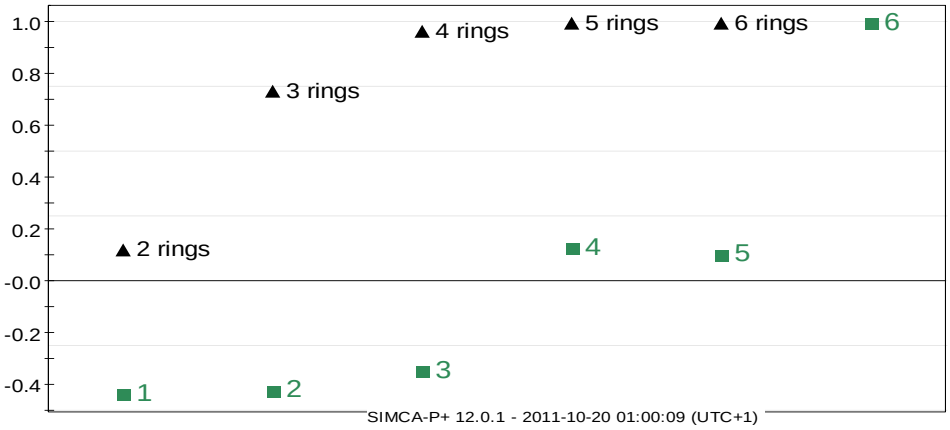

Figure 10: PCA Loadings Bi Plot for PAHs grouped by number of rings. Observations (soil samples) are presented with (■) while PAHs grouped by number of rings are presented with (▲). Only one principal component is significant and is presented in the Y-axis as correlation coefficients for both loadings and scores. Variables positively influencing observations are located close to each other in the plot with respect to the Y-axis.

The PCA of the PAH-concentrations (Figure 10) resulted in only one significant principal component with R^2X 70%. This first principal component emphasizes the difference between lightly polluted (1, 2 and 3) and highly polluted soils (soil 6). There is a higher predominance of HMW PAHs in the most polluted samples, meaning that as the contamination level in the soil sample increased also the content of HMW PAHs increased, being soil 6 the one with highest amount of 4-, 5-, and 6-ring PAHs.

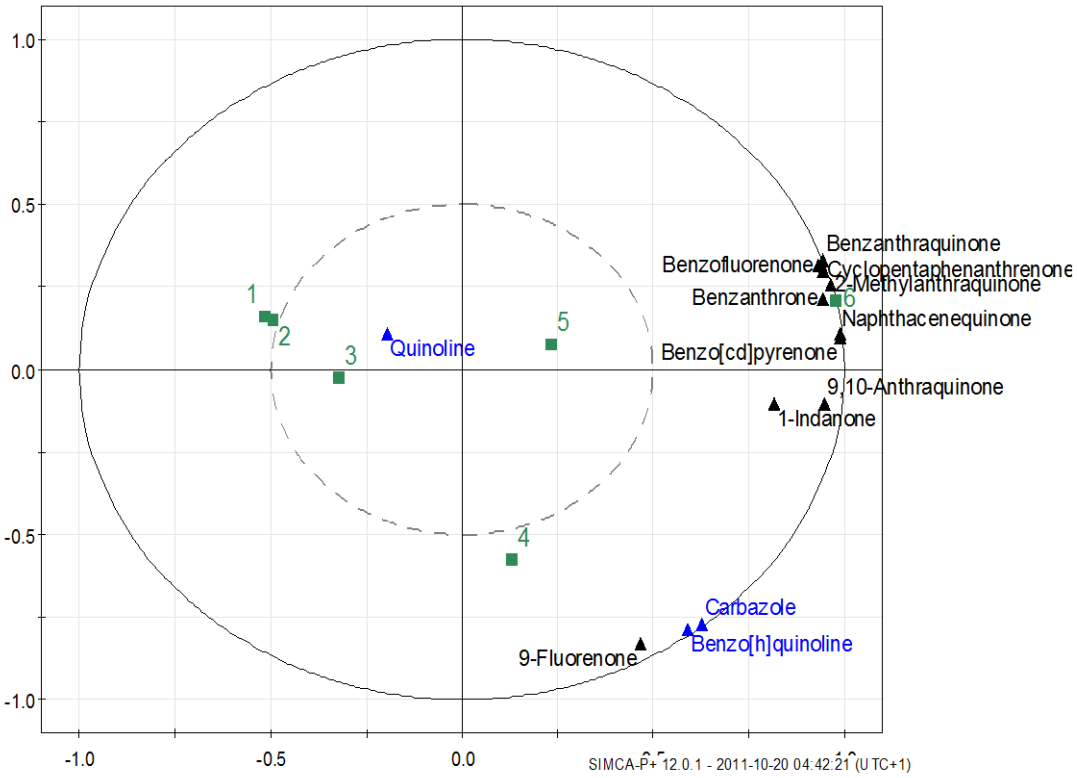

Figure 11: PCA Loading Bi Plot relating Oxy-PAHs and azaarenes with the soil samples. Observations (soil samples) are presented with (■). Oxy-PAHs and azaarenes are presented with (▲) in color black and blue respectively. Two principal components are plotted; the first component in the X-axis and the second component in the Y-axis. Variables located close to an observation are present in high amounts in such observation.

The PCA loading Bi Plot for oxy-PAHs and azaarenes, in Figure 11, shows two significant principal components. The first component (plotted in the X-axis with R^2X 69%) differentiates between highly polluted soils (4, 5 and 6) and the less polluted ones (1, 2 and 3), meaning that higher amounts of oxy-PAHs and azaarenes (except Quinoline) are present in the highly polluted soils compared to the less polluted soils. The second principal component (plotted in the Y-axis and R^2X 18%) explains the difference between soil 4 and the other soil samples. Soil 4, unlike the other soil samples, has a higher content of 9-Fluorenone (oxy-PAH), Carbazole and Benzo(h)quinoline (azaarenes). Quinoline seems to have no predominance in any of the samples (a correlation coefficient close to zero in both components).

Bioavailable fraction estimated with the butanol extraction

The bioavailable content was estimated using soft extraction with butanol as an abiotic method to predict bioavailability.

The bioavailable fraction (%) was calculated as the ratio of the concentrations measured in the butanol extracts and the concentrations found in the total extracts. The bioavailable fractions of the sum of PAHs (16 US EPA) in soils 2, 3, 5 and 6 are presented in Figure 12. Due to a probable cross contamination during the analytical process, soil 1 was excluded from the comparison. For the same reason, Bionaphtalene (bioavailable naphthalene) and Bioquinoline (bioavailable quinoline) were excluded in all samples. Furthermore, soil 4 was not analyzed using the soft butanol extraction method due to shortage of soil.

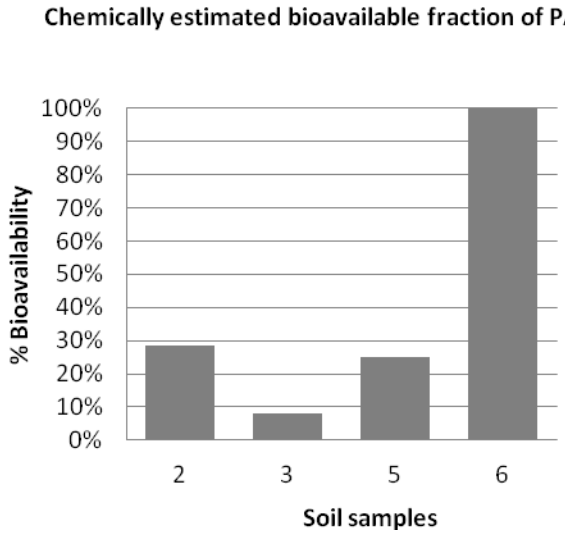

Figure 12: Bioavailable fraction (%) of the total sum of PAHs in soils 2, 3, 4, and 6, estimated using mild extraction with butanol.

The contaminants in soil 6 seem to be completely bioavailable while those in soil 3 seem to have a much lower bioavailability (see Figure 12). The reason for the high estimated bioavailability of soil 6 could be that, being this soil the most heavily polluted with PAHs, a large fraction of the contamination in this soil were loosely bound to the surface of the particles. Soil 3 on the other hand, showed very low

availability of the contaminants. This could probably be explained by the high content of OM in this soil, which leads to a strong sorption of the contaminants.

Trace Metal Analysis

The multivariate evaluation of the results from the metal analysis are presented in a PCA in Figure 13, while the complete list of determined metal concentrations can be found in Annex 2.

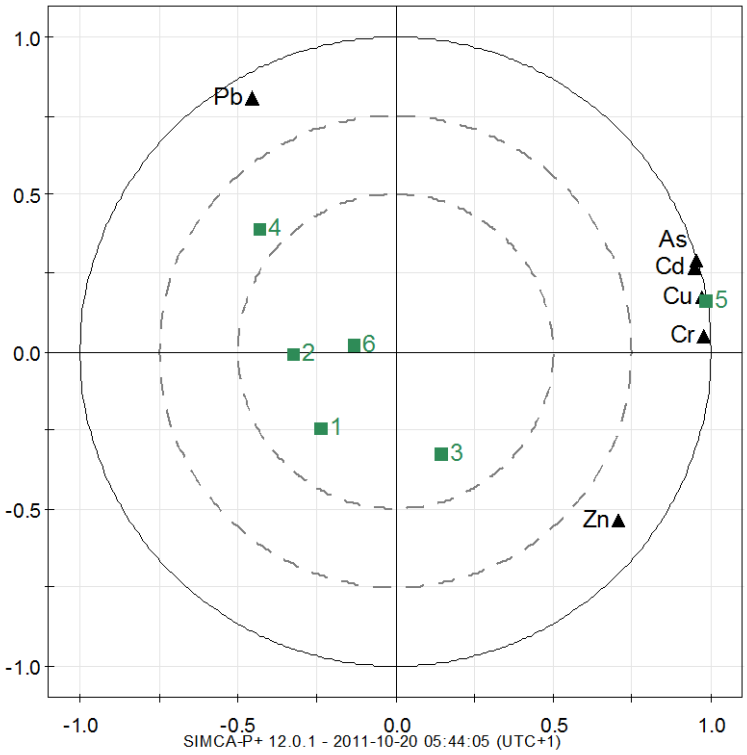

Figure 13: PCA Loading Bi Plot relating metal contents with the soil samples. Observations (soil samples) are presented with (■) while metals are presented with (▲). The first principal component is plotted in the X-axis and the second principal component in the Y-axis. Variables positively influencing any observation are located close to that one in the plot.

Two principal components were considered for the PCA plot, the first component is plotted in the X-axis and the second component in the Y-axis, with R^2X of 73% and 19% respectively (see Figure 13). Soil 5 had the highest total content of metals (see Annex 2), and had also the highest content of almost all individual metals analyzed in this study except for Pb and Zn. According to the first principal soil 5 has the highest content of As, Cd, Cu, Cr and Zn while Soil 4 has the highest content of Pb. The second component differentiates soils 1 and 3 that have higher amounts of Zn, from soil 4.

Two-phase Ecotoxicological Tests

Dose-response curves obtained in the Two-phase tests for the all soils are shown in Figure 14. Results from soil 4 had a great variability among different trials for final ecotoxicity tests. Due to the shortage of sample 4 no more tests could be performed to confirm the toxic response and therefore the result for this soil is highly uncertain.

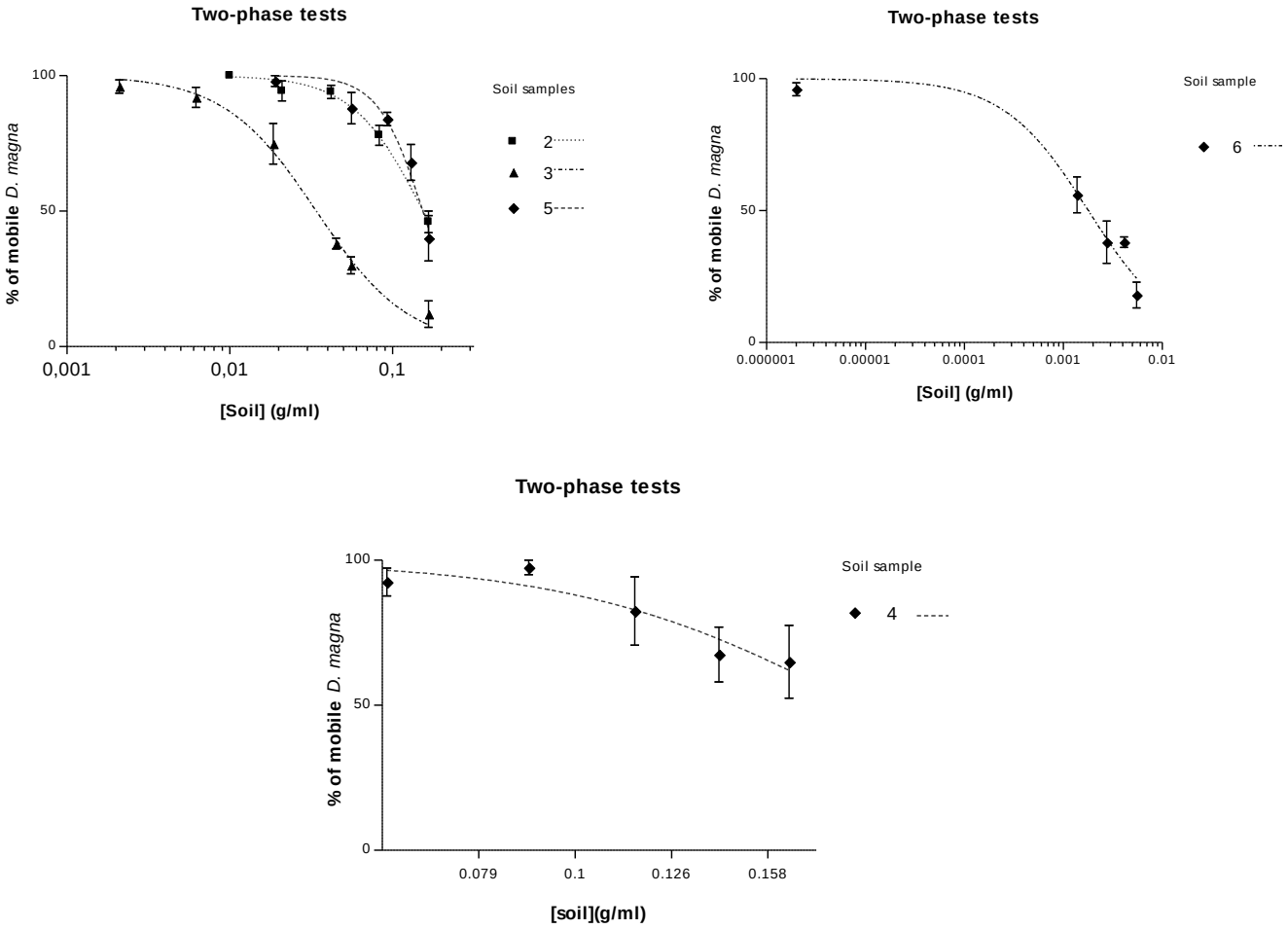

Figure 14: Dose-response curves obtained in the Two-phase test with *Daphnia magna*. Top left: dose-response curves for soils 2, 3 and 5. Top right: dose-response curve for soil 6. Bottom: dose-response curve for soil 4.

There was no toxic response (mobility inhibition of daphnids) for soil 1, even tested at the highest concentration (0.17 g/ml), so no EC50 could be estimated; this sample was just considered not toxic to *Daphnia magna* using the two-phase test. Response of daphnids to soils 2 and 5 were very similar (see Figure 14); the dose-response curve in both samples is interrupted by the highest concentration tested (0.17 g/ml). Soil 3 showed a somewhat higher toxicity, while soil 6 showed the highest toxicity to daphnids of all soils in the two-phase test.

In order to obtain comparable EC50 values for curves of soils 2, 3, 5 and 6, their data was fitted to a sigmoidal curve with variable slope and top and bottom plateaus set constant. The software used was GraphPad Prism 5.0. The top plateau was set to 100%, and the bottom plateau was set to 0% of mobile daphnids (assuming that all daphnids would be completely immobilized eventually when increasing the soil concentration even over 0.17 g/ml, as in soils 2, 5 and 4).

As it was mentioned before, it was not possible to obtain more accurate values of the toxic response to soil 4, nevertheless in order to make a comparison of EC50 with the other soils, the same sigmoidal dose-response model (as for samples 2, 3, 5 and 6) was used, and the EC50 was estimated outside of the measured data range.

The calculated EC50 for all soils, as well as their uncertainties are listed in Table 3:

Table 3: Overall results of the two-phase tests - EC50

Sample	EC50 (g/ml)	95 % CI
1	nd	--
2[a]	0.15	0.14 to 0.17
3[a]	0.034	0.029 to 0.039
4[a]	0.20	0.14 to 0.28
5[a]	0.15	0.13 to 0.17
6[a]	0.0018	0.0013 to 0.0025

a.- values calculated fitting a sigmoidal curve, and setting the top and bottom plateaus to 100% and 0% respectively.
nd: no toxic response detected at the highest concentration tested 0.17 g/ml (no toxicity)

In this study the EC50 values of the two-phase ecotoxicological test had a moderate negative correlation with the total sum of PAHs in the soil (r= -0.53) and the total sum of oxy-PAHs (r=-0.56). Soil 1 was excluded from this correlation analysis due to lack of EC50. The reason of the low correlation coefficient could be that many other factors were also affecting the response and were not parameterized in this study e.g. the high content of organic matter in soil 3. There is no correlation with the total content of metals (r=0.03) and a weak positive correlation with the sum of azaarenes (r=0.27). Also more data would be needed to conclude that the correlations do not occur by chance.

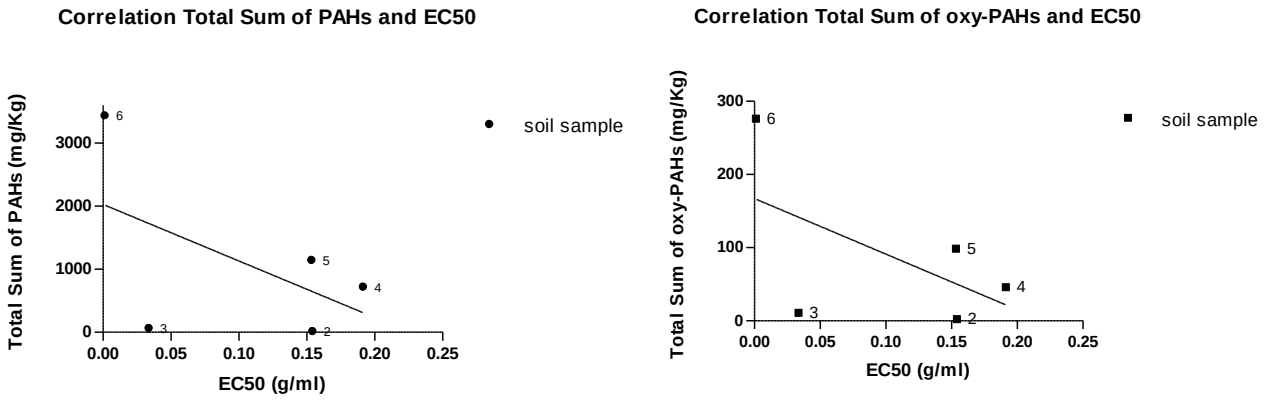

Figure 15: Graphs showing the correlation between the total sum PAHs and EC50 (left) and the total sum of oxy-PAHs and EC50 (right).

The correlation between the toxic response (EC50) and the measured concentrations of all individual contaminants was investigated with OPLS in the SIMCA-P+ software. The OPLS model has only one predictive component. The loading plot in Figure 16 shows how the variables correlate either positively or negatively with the EC50 value. The EC50 values is projected above the (0.00) line, while the contaminants project in the opposite direction (downwards) with negative correlation coefficients, this is a reasonable behavior as the higher amount of pollutants causes a lower EC50 value (increased toxicity). All variables are listed along the X-axis; they are colored according to different groups as metals (yellow), PAHs (red), oxy-PAHs (blue), azaarenes (green) and OM (black).

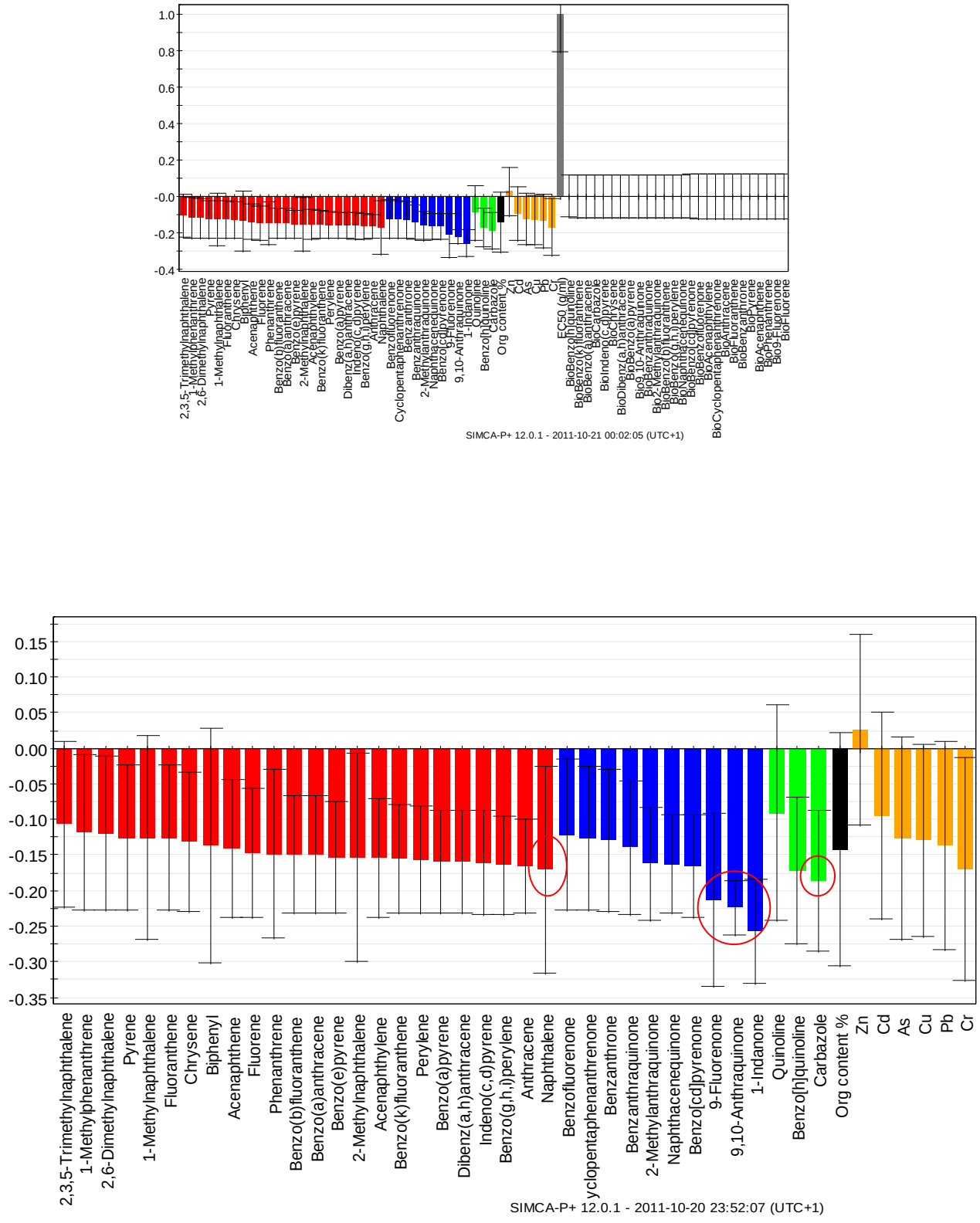

Figure 16: Column loading plot of the first predictive component of the OPLS model, on the top the complete plot, and below an extract of the most important variables. Bioavailable fractions are named with the prefix Bio, for example BioNaphtalene. On the plot at the bottom, variables PAHs, Oxy-PAHs, azaarenes, OM and metals are listed in the X-axis in different colors and sorted in descending order. Variables with higher negative correlation to the EC50 are listed to the right in each group (larger columns).

In the multivariate analysis all the six samples were included. However, since no EC50 was estimated for soil 1, a very high EC50-value was given to this soil. Chemically estimated bioavailable

concentrations are named with the prefix Bio. BioNaphtalene and BioQuinoline were excluded from all samples due to suspected cross contamination during analytical procedure, and for the same reason all the measurements of the bioavailable content of sample 1 were also excluded from the model.

The loading plot of the OPLS suggests no correlation between the bioavailable concentration and the EC50. The model determined similar correlation coefficients for almost all of the PAHs, which could mean that all PAHs were equally toxic to *Daphnia magna*. However, this is not very likely as some individual compounds are known to be more toxic than others. Naphthalene was the PAH that showed the highest negative correlation with the EC50, and seeing that soil 3 contained the highest concentration of naphthalene of all soils (see annex 1), it is probable that this compound was the underlying cause for the toxicity in soil 3.

The graph in Figure 16 indicates that the toxicity had the highest positive correlation with the content of 1-indanone, 9, 10-anthraquinone and 9-Fluorenone (oxy-PAHs), and carbazole (N-PAHs) in the soils. This confirms the theory that oxy-PAHs are in general more toxic than unsubstituted PAHs due to their higher solubility[18]. We should also consider that the OPLS model cannot take into account synergistic effects of the mixture of compounds which could be influencing the established correlation.

In general, the toxicity was less correlated to the metal content than to the content of organic contaminants. However, an exception was chromium, Cr, which showed a fairly high negative correlation with the EC50. Also the confidence intervals for Cd, As, Cu and Pb correlation coefficients were fluctuating from positive to negative, suggesting that these variables may not be relevant to the model, still more data would be needed to refine the model and its uncertainties. Recalling the metal content of soil 5, which had the highest amounts of all metals except Pb and Zn, its toxic response was low compared to the other soils. Soil 4 was characterized by a higher amount of Pb but also a very low toxic response compared to the other soils. Soil 3 had a higher toxic response and also a high content of Zn. However, the Zn-content was also high in soils 1 and 5, which both showed lower toxic responses than soil 3. This analysis could suggest that metals are not directly associated with the toxicity of the soils in this study.

To generate reliable results from an OPLS model around 10-20 samples could be considered minimum. Hence, the 6 samples used in this study are too few to draw precise conclusions, therefore the model is weak and in this sense it should not be used for prediction[2].

Standard Ecotoxicological Tests

The purpose of these tests was to elucidate if the toxicity was drawn with the aqueous leachate or if it was mainly particle bound.

Soils 3 and 6 were used in this section, and all experimental set ups were carried out at a single soil suspension concentration level of 0.06 g/ml for soil 3 and 0.005 g/ml for soil 6. The three tests performed in this section were:

- Two-phase test
- Standard test (aqueous leachate)
- Test of the resuspended residual particles in a two-phase system.

The comparison of overall results of the three experimental tests performed in this section is presented in Figure 17.

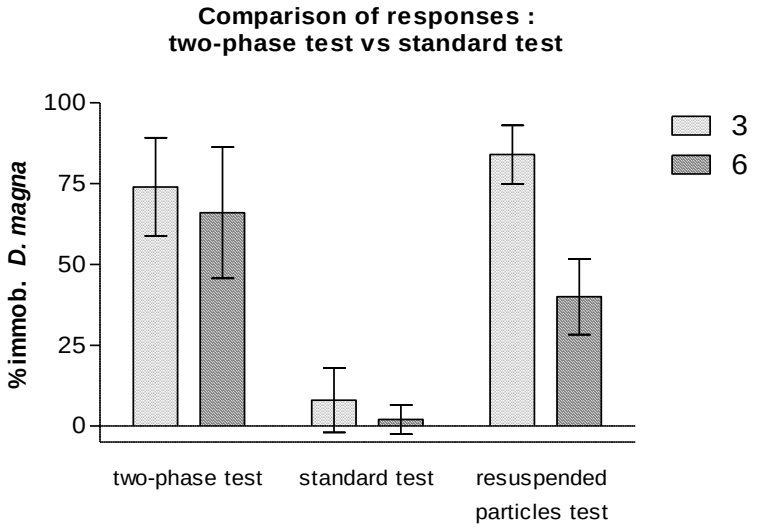

Figure 17: Comparison of the toxic response of *Daphnia magna* to the two-phase test (left), the standard test (middle), and the resuspended particles remaining from the standard test procedure (right), all of them at a single concentration level in g/ml.

The toxicity seems to follow the particles i.e the toxicity disappears when the particles are removed and reappears when the particles are put back in the system again (resuspended particles test). This suggests that the standard *Daphnia magna* test is unable to describe the toxicity of these soils (Figure 17).

For soil 3, the toxic response corresponding to the two-phase test was completely recuperated when the washed out particles were resuspended and tested. However, for soil 6 the response in the resuspended particles test was less than in the two-phase test. Nevertheless, there is a significantly higher response in the resuspended particles test for both samples as compared to the response in the aqueous leachate.

Toxicity tests after extraction with cyclohexane

In the standard tests in the previous section, it was observed that the toxicity was drawn with the particles and didn't follow the aqueous leachates. The objective of testing the toxicity after extraction with cyclohexane was to see if the toxicity was due to the particles themselves or to the lipophilic contaminants attached to them.

Lipophilic contaminants were extracted from the soil particles with cyclohexane. The extracted soil was then tested using the two-phase test method. Later on, the lipophilic contaminants previously extracted with cyclohexane were reapplied on the extracted soil, after which it was tested again in two-phase test.

After extraction with cyclohexane it was expected that the organic contaminants bound to the particles had been removed, and that the extracted soil could be tested to show if the extracted particles alone would induce any toxic response.

The extracted soil was tested at a single concentration. The results of the extracted soil tests were compared to the estimated response according to the dose-response curve of the Two-phase tests section. The comparison of results is shown in Table 4

Table 4: Comparison of responses obtained in the Extracted soil test and in the Two-phase test.

Sample	Conc. tested (g/ml)	% immobilized daphnids	
		Two-phase test	Extracted soil test
Soil 3	0.038	54.2 ± 5.4	46 ± 11.8
Soil 6	0.06	97.2	4 ± 5.2

Surprisingly, for soil 3, the toxic response of the extracted soil was similar to the original untreated soil, which means that almost all of the toxicity in soil 3 remained in the particles after the cyclohexane extraction. This could indicate that the soil particles themselves were somewhat toxic to the daphnids or at least affected them negatively in some way. However, another explanation could be that the cyclohexane extraction method used didn't remove the contaminants from the soil particles, thereby also leaving most of its toxicity. The high levels of organic matter in soil 3 may be involved in this process, by retaining PAHs, oxy-PAHs and azaarenes, and thereby reducing their availability. Lehnik-Habrink et al. observed that PLE using cyclohexane at 100°C yielded 49% efficiency in extracting PAHs from soils with high content of organic matter[38]. Perhaps a more powerful extraction method (using higher temperatures or another solvent) should have been used to extract the lipophilic contaminants.

Unlike soil 3, soil 6 showed no toxicity in the extracted soil, indicating that the toxicity was due to the lipophilic contaminants extracted with cyclohexane. This behavior was also observed in former studies during the development of the two-phase test[39].

In the second part of the Toxicity tests after extraction with cyclohexane, the cyclohexane extract containing the lipophilic contaminants was reapplied onto the extracted soil. The reapplied soil was then tested again, following the procedure of the two-phase tests.

The dose-response curve for the reapplied soil was compared to the dose-response curves obtained previously with untreated soil in the Two-phase tests section, see Figure 18. For soil 6 the tested range was narrow, explaining why the EC50 estimated from the dose-response curve has a very high uncertainty, see Table 5.

Table 5: Comparison of EC50 obtained from the Reapplied soil test and the untreated soil from the Two-phase test section.

	Untreated soil EC50 (g/ml)	Reapplied soil	
		EC50 (g/ml)	95%CI
3	0.034	0.027	0.022 to 0.033
6	0.0018	0.0009	0.000015 to 0.047

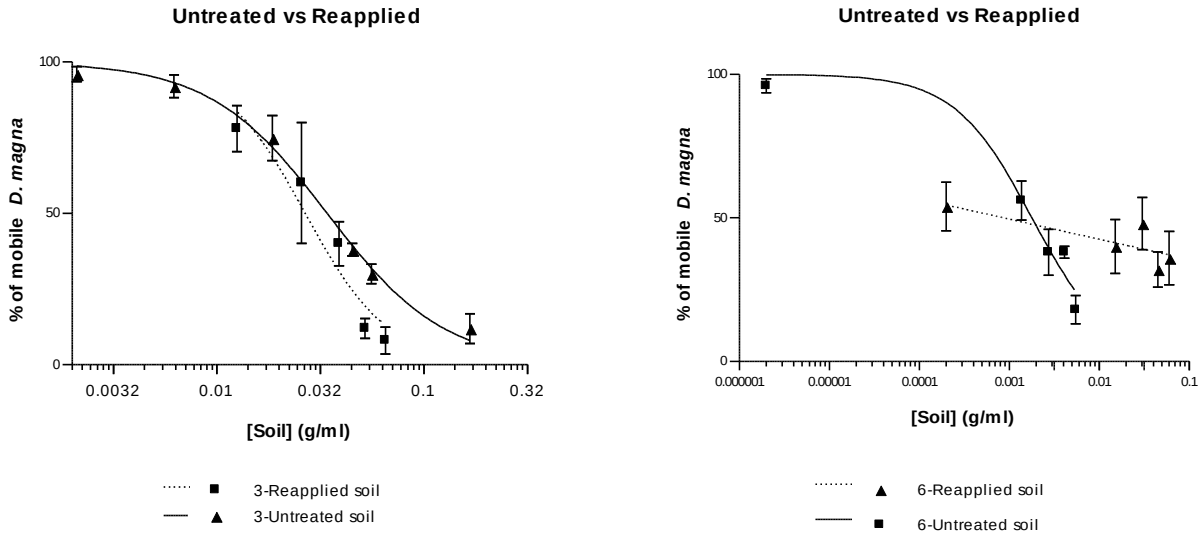

Figure 18: Comparison of dose-response curve for untreated soil and Reapplied soil. Both soils tested following the procedure of the two-phase acute toxicity method. Untreated soil was not pre-treated before the two-phase method, while reapplied soil stands for soil whose lipophilic contaminants were extracted with cyclohexane and then reapplied on the soil before proceeding with the two-phase acute toxicity method.

A comparison between the previously determined EC50 for untreated soil in the Two-phase tests section, and the Reapplied soil EC50 is presented in Table 5.

By reapplying the extracted contaminants we expect to see if the toxicity returned or changed. It was observed previously that reapplication of extracted lipophilic contaminants may lead to an increase of the toxicity, probably due to an increase in bioavailability, since freshly added contaminants are more loosely bound[39].

The reapplied soil 3 EC50 turned out to be very similar to the untreated soil EC50, just slightly lower EC50 (higher toxicity). Recalling the results in the extracted soil (see Table 4), we could say that a great part of the toxicity was particle bound that was present in the extracted soil before the reapplication of the lipophilic contaminants.

Soil 6 showed no toxicity in the extracted soil (see Table 4), but the toxicity was recovered and increased in the reapplied soil. This result confirms that the extracted particles were not toxic to *Daphnia magna* as it was observed in former studies at Pelagia and Umeå University[39]. The lipophilic contaminants extracted with cyclohexane are most likely to be the cause of the toxic response since the toxicity completely reappeared and increased after reapplication of the extract on the soil.

CONCLUSIONS

The two-phase modification of the acute toxicity test with *Daphnia magna* gives a better approximation of the risk posed by soils than the standard testing of the water leachates. This is especially true for soils polluted with lipophilic contaminants such as PAHs and oxy-PAHs as it is demonstrated in this study.

The toxic response in the two-phase test, indicated as EC50-values, had a moderate negative correlation with the amount of PAHs (r= -0.53) and the total sum of oxy-PAHs (r=-0.56), but there was no correlation found between the toxic response and the amount of metals in the soils used in this study.

Daphnia magna showed no sensitivity to the aqueous leachates of the soils in this study, but a significant toxic response is appreciated when testing the soil suspensions.

After extracting the lipophilic contaminants with cyclohexane, the extracted particles exerted no toxicity on *Daphnia magna*. But after reapplication of the extracted organic contaminants on the soil, the toxicity was recovered or even increased.

The high OM content seems to be highly associated with the bioavailability of the PAHs in this study; soil 3 presented the highest amount of OM and the lowest bioavailable fraction estimated with the butanol extraction.

The multivariate analysis show that the toxic response (EC50) is somewhat more correlated with some Oxy-PAHs (1-indanone, 9,10-anthraquinone) and azaarenes (carbazole) than with all other compounds analyzed in this study. From the group of PAHs, toxicity seems to be more associated with Naphtalene. However the multivariate analysis doesn't count for synergistic or additive effects.

The results of this study shows that the developed two-phase variant of the acute toxicity test with *Daphnia magna* truly exhibits the toxicity of particle bound contaminants, and as being a relatively simple and cost effective technique it is a promising tool in future screening activities.

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Annex 1

CONCENTRATION OF ANALYZED ORGANIC COMPOUNDS

Concentrations obtained as Total content of PAHs, alkylated PAHs, Oxy-PAHs and azaarenes are presented in Tables 1-3. Recoveries for the determination of the total contents are presented in Tables 4-5. N.D. stands for Not Detected, and Bk stands for Blank sample.

Table 1: Total concentration of PAHs and alkylated PAHs

Concentrations mg/Kg	1	2	3	4	5	6	Bk
Naphthalene	0.2	0.9	15	2	3	7	0.5
2-Methylnaphthalene	0.1	0.5	5	12	0.6	2	0.3
1-Methylnaphthalene	0.05	0.3	2	11	0.6	2	0.7
2,6-Dimethylnaphthalene	N.D.	0.1	0.6	4	0.3	15	N.D.
Acenaphthylene	0.02	0.2	0.4	2	1	6	N.D.
Acenaphthene	0.01	0.1	0.4	70	4	60	N.D.
2,3,5-Trimethylnaphthalene	N.D.	0.1	0.2	2	2	22	N.D.
Fluorene	0.01	0.1	0.5	90	11	84	0.01
Phenanthrene	0.2	0.9	21	143	10	63	0.02
Anthracene	0.3	0.1	1	34	17	52	N.D.
1-Methylphenanthrene	0.1	0.2	4	6	24	103	0.02
Fluoranthene	0.2	0.7	6	146	502	1526	0.1
Pyrene	0.2	0.5	3	82	336	973	0.03
Benzo(a)anthracene	0.1	0.2	0.8	38	68	154	0.01
Chrysene	0.3	0.5	3	41	92	289	0.01
Benzo(b)fluoranthene	0.2	0.5	1	19	32	79	0.01
Benzo(k)fluoranthene	0.1	0.2	1	19	29	64	0.002
Benzo(e)pyrene	0.1	0.004	0.8	12	19	44	0.002
Benzo(a)pyrene	0.1	0.2	0.4	13	15	35	N.D.
Perylene	0.01	0.05	0.1	3	4	9	N.D.
Dibenz(a,h)anthracene	0.02	0.04	0.1	2	2	5	N.D.
Indeno(1,2,3-c,d)pyrene	0.1	0.2	0.4	6	9	18	N.D.
Benzo(g,h,i)perylene	0.05	0.2	0.3	3	5	10	0.01
Sum PAH	2.3	6.7	69	762	1186	3621	1.7
Sum PAH (16 US-EPA)	1.9	5.4	54	710	1136	3425	0.7

Table 2: Total concentration of Oxy-PAHs

Concentration (mg/Kg)	1	2	3	4	5	6	Bk
1-Indanone	0.003	0.006	0.086	0.076	0.13	0.13	N.D.
9-Fluorenone	0.047	0.18	3.5	6.6	1.7	3.4	N.D.
9,10-Anthraquinone	0.049	0.34	2.2	5.3	7.6	8.8	N.D.
2-Methylanthracene-9,10-dione	0.014	0.092	0.51	1.03	3.1	5.6	N.D.
7H-Benz[de]anthracen-7-one	0.006	0.027	0.15	0.94	1.1	5.1	N.D.
Benz[a]anthracene-7,12-dione	0.048	0.064	0.50	1.6	8.5	17	N.D.
Naphthacene-5,12-dione	0.040	0.18	0.39	7.7	12	24	N.D.
4H-Cyclopenta[def]phenanthren-4-one	0.025	0.15	1.4	16.9	48	160	0.003
Benzo[a]fluorenone	0.037	0.085	0.86	4.5	14	53	0.002
6H-Benzo[cd]pyren-6-one	0.007	0.025	0.069	0.31	0.38	0.94	0.002
Sum Oxy-PAH	0.28	1.2	9.7	44	97	270	0.01

Table 3: Total concentration of azaarenes

Concentration (mg/Kg)	1	2	3	4	5	6	Bk
Quinoline	0.042	0.023	0.361	N.D.	0.131	0.045	0.021
Benzo[h]quinoline	N.D.	0.010	0.217	2.253	0.686	1.130	N.D.
Carbazole	0.019	0.051	0.511	3.139	1.095	1.731	N.D.
Acridine	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Sum N-PAH	0.06	0.08	1.09	5.39	1.91	2.91	0.02

Table 4: Recovery (%) for total amount determination of PAHs

	1	2	3	4	5	6	Bk
Naphthalene	44	29	44	21	25	15	2
Acenaphthylene	45	45	50	33	29	25	6
Acenaphthene	61	63	62	43	39	29	15
Fluorene	69	80	71	43	50	42	34
Anthracene	30	69	67	76	50	41	15
Pyrene	87	86	87	98	104	77	75
Chrysene	83	58	71	73	83	54	78
Benzo(k)fluoranthene	77	53	59	53	69	37	64
Benzo(g,h,i)perylene	64	50	50	43	58	31	55

Table 5: Recovery (%) for total amount determination of Oxy-PAHs and azaarenes

	1	2	3	4	5	6	Bk
Dimethylanthraquinone	106	118	115	105	111	93	91

The very low recovery of Naphthalene in the soil samples 2, 4, 5, 6 and Blank could be due to evaporation of the sample at some point during the sample preparation process. In general low recoveries were obtained for the LMW PAHs (14-75%), while for HMW PAHs the recovery range is 31-103%. Low recoveries for Anthracene couldn't be explained. Even though the recoveries were low, it was still possible to quantify the PAHs as the IS was lost at a similar extent.

The recovery for oxy-PAHs was above 90% for all samples, the values over 100% were probably due to coeluting interferences that increased the area of some peaks.

The proportion of Oxy-PAHs and azaarenes to the total sum of PAHs decreased as the total amount of PAHs increased.

Table 6: Proportion of Oxy-PAHs and azaarenes to the total sum of PAHs

%	1	2	3	4	5	6	Bk
ΣOxy-PAHs : ΣUS-EPA PAHs	14	21	18	6	8.6	8	1.2
ΣNPAHs : ΣUS-EPA PAHs	3.1	1.6	2.0	0.76	0.17	0.08	3.2

Concentrations of the chemically estimated bioavailable content of PAHs, Oxy-PAHs and azaarenes in the soil samples 2, 3, 5 and 6 are presented in Tables 7-9. Results obtained for Soil 1 were excluded due to suspicion of cross contamination. For the same reason Naphtalene and Quinoline were also

excluded. Soil 4 was not analyzed with the butanol extraction method due to shortage of the soil sample.

Recoveries for the determination of the Bioavailable fraction are presented in Tables 10-11.

Table 7: Concentrations of PAHs in the chemically estimated Bioavailable fraction

Concentrations mg/Kg	2	3	5	6	Bk
Acenaphthylene	0.10	N.D.	0.29	2.3	N.D.
Acenaphthene	N.D.	N.D.	0.30	28	0.02
Fluorene	N.D.	N.D.	0.18	45	0.01
Phenanthrene	0.04	0.12	0.11	29	N.D.
Anthracene	0.01	0.03	1.8	16	N.D.
Fluoranthene	0.22	1.0	110	2100	0.06
Pyrene	0.27	0.81	N.D.	1100	0.02
Benzo(a)anthracene	0.07	0.30	58	120	0.01
Chrysene	0.17	0.55	44	93	0.00
Benzo(b)fluoranthene	0.28	0.65	27	65	0.01
Benzo(k)fluoranthene	0.11	0.39	18	33	N.D.
Benzo(a)pyrene	0.06	0.22	11	21	N.D.
Dibenz(a,h)anthracene	N.D.	N.D.	2.2	4.5	N.D.
Indeno(c,d)pyrene	0.12	0.14	5.4	12	N.D.
Benzo(g,h,i)perylene	0.09	0.08	2.7	5.7	N.D.
Summa PAH	1.6	4.3	285	3700	0.13

Table 8: Concentrations of Oxy-PAHs in the chemically estimated Bioavailable fraction

Concentrations mg/Kg	2	3	5	6	Bk
1-Indanone	N.D.	N.D.	N.D.	N.D.	N.D.
9-Fluorenone	N.D.	N.D.	0.17	1.7	N.D.
9,10-Anthraquinone	N.D.	0.15	3.2	6.02	N.D.
2-Methylanthraquinone	0.03	N.D.	2.2	4.9	N.D.
Benzanthrone	N.D.	N.D.	0.52	4.3	N.D.
Benzanthraquinone	N.D.	N.D.	6.4	15	N.D.
Naphthacenequinone	0.06	0.04	8.8	22	N.D.
Cyclopentaphenanthrenone	0.05	0.04	22	120	N.D.
Benzofluorenone	0.02	0.17	14	55	N.D.
Benzo[cd]pyrenone	N.D.	N.D.	0.19	0.56	N.D.
Sum Oxy-PAH	0.16	0.40	58	230	N.D

Table 9: Concentrations of azaarenes in the chemically estimated Bioavailable fraction

Concentrations mg/Kg	2	3	5	6	Bk
Benzo[h]quinoline	N.D.	N.D.	0.08	0.13	N.D.
Carbazole	N.D.	N.D.	0.05	0.09	N.D.
Acridine	N.D.	N.D.	N.D.	N.D.	N.D.
Sum N-PAH	0.32	0.36	0.42	0.51	0.32

Table 10: Recovery of PAHs in the chemically estimated Bioavailable fraction

Recovery (%)	2	3	5	6	Bk
Naphthalene	15	18	14	33	36
Acenaphthylene	23	36	25	83	39

Recovery (%)	2	3	5	6	Bk
Acenaphthene	33	52	30	98	62
Fluorene	46	63	40	170	69
Anthracene	72	69	74	290	83
Pyrene	89	84	120	260	68
Chrysene	83	110	150	470	97
Benzo(k)fluoranthene	74	99	120	390	92
Benzo(g,h,i)perylene	72	92	79	290	80

The high recoveries for soil 6 are marked in red color; the peak area for the RS in this sample was smaller compared to the other samples, and this has caused the recovery calculations to be higher.

Unlike the determination of total amounts of oxy-PAHs and azaarenes, in the butanol extraction D8 anthracene dione was used as IS. The recovery for Oxy-PAHs and azaarenes is presented in Table 11.

Table 11: Recovery of Oxy-PAHs and azaarenes in the chemically estimated Bioavailable fraction

Recovery %	2	3	5	6	Bk
Oxy-PAHs	76	74	81	62	69
azaarenes	99	96	105	80	89

Annex 2
CONCENTRATION OF ANALYZED TRACE METAL COMPOUNDS

Table 1: Concentration of trace metal compounds analyzed. Bk stands for Blank, and N.D. for not detected.

ug/g dw	Sample						
	1	2	3	4	5	6	Bk
Zn	230	97	400	88	330	103	0.67
Cu	45	38	100	41	305	120	0.51
Cr	28	84	370	72	690	170	1.5
As	0.10	24	120	57	910	150	N.D.
Cd	N.D.	N.D.	1.02	N.D.	17	0.47	N.D.
Pb	15	23	15	51	18	21	N.D.
Hg	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Some limitations must be observed about the utilization of the reference materials: the matrix (marine sediments) is different from the samples in this study (soil), which could affect the comparison to certified values. The certified values were obtained after a total decomposition method (prolonged digestion with hydrofluoric, sulfuric and perchloric acid); which could estimate higher values than the method 3051A used in this study. Hg in the reference material was not certified for measurements with ICP OES, nevertheless the method 3051A is validated also for analysis of Hg using ICP OES.

Despite these limitations accurate metal measurements could be obtained for Zn, Cu, Cr, As and Pb, while the measurements for Cd differed significantly from the certified values of the reference materials. One possible explanation could be that the calibration curve for Cd was in this case more representative of the samples than of the reference materials i.e. the reference material concentrations were in the lower part of the range of the calibration curve, where a lot more of uncertainty might be expected, another reason that is also likely to be is the difference in the matrix of the reference materials.

Annex 3
QUALITY CONTROL ECOTOXICOLOGICAL TESTS

Potassium dichromate ($K_2Cr_2O_7$) was used as reference toxicant. The certified EC50 (24h) in the specification sheet for toxkit ephippia is 1.20 mg/l, but the acceptability range according to ISO 6341 is 0.6 – 2.1 mg/l.

Table 1: Quality Control test with $K_2Cr_2O_7$

Conc mg/l	Rep1	Rep2	Rep3	Rep4	Rep5	Immobilized Daphnia %
3.2	100	90	100	100	100	98
1.8	100	100	100	100	100	100
1.4	90	100	100	100	100	98
1.2	90	100	70	100	60	84
1.0	70	100	100	90	80	88
0.8	90	60	70	60	40	64
0.56	60	30	20	0	30	28
0.32	0	0	10	20	0	6

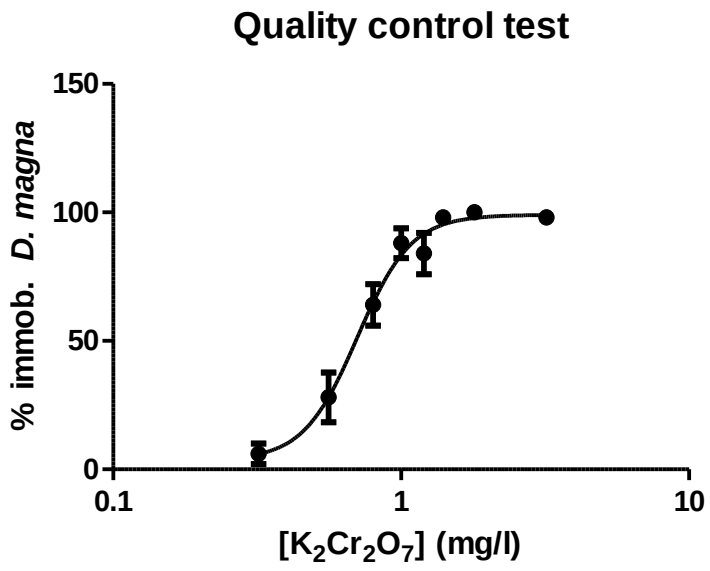

Figure **1: Dose-response curve for the Quality Control test, estimated using a non linear model, sigmoidal curve, draw using GraphPad Prism 5.0.**

The calculated EC50 with a nonlinear regression model, sigmoidal curve with variable slope, is 0.7 g/ml with 95% CI 0.62 to 0.8 g/ml, which is within the acceptable range.

A control blank was also tested (using just medium with 10 daphnids in 5 replicates), resulting in 12 ± 3.9% immobilized daphnids.

Physicochemical Characteristics at the highest concentration tested of the soil suspensions are listed in Table 2. The dilution medium alone and $K_2Cr_2O_7$ at the highest concentrations were also tested.

Table 2: Physicochemical characteristics measured in the soil suspension used for the ecotoxicological tests, also medium and $K_2Cr_2O_7$ are listed in the table. N.M. stands for not measured.

Soil sample	Conc. (g/l)	pH	Cond (mS)	DO %	DO (mg/l)	Temp °C	Hardness (mg/l $CaCO_3$)
1	0.16	6.8	1.2	N.M.	N.M	21.8	
2	0.16	6.0	1.1	64	5.7	22.1	252
3	0.16	5.0	1.2	62.9	5.5	21.9	306
4	0.16	6.3	1.3	52	4.5	22.4	270
5	0.16	5.3	1.3	49.5	4.5	21.5	270
6	0.005	7	1.2	68.9	6	22	216
Medium		7.4	1.4	70.5	6.2	22.5	288
$K_2Cr_2O_7$	3.2 mg/l	7.5	0.4	63.5	5.4	22.6	126

The medium complies with the ISO 6341 requirement for pH = 7.8 ± 0.5, hardness is slightly over the standard which is 225 ± 50 mg/l $CaCO_3$, while the DO was around 6 mg/l (below the standard).

DO was measured as %, and it was calculated in mg/l using a saturation chart that assumes altitude to be sea level.