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Ecotoxicity Assessment of the Water Extracts from Metal-Contaminated and Hydrocarbon-Contaminated Soils

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Abstract: Soil water extracts could help to quickly assess the potential hazard of contaminants in soil, groundwater, and surrounding receiving water. In this study, the adverse effects of water extracts obtained from soils contaminated by heavy metals (sites A and B) or hydrocarbons (sites C, D and E) were evaluated using wheat, pak choi, and zebrafish. The test results obtained with freeze-dried soil samples showed a good correlation with those obtained from fresh wet soil samples. Phytotoxicity level was found to be greater in samples obtained from the metal-contaminated site B as compared to those from site A, whereas the opposite was observed for the zebrafish embryo acute toxicity. The water-soluble fractions of petroleum hydrocarbons in soils from sites C and D pose ecological risks to the environment, even though the concentrations of total petroleum hydrocarbon were below the established screening value. The results obtained with our battery of biological assays could complement the ecological risk estimation of a complex mixture of contaminants in soils. Site-specific ecological risk assessment using chemical analytical data, screening values, and ecotoxicity testing with soil water extracts could serve as a screening approach to identify the impact of contaminated soils on the freshwater environment.

Keywords: soil extraction; contaminated sites; ecotoxicity assays; plants; fish

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1. Introduction

Soil is a sink for a wide range of contaminants generated by human activities [1]. Soils at construction land sites can be polluted with many hazardous substances, such as heavy metals (HMs), petroleum hydrocarbons (PHs), polycyclic aromatic hydrocarbons (PAHs), and pesticides as a result of chemical leakage, waste treatment, or sewage production [2]. Contaminated sites present a plausible pollution threat to the well-being of adjacent inhabitants and the aquatic ecosystem, given the potential for toxic chemicals to permeate the soil, groundwater and surface water, particularly if a flooding event or groundwater table rise causes an increase in dissolved contaminant concentrations [3,4]. Therefore, soil and soil leachate are acknowledged as important environmental hazards, and the risk assessment and management are considered imperative.

Environmental risk assessment in soils needs to be conceived on the basis of a joint perception of chemical analysis and toxicological assessment [5,6]. A series of toxicity bioassays could provide a more realistic risk assessment of specific polluted sites by quantifying the overall toxic effects on selected organisms, which might reveal the bioavailable fraction of the complete mixture of pollutants in soil [7]. Direct toxicity bioassays were performed using the whole soil, and indirect exposure bioassays were performed using soil water-leachable extracts, which were proposed by ISO guidelines for assessing the ecotoxic potential of soils on soil-dwelling and aquatic organisms [8,9]. Most direct bioassays utilized for contaminated soils involve assessing the adverse impact of the soil on a living organism (e.g., *Eisenia fetida*, *Folsomia candida*, *Triticum aestivum*, *Lactuca sativa*, *Vibrio*

fischeri, etc.) [10–15]. These tests indicate the potential effects of contaminants on the habitat function of soil, which are relatively expensive and time consuming [16]. Indirect bioassays were employed to assess the potential toxicity of the soil to neighboring regions by serving as a source of contamination [5,16]. For example, the investigation of water-soluble heavy metals facilitates accurate risk assessment of metal-contaminated soils as water-soluble metals are the most mobile and readily available fractions. They may suggest potential effects of pollutants on the filter function of soil. Extraction procedures for indirect bioassays are not yet standardized to a high level [16]. There are some tests, already standardized by the Organization for Economic Co-operation and Development (OECD), International Organization for Standardization (ISO) and the European Union, to evaluate the toxicity in water that can be used to test soil elutriates. Water elutriates toxicity tests, using mustard seeds *Sinapis alba* in Petri dishes filled with filter paper and the chlorococcal algae *Desmodesmus subspicatus*, were defined in current Czech legislation for characterization of waste and contaminated soils [17]. The risk assessment should contain different species under the consideration of diverse exposure pathways and toxicity endpoints.

Plant species are indicators of soil toxicity of leachable elements in assessing harm and risk to contaminated soil ecosystems. Bioassays with vascular plants at the early life history stages offer a range of merits to evaluate soil ecotoxicity, allowing the assessment of sensitive parameters such as seed germination and seedling growth [18]. Phytotoxicity evaluation can be conducted using seed germination in a Petri dish with filter paper [19]. The examination is predicated upon an evaluation of the phytotoxic consequences resulting from the presence of pollutants during the germination stage of seeds and the subsequent growth of seedlings. Wheat (*Triticum aestivum* L.) and pak choi (*Brassica chinensis* L.) are commonly recommended species used in seedling emergence and seedling growth tests [20]. Wheat is a monocotyledonous plant and belongs to the Poaceae Family. It is a primary cereal crop and a staple food source for over half of the world's population [21]. Pak choi is a dicotyledonous plant that belongs to the Brassicaceae family, which is rapidly growing and playing an important role in China's annual leafy vegetable supply [22].

Aquatic bioassays have been suggested as a suitable method for assessing the potential toxicity of soil pollutants' water-extractable fraction, posing a risk to both surface and ground waters [23,24]. Zebrafish (*Danio rerio*) embryo bioassays are applied to testing chemical toxicity of freshwater and sediment [25–27]. Zebrafish is rapidly becoming an embryonic testing model due to its high fecundity, rapid development, low cost, ease of handling and high genetic homology to humans [28,29]. Thus, zebrafish embryo standardized bioassay has become an ideal screening tool of evaluating the toxicity of real soil samples for the aquatic compartment. Embryonic zebrafish assay has been applied to investigate the soil toxicity from multiple industrial sites, including PAHs-contaminated soil [30], gas-contaminated soil [31], creosote-contaminated superfund soil [32], uranium mining wastes [33], Basamid®-contaminated soil [34] and soil samples from the gangue stacking areas [35].

The Yangtze River Delta (YRD), which includes the municipality of Shanghai, Jiangsu province, Zhejiang province, and Anhui province, is one of the largest regional economic zones in China. Massive industrial and commercial enterprises contribute to its economic development [36]. However, the emission of excessive toxic chemicals from industrial sites can pose a serious threat to the groundwater and neighboring rivers, especially the highly permeable sites located along the lower Yangtze River and Qiantang River [37]. The aim of this study was to develop screening toxicity bioassays with soil water extracts to assess ecological risk of contaminated sites to the aquatic compartment, which could support the risk-based environmental management decisions for remediation strategies. A battery of toxicity tests was selected to represent different exposure routes and toxicity endpoints. For wheat and pak choi, the roots are the main pathway for the entrance of contaminants [38]. Pollutants can be absorbed into fish through various routes of exposure, including dermal, oral or inhalation [39]. Site-specific risk assessments were conducted using ecotoxicity testing and correlation of the toxicity in conjunction with contaminate

concentration. Soil toxicity is influenced by soil physicochemical properties and chemical species of the contaminants, which might be both altered when saturated field soils are air-dried in preparation for risk assessment tests. However, it is unclear whether the handling of soil by drying would alter the toxicity results of soil elutriates. The aims of the present study were to: (1) determine the toxicity difference between elutriates from the freeze-dried soil and fresh wet soil samples; (2) evaluate the comparative toxicity response of plants and zebrafish to water extracts from metal-contaminated and hydrocarbon contaminated soils; and (3) investigate the applicability of the acute bioassay methods for the ecological risk assessment of field contaminated soils.

2. Materials and Methods

2.1. Sites and Soil Characterization

Soil samples from sites A and B were obtained from abandoned factories that had been polluted with heavy metals. Soil samples from sites C and D were collected from abandoned gas stations that had been contaminated with petroleum hydrocarbons. Contaminated sites A, B, C, and D were located in Shanghai and the soil is predominantly classified as sand clay loam that developed from the soil-forming parent material of river-sea phase sediment [40]. Soil sample from site E was obtained from a closed pesticide factory located at the Jiangsu province, which had produced pesticide and other chemicals for 50 years. All soil samples from five contaminated sites were collected using a clean stainless-steel spade and placed in zip-lock polythene bags. They were then taken back to laboratory immediately. Each sample were split into two parts. One part (original moist soil samples) was directly used in the soil extraction procedure. The other part was freeze-dried, passed through a 2 mm sieve to remove stones and other debris, and pestled into sizes less than 0.15 mm. Therefore, a total of 10 soil samples were used for water extraction and bioassays.

For metal-contaminated soils, the sieved and freeze-dried samples were microwave digested in Teflon vessels with a mixture of concentrated HNO_3, HCl and HF. $HClO_4$ was added to remove HF and then adjust to a volume of 10.0 mL with 2% (v/v) HNO_3 before instrumental analysis. Target elements (Cr, Cd, Ni, As, Zn, Pb, and Cu) were analyzed after soil digestion using inductively coupled plasma atomic emission spectroscopy (Agilent 720ES, Agilent, Santa Clara, CA, USA). For hydrocarbon-contaminated soils, the EC6-EC9 fractions and EC10-EC40 fractions of total petroleum hydrocarbons, polycyclic aromatic hydrocarbons and monoaromatic hydrocarbons were determined using a gas chromatograph/mass spectrometer (GC-MS Clarus680-SQ8T, PerkinElmer, Hong Kong) and a gas chromatograph with a flame ionization detector (GC-FID, Shimadzu, Kyoto, Japan). Soil samples from site E were tested for volatile organic compounds and semi-volatile organic compounds by gas chromatograph/mass spectrometer. Chemical analysis of polluted soils was conducted and the procedures have been published in detail elsewhere [37,41,42].

2.2. Water Extraction Procedure

Soil was extracted with pure water using a soil/water ratio of 1:10 (mass/volume) for both freeze-dried and original moist soil samples [43–45]. The batch test with water was carried out following the instruction from HJ 557-2010, which is a Chinese standard leaching method designed to simulate the leaching of solid waste into groundwater or surrounding surface water under certain circumstances [45]. Soil suspensions were horizontally vibrated at 110 ± 10 times/min for eight hours at room temperature. The mixtures were allowed to stand for 16 h to separate solid and liquid phases and then filtered through a nylon membrane filter with a pore size 0.45 μm. The filtrate was kept at 4 °C until subsequent bioassays.

2.3. Bioassays

Ecotoxicological assessment of soil water extracts were conducted with two plant species (monocot-wheat, and dicot-pak choi) and a model fish zebrafish. These bioassays

were utilized to evaluate the potential toxicity risks of contaminated soils as a source of contamination to adjacent water ecosystems [16].

2.3.1. Plants

Seeds of wheat (*Triticum aestivum* L, Yannong 19) and pak choi (*Brassica chinensis* L., Shanghaiqing) purchased from the agricultural seed market of Shanghai were surface-sterilized in a 10% Na-hypochlorite solution for 20 min, and washed with distilled water for three times. The seeds were then covered by a clingfilm with needle-like holes for ventilation and retained in a dark artificial climate incubator at 25 °C and 70% humidity for 24 h. The toxicity towards both plant species was assessed in accordance with the OECD Guideline 208 [20] by evaluating the effects on root and shoot elongation, and shoot fresh and dry biomass of wheat and pak choi, following the exposure to soil water extracts. The filter paper petri dish test was performed. A 9 mL of soil extracts was added to the pre-sterilized petri dish to wet the filter paper, and then 15 seeds were evenly distributed in each petri dish with double-layer hydrating filter paper (45 seeds in each control and treatment group). Distilled water was used as control. All of the treatments were in triplicate. Petri dishes were sealed with plastic wrap and incubated in an acclimated chamber under supervised conditions (25 ± 0.5 °C, 70% humidity, and in the dark). The filters were kept moist and the numbers of germinated seeds were counted each day. The germination criterion was the emergence of a radicle (at least 5 mm) ruptured through the seed coat. When the seed germination rate was above 90% and both the length of shoots and roots exceeded 20 mm in the control group, the shoot and root parts of the seedlings were collected individually. Endpoints including seedling emergence (length of root and shoot), and biomass (fresh and dry shoot weight) were measured.

2.3.2. Zebrafish (*Danio rerio*)

Mature wild-type (AB strain) zebrafish (*Danio rerio*) obtained from the China Zebrafish Resource Center (CZRC) were maintained in a fish breeding circulating system (Hai Sheng, Shanghai, China) at 28 ± 0.5 °C with a light: dark photoperiod of 14/10 h. Zebrafish were fed three times per day. A breeding pair of adult male and female fish was kept in a breeding tank overnight and separated by a partition which was taken away for mating in the next morning. Fertilized eggs were collected, washed with Holtfreter's medium in a petri dish and incubated at 28 °C for subsequent exposure tests. Healthy embryos were immediately selected after fertilization (2 h post fertilization (hpf)) and randomly delivered into individual wells of a 96-well plate containing 90 µL of soil extracts. Treatment and control groups (n = 32 embryos/group) were equally distributed into multiple 96-well plates to control for intra- and inter-plate variability. The embryos were held in a humidified incubator with a 14 h/10 h light/dark cycle at 28 ± 0.5 °C, and dead embryos were removed. Developmental toxicity was assessed at 96 hpf for morphological alterations as well as lethal rates in all treatment and control groups. Mortality and egg hatching success were visually assessed under a dissecting microscope (Olympus-SZ61, Olympus Ltd., Tokyo, Japan). All zebrafish-related tests were conducted with fertilized embryos in accordance with the Tongji University Animal Care and Use Protocol (Protocol #TJLAC-019-113).

2.4. Data Analysis

Initial analysis of bioassay results was conducted according to the different contaminated sites and different experimental species for each soil sample. Data were statistically analyzed using a one-way ANOVA, and means were separated by the Tukey test ($p < 0.05$) using the statistical software SPSS v18.0 for Windows. In all figures, mean values labeled with different letters are significantly different ($p < 0.05$) based on Tukey's range test.

3. Results

3.1. Chemical Analysis of Contaminated Soils

Chemical characterization of soil samples from the contaminated sites was shown in Table 1. Sampling site A was polluted with multiple heavy metals, with amounts of Ni and Pb in the soil samples exceeding the limiting concentration in Chinese standard Soil environmental quality—Risk control standard for soil contamination of development land (GB 36600-2018) [46], the ceiling concentrations of Cu, As, Ni, Pb, Cr, and Cd were 2000 mg/kg, 20 mg/kg, 150 mg/kg, 400 mg/kg, 3 mg/kg, and 20 mg/kg, respectively. Sampling site B was highly polluted with hexavalent chromium (Cr(VI)), which is a primary toxic metal and a class-one carcinogen [47,48]. Sampling sites C and D were located in abandoned gas stations that have been contaminated with petroleum hydrocarbons. Sampling site E was a closed pesticide plant and chlorinated hydrocarbons were the main characteristic pollutants [49,50]. The amounts of 1,4-dichlorobenzene in soil sample E exceeded limiting concentrations in GB 36600-2018.

Table 1. Total concentrations of chemicals in contaminated sites.

Chemicals	Element Concentration (mg/kg)		Risk Screening Values for Soil Contamination of Development Land (mg/kg) [1]	
	Site A	Site B	Class I	Class II
Cr^{6+}	<0.2	25	3.0	5.7
Cd	0.593	0.87	20	65
Ni	254	49.08	150	900
As	17.2	1.81	20	60
Zn	3580	136.12	-	-
Pb	939	38.34	400	800
Cu	1380	20.19	2000	18,000
Chemicals	**Chemical Concentration (mg/kg)**		**Risk Screening Values for Soil Contamination of Development Land (mg/kg) [1]**	
	Site C	**Site D**	**Class I**	**Class II**
Total petroleum hydrocarbons (C6-C9)	17.9	30.7	-	-
Total petroleum hydrocarbons (C10-C40)	150	180	826	4500
Benzene	<0.05	0.06	1	4
Methylbenzene	0.08	0.53	1200	1200
Ethylbenzene	0.21	1.02	7.2	28
M-xylene and p-xylene	0.38	1.15	163	570
O-xylene	0.14	0.49	222	640
Naphthalene	<0.10	0.10	25	70
Phenanthrene	<0.10	0.22	-	-
Fluoranthene	<0.10	0.26	-	-
Pyrene	<0.10	0.23	-	-
Benzanthracene	<0.10	0.10	5.5	15
Chrysene	<0.10	0.13	490	1293
Benzofluoranthene	<0.10	0.15	5.5	15

Table 1. *Cont.*

Chemicals	Chemical Elements Concentrations (mg/kg)	Risk Screening Values for Soil Contamination of Development Land (mg/kg) [1]	
	Site E	Class I	Class II
Chlorobenzene	0.19	68	270
Bromobenzene	<0.05	-	-
2-chlorotoluene	0.12	-	-
4-chlorotoluene	0.08	-	-
1,2-dichlorobenzene	403	560	560
1,3-dichlorobenzene	5.29	-	-
1,4-dichlorobenzene	21.7	5.6	20
1,2,4-dichlorobenzene	0.81	-	-

Notes: [1] Risk screening values for soil contamination of development land (Class I for residential/parkland and Class II for commercial and industrial use), according to Chinese standard Soil environmental quality—Risk control standard for soil contamination of development land (GB 36600-2018).

3.2. Plants Bioassays on Metal-Contaminated Soils

The inhibition rates of root and shoot elongation, shoot fresh weight, and shoot dry weight of wheat and pak choi were assessed after exposure to water extracts from wet and freeze-dried soils. Representative images of wheat and pak choi seedlings were shown in Figure 1e,f, respectively. The toxicity results for metal-contaminated soils were presented in Figure 1a,b. In general, heavy metals caused more pronounced reduction in root length than shoot length, and the degree of inhibition followed the order of root length > shoot length.

For wheat, the most sensitive parameter at site A was shoot fresh weight, with the inhibition rates of 40.7 ± 7.5% and 43.7 ± 3.0% after exposure to water extracts from freeze-dried and wet soils, respectively. The inhibition rates of shoot dry weight showed significant difference ($p < 0.05$) between A-dry and A-wet soils. At site B, root length was the most sensitive parameter, with inhibition rates of 87.9 ± 3.3% and 83.6 ± 2.3% after exposure to water extracts from freeze-dried and wet soils, respectively. The inhibition rates of four parameters showed no significant difference between B-dry and B-wet soils.

For pak choi, shoot dry weight was the most sensitive parameter for site A, and the inhibition rates of shoot dry biomass exposed to water extracts from freeze-dried soil and wet soil reached 41.4 ± 0.3% and 67.8 ± 3.0%, respectively. The inhibition rates of root length, shoot length, and shoot dry weight presented significant difference between A-dry and A-wet soils. For site B, shoot fresh weight was the most sensitive parameter, and the inhibition rates of shoot fresh weight exposed to water extracts from freeze-dried soil and wet soil achieved 90.1 ± 5.2% and 91.9 ± 3.3%, respectively. The inhibition rates of shoot dry weight showed significant difference ($p < 0.01$) between B-dry and B-wet soils.

3.3. Plants Bioassays on Hydrocarbon-Contaminated Soils

As shown in Figure 1c, root length was the most sensitive parameter for wheat at site C, with the inhibition rates of 18.1 ± 3.9% and 19.6 ± 6.8% for water extracts from freeze-dried and wet soils, respectively. The inhibition rates of the four parameters showed no significant difference between C-dry and C-wet soils. For site E, shoot dry weight was the most sensitive parameter, and the inhibition rates of shoot dry weight exposed to water extracts from freeze-dried soil and wet soil achieved 30.7 ± 2.7% and 38.1 ± 5.4%, respectively. The inhibition rates of shoot fresh weight showed significant difference ($p < 0.01$) between E-dry and E-wet soils.

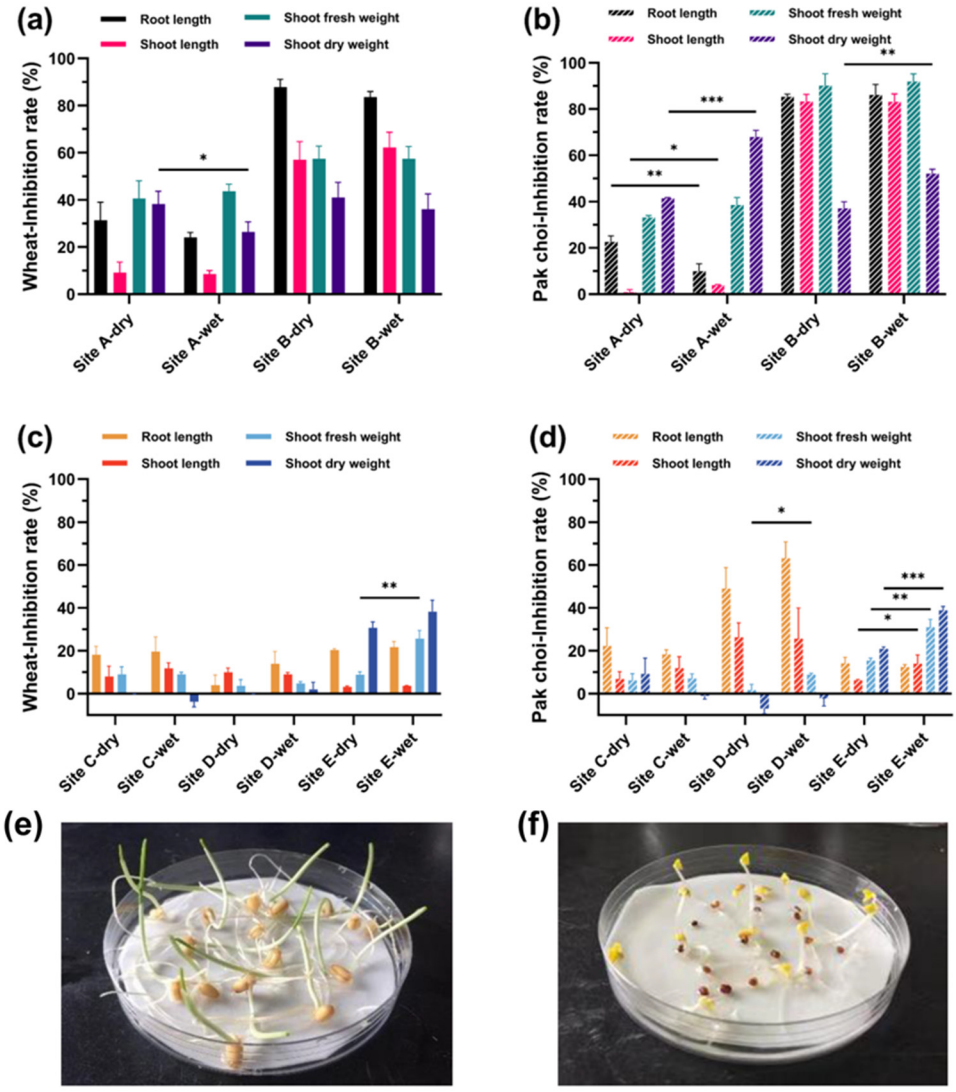


Figure 1. Inhibition rates of root and shoot elongation, shoot fresh biomass, and shoot dry biomass of (**a**) wheat and (**b**) pak choi exposed to water extracts from metal-contaminated soils in sites A and B. Inhibition rates of root and shoot elongation, shoot fresh biomass, and shoot dry biomass of (**c**) wheat and (**d**) pak choi exposed to water extracts from hydrocarbon-contaminated soils in sites C, D and E. The error bars represent the standard deviations. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicates a significant difference. Representative images of (**e**) wheat and (**f**) pak choi seedlings.

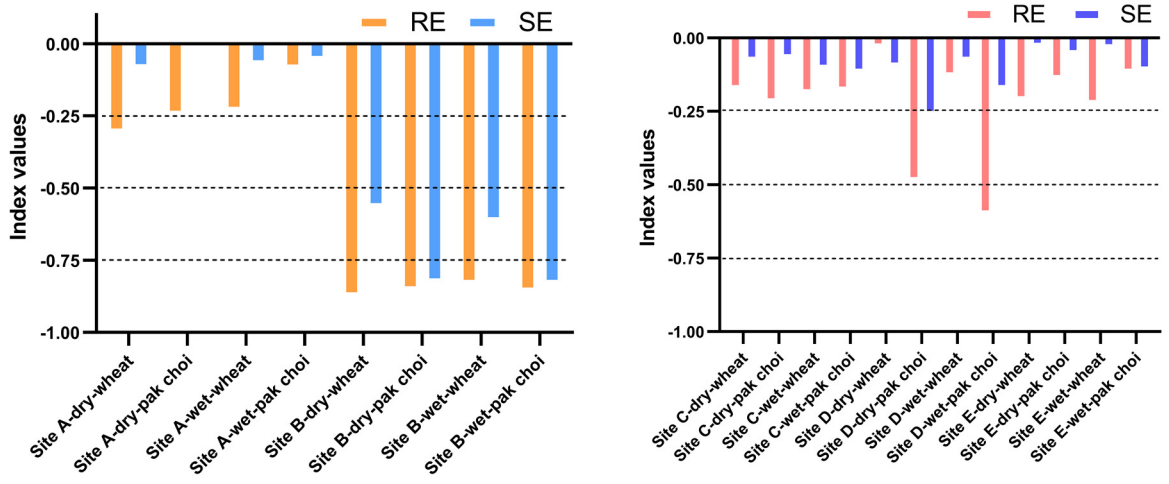
For pak choi, root length was the most sensitive parameter for site C, with the inhibition rates of 22.2 ± 8.5% and 18.3 ± 2.1% after exposure to water extracts from freeze-dried and wet soils, respectively (Figure 1d). The inhibition rates of all four parameters showed no significant difference between C-dry and C-wet soils. For site D, root length was the most sensitive parameter, with the inhibition rates of 49.0 ± 9.8% and 63.2 ± 7.6% for elutriates from freeze-dried and wet soils, respectively. The inhibition rates of shoot fresh weight showed significant difference ($p < 0.05$) between D-dry and D-wet soils. For site E, shoot dry weight was the most sensitive parameter, with the inhibition rates of 20.8 ± 0.9% and 38.8 ± 1.8% for elutriates from freeze-dried and wet soils, respectively. The inhibition rates of shoot length, shoot fresh weight, and shoot dry weight presented significant difference between E-dry and E-wet soils.

3.4. Phytotoxicity Evaluation of Soil Water Extracts

Root elongation (RE) and shoot elongation (SE) acute toxicity test was utilized to indicate the toxicity of the soluble-in-water fractions of pollutants that represent the most mobile, soluble and bioavailable pollutants in soil. RE and SE stand for the normalized residual elongation of the root and shoot of the germinated seeds in each treatment group, respectively.

$$RE/SE = (Elong_{sample(i)} - Elong_{control})/Elong_{control} \quad (1)$$

where $Elong_{sample(i)}$ is the average length of the seed roots or shoots after exposure to the soil extract i (cm), and $Elong_{control}$ is the average length of the seed roots or shoots in the blank control (cm) [51]. The values of these indicators differ from −1 to >0. The toxicity evaluation has been performed following the classification introduced by Bagur-González et al. [51]. The values in range of 0 and −0.25, −0.25 and −0.5, and −0.5 and −0.75 represent low toxicity, moderate toxicity and high toxicity, respectively. Meanwhile, the values range from −0.75 to −1 indicate very high toxicity. The growth of seed was stimulated when RE or SE values were above zero. As shown in Figure 2a, wheat root elongation (index values from −0.25 to −0.5) was more sensitive than pak choi (index values from 0 to −0.25) for water extract from site A freeze-dried soil. Indexes of shoot and root elongations for wheat and pak choi exposed to water extract from site A wet soil did not propose a great toxicity result (never more severe than low toxicity). Soil water extract from site B showed very high toxicity (index values from −0.75 to −1) for both wheat and pak choi. As seen in Figure 2b, soil water extracts from sites C and E presented low toxicity (index values from 0 to −0.25) for both wheat and pak choi, which could indicate that the possible soil water extracts from sites C and E are not a severe environmental issue in terms of acute toxicity. Pak choi showed sensitive responses to both dry and wet soil extracts from site D, and the RE index values were from −0.25 to −0.5 and from −0.5 to −0.75, respectively. Indexes of shoot and root elongations for wheat exposed to soil extracts from site D varied from 0 to −0.25, indicating low toxicity.



(**a**) (**b**)

Figure 2. Toxicity index of different metal-contaminated sites and hydrocarbon-contaminated sites analyzed in (**a**) wheat and (**b**) pak choi. Root elongation (RE) and shoot elongation (SE) stand for the normalized residual elongation of the root and shoot of the germinated seeds in each treatment group, respectively.

3.5. Toxicity Evaluation of Soil Water Extracts by Zebrafish Embryos

After being exposed for 96 h, soil water extracts affected developmental parameters including hatching rate and lethality (Figure 3). The hatching rates in the soil water extract from Site A were zero, indicating that soil extract from site A severely delayed the hatching time. As shown in Figure 3d,e, the hatching rates were significantly decreased, and the lethality were significantly increased when zebrafish embryos exposed to soil water extracts from site C and E. The hatching rates in C-dry and C-wet soil water extracts were 86.1 ± 4.8% and 88.9 ± 4.8%, respectively. The lethality rates in C-dry and C-wet soil extracts achieved 13.9 ± 4.8% and 11.1 ± 4.8%, respectively. Soil water extracts from site B and D did not significantly affect the hatching rate and lethality.

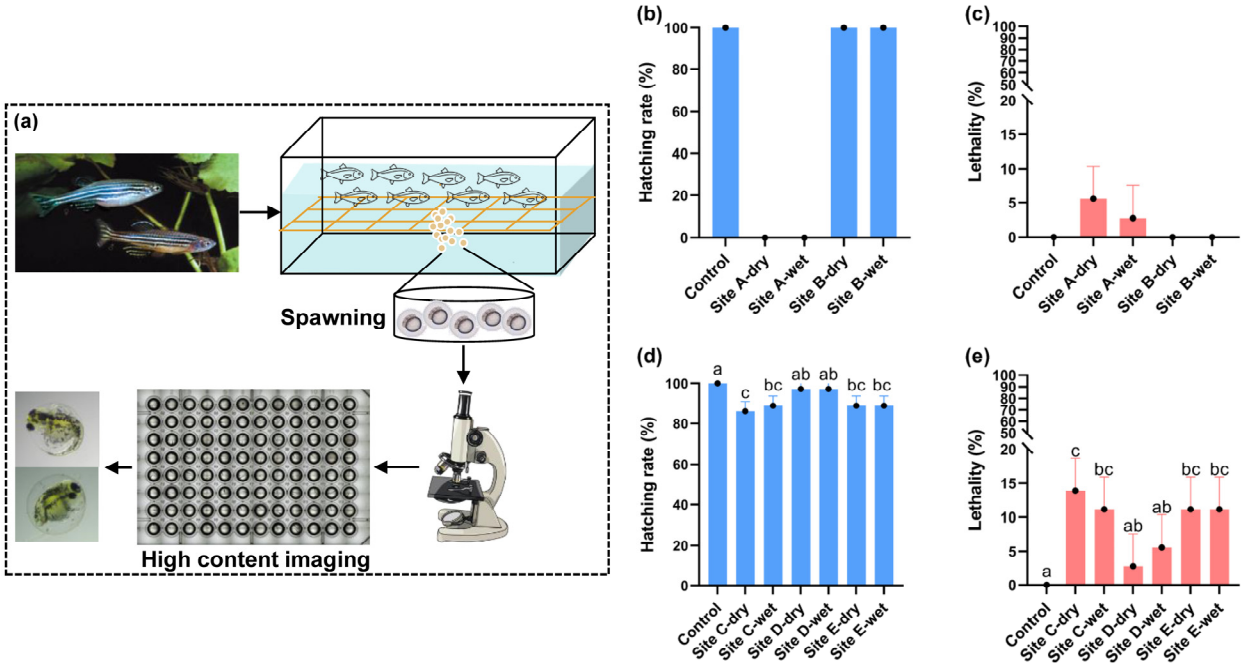


Figure 3. The experimental scheme of zebrafish developmental toxicity (**a**). Effects of soil water extracts from metal-contaminated soils (**b**,**c**) and hydrocarbon-contaminated soils (**d**,**e**) on the developmental parameters of zebrafish embryos. The error bars represent the standard deviations. Means with the same letters were not statistically different ($p < 0.05$).

4. Discussion

Soil quality assessment is often based on chemical analysis, but ecotoxicological data are also essential for properly assessing the ecological risk in the management and remediation of contaminated sites. Many studies have attempted to use bioassays for both aquatic and terrestrial compartment to characterize contaminated soils (Table S1). Lors et al. [52] demonstrated that liquid-phase bioassays allowed classing the contaminated soils in the same order than soil-phase bioassays. The risk of the contaminants on soil and groundwater is estimated from the test-results of soil extracts [53]. The use of water extracts of soil samples for the assessment of environmental hazards of water-mobilised soil pollutants has been critically examined [53,54]. In this study, integrated chemical parameters and ecotoxicity bioassays were conducted in site-specific risk assessments for five sites contaminated with heavy metals (sites A and B) or hydrocarbons (sites C, D and E). A phytotoxicity bioassay based on wheat and pak choi was conducted to evaluate the effect of soil water extracts on seedling growth. The plant responses to soil leachates toxicity could be related to different plant characteristics such as seed properties, root physiology and sensitiveness [55]. Compared to other standardized methods, the plant bioassay is simple, fast, inexpensive and accurate. Zebrafish embryo was selected as a standard test

and a new alternative bioassay. The utilization of zebrafish testing can not only ascertain mortality rates but also identify potential developmental abnormalities that may arise from short-term exposures. In contrast to other screening tests that are recognized for their rapidity and cost-effectiveness, such as the Microtox® test, this test would provide a greater amount of valuable information since zebrafish share common features with humans in development and organ responses induced by chemicals [56]. We aim to improve the knowledge about toxicological protocols that would enable the fast and first screening and identification of hazardous contaminated soil samples for the aquatic compartment using different organisms based on diverse exposure routes and toxicological endpoints.

Soil samples collected from contaminated sites can be conserved using wet storage or dry storage such as freeze-drying and air-drying. The fresh wet samples tend to preserve the initial chemical forms of elements in the soil compared to dried samples. The speciation of heavy metals may be altered when the soil is homogenized, sieved and dried compared to the original field situation [57]. Heavy metals present in different chemical speciation might affect their mobility, bioavailability and toxicity [58]. Therefore, soil toxicity might be altered when saturated field soils are air-dried in preparation for risk assessment tests [59]. In this study, acute bioassays with wheat and pak choi were applied to both types of soil water extraction samples, and the results were quite similar. The results of phytotoxicity evaluation indicated that the toxicity of both freeze-dried and wet soil water extracts on wheat differed depending on the contaminated site, with site A exhibiting low to moderate toxicity, site B showing very high toxicity, and sites C, D, and E displaying low toxicity. Similarly, the results of phytotoxicity evaluation revealed that the toxicity of both freeze-dried and wet soil water extracts on pak choi differed depending on the contaminated site, with sites A, C, and E exhibiting low toxicity, site B showing very high toxicity, and site D displaying moderate to high toxicity. The test results obtained with dry soil samples showed a good correlation with those obtained from fresh wet soil samples.

In general, all variables including root and shoot elongation, shoot freshness, and dry biomass were reduced after exposure to water extracts from metals-contaminated soils. Among heavy metals, zinc, copper, chromium, and nickel are fundamental micronutrients for most living organisms, but they may cause harm when are largely taken in [60,61]. For example, the rice biomass and yield production were decreased and the root-to-shoot translocation of nutrient element phosphorus was impeded due to excess Zn [62,63]. In this study, the roots growth was retarded more than shoots growth when exposed to metal-contaminated soils. The results are in agreement with other studies that demonstrated that root growth was a more sensitive indicator than shoot length after exposure to heavy metals, such as lead [64]. The explanation for this phenomenon is that roots are the first target tissue to encounter high concentrations of pollutants and metals tend to be retained in root tissues.

Soils water extracts from site A revealed different toxicity profiles and severity on plants and zebrafish. The soil water extracts from site A showed low to moderate toxicity on wheat, and growth inhibition by metals may be due to the high metal accumulation by seedlings [65,66]. Wheat root elongation was suppressed after exposure to certain concentrations of individual metals such as 2 mM Pb [64]. Ni at a concentration of 200 mM was reported to inhibit the shoot growth of wheat plants and exert toxic effects on wheat by influencing the metabolic processes of plants, disrupting plant mineral nutrition balance, inhibiting plant transpiration and photosynthesis, and interfering with common cell responses for heavy metal detoxification [67]. The potential pollutant Zn might also pose some risks, as it has no established screening value. Although Zn is an essential metal for all cells in all known organisms, it was demonstrated to induce a hermetic dose-response relationship in wheat seedlings [68]. Significant inhibitions were observed for the root growth and shoot elongation of wheat when the concentration of Zn was higher than 50 mg/L [69]. Soil water extracts from site A inhibited the hatching rates of Zebrafish embryos, which might be explained by its high concentration and bioavailability of Zn. Zn is regarded as an important pollutant for fish and other aquatic biota [70]. It was

demonstrated that the concentration of Zn in soil water extracts (soil:water = 1:2 ratio) was 290 µg/L when the soil is polluted with 33 mg/kg Zn [23]. The released free Zn(II) showed a hatching interference and only 7.1 ± 9.4% and 2.0 ± 2.7% zebrafish embryos hatched at 96 hpf when the Zn(II) concentration reached 0.5 and 2.2 mg/L, respectively [71]. Dissolved Zn ions were found to interfere in the functioning of the zebrafish hatching enzyme 1 (ZHE1) [72]. Besides additive interaction, other interactions may occur among various heavy metal species present in soils and elutriates, which often cause higher toxicity. Zebrafish larvae hatch from the entire egg at 48–72 hpf, but fish exposed to over dose (5.0 and 15.0 mg/L) of $NiCl_2$ took up to 96 hpf to complete the hatching process, indicating that nickel at high concentrations could lead to delayed hatching in Ni-treated larvae [73].

Soil water extracts from site B exhibited very high toxicity to wheat and pak choi, implying a significant environmental risk in terms of acute toxicity, which highlighted the urgency to remediate this site in the short term. The plant bioassays identified that soil from site B was more toxic than site A. These responses could be provisionally attributed to the evidence that soil B had higher bioavailable Cr(VI) than soil A, or to some overlooked interactive toxicity. Seedling growth could be significantly retarded when confronted with excessive mobile and soluble Cr(VI). The root elongation and shoot growth of wheat were significantly inhibited when the chromium level was 20 ppm [74]. Cr(VI) has a relatively high soil-plant transfer index, faster adsorption and uptake by root cells, and higher toxicity than Cr(III) [75]. The toxicity mechanism is that Cr(VI) might erupt the surfaces of plant root cells and inhibit the cell division and elongation. The inhibition of plant root growth would further disturb nutrient absorption and transportation, leading to suppression of plant shoot growth [76]. Thus, the reduction ratio of the root length was higher than that of the shoot length after exposure to high concentrations of Cr(VI). Excessive accumulation of chromium in the root influences plant metabolism, resulting in stunted lower biomass. Besides, Cr(VI) inhibits photosynthesis by interfering with electron transport and creates oxidative stress in plants by generating ROS [77]. Chromium enters the ecosystem via anthropogenic activities or natural processes, and the major anthropogenic activities include fertilizer application and the excessive use of Cr in alloys and chrome plating. Wheat is one of universally consumed cereals, and its production is noted to be damaged due to excessive Cr in soils [78,79]. Soil water extracts from site B showed no toxic effects on zebrafish embryos, while the water extracts from site A exhibited higher toxicity. These differences in species sensitivity underline the need to incorporate different species in a battery of bioassay methods [16,80].

Hydrocarbon-contaminated soil containing toxic and persistent compounds pose harmful effects on the ecosystem [81,82]. Petroleum hydrocarbons are complex mixtures of diverse hydrocarbon compounds, including aliphatic saturated compounds or paraffins, such as straight and branched chain alkanes, cycloalkanes, unsaturated alkenes, and alkynes, as well as aromatic compounds such as polycyclic aromatic hydrocarbons (PAHs) and monoaromatic hydrocarbons (MAHs) such as BTEX (benzene, toluene, ethylbenzene, xylene), asphaltenes, resins, waxes and tars [48,83]. Among all petroleum hydrocarbon pollutants, MAH and PAH pollutants are potent environment pollutants, also known as persistent organic pollutants [84]. Total petroleum hydrocarbon (TPH), BTEX and PAHs were investigated in soils from sites C and D. Petroleum hydrocarbon interfere with the plant intake of water and mineral salt, which lead to the failure of metabolic processes including the retention of chlorophyll and nutrients. The injured plant shows inhibited growth, contorted roots, leaves, and flowers with chlorosis and necroses [85]. The monoaromatic fraction (BTEX) is a water-soluble hydrocarbon with low octanol: water partition coefficient (K_{OW}), indicating high polarity, and is classified as a priority toxic pollutant by the US Environmental Protection Agency [86]. The existence of BTEX deserves serious attention as it can have a significant burden on humans and other organisms of the environment through migration to vadose zone and groundwater environments [87,88]. Thus, petroleum hydrocarbon-contaminated soils cause high potential ecological risks and hazardous impacts on humans and other living organisms surrounding the contaminated

aquatic and terrestrial ecosystems. Most organic contaminants (with log K_{ow} varying from −0.77 to 8.27) are preferentially embraced by plant roots and translocated to shoots, since chemicals with higher hydrophobicity are more readily bound to the root surfaces but more difficult to be translocated within the plant tissues [38]. Thus, the responses of roots were higher than shoots, which was especially shown in pak choi after exposure to organic pollutants extracted from site D.

Both freeze-dried and moisture soil water extracts from site C were found to have low toxicity towards wheat and pak choi. Soil water extracts from site D showed moderate or high toxicity towards pak choi and low toxicity towards wheat, affecting both root and shoot elongation. The concentrations of total petroleum hydrocarbon were 167.9 and 210.7 mg/kg in soils from site C and D, respectively. Soil from site D had higher concentrations of MAHs and PAHs than site C. Soil water extracts from site C decreased the hatching rate significantly and increased the lethality significantly, while soil water extracts from site D presented no toxic effects on zebrafish embryos, indicating that pollution mixtures might provoke interactive effects on organisms. Therefore, water extracts from soil C and D caused significant effects on zebrafish embryos and pak choi, respectively, underlying the difference between species bioavailability and sensitivity after exposure to the same contaminated medium. The different test species showed differential responses to the hydrocarbon mixture, and species responses of particular bioassays depended upon different contaminated site. For example, pak choi root elongation at the site D showed remarkable responses at a given site for specific endpoints. It is worth noting that sites C and D would probably not be categorized as contaminated sites since the soil is considered non-toxic when aromatic compounds having carbon numbers in the range of C10–C40 is below 826 mg/kg according to GB 36600-2018 [89]. The results of this study emphasize the need to apply a multiple test battery to screen the toxicity of soil in association with chemical analysis and soil characteristics, in order to provide accurate recommendations for remediation processes or interventions. Ecotoxicological testing of soil water extracts might be an excellent tool to shed light on unexpected conclusions.

The contaminated site E, located in Jiangsu province, China, is adjacent to surface rivers, and the groundwater table is shallow (3.5–5.0 m below the ground surface) [90]. Dichlorobenzene (DCBs) are widely applied as raw materials, organic solvents and intermediates in pesticidal industries [91]. Due to their high toxicity, the US Environmental Protection Agency has listed DCBs as priority pollutants. The maximal contaminant levels in drinking water for 1,2-DCB and 1,4-DCB were reported as 0.6 mg/L and 0.075 mg/L, respectively [92,93]. The concentrations of 1,2-DCB and 1,4-DCB were 403.0 and 21.7 mg/kg in soils from site E, respectively, which would inevitably lead to the release of great quantities of these chemicals into the groundwater. For site E, the inhibition rates of freeze-dried and moisture soil extracts on the wheat shoot dry weight were 30.7 ± 2.7% and 38.1 ± 5.4%, respectively. Wheat plants exposed to DCBs had reduced biomass since DCBs inhibit cell division [94,95]. The inhibition rates of freeze-dried and moisture soil extracts on the pak choi shoot dry weight were 20.8 ± 0.9% and 38.8 ± 1.8%, respectively. The different levels of interference of the contaminated soil in the shoot development can be attributed to the specific species [96]. The hatching rate and lethality of zebrafish embryos were also significantly affected by soil extract E. The toxicity test of soil water extracts using wheat, pak choi, and zebrafish indicated the presence of water-soluble toxic substances in soils from site E.

5. Conclusions

Elutriate toxicity tests were conducted using wheat, pak choi, and zebrafish to perform site-specific risk assessments on soils contaminated with metals and hydrocarbons. Soil water extract from site A showed low to moderate toxicity on wheat. The soil water extract from site A completely inhibited zebrafish embryo hatching rates, which might be explained by its high concentration and bioavailability of Zn, a potential pollutant without an established screening value. Soil water extracts from site B, containing high

concentrations of Cr(VI), exhibited very high toxicity to wheat and pak choi, while no significant toxicity was shown on zebrafish embryo. Soil water extracts from site C were only found to have low toxicity to wheat and pak choi but exhibited significant toxicity to zebrafish. Soil elutriates from site D showed moderate or high toxicity to pak choi and low toxicity to wheat, affecting root and shoot elongation. Therefore, soils from sites C and D pose ecological risks to the environment, even though the concentrations of TPH are below the established screening value. Soil extracts from site E containing excessive dichlorobenzene presented toxicity to plants shoot biomass and zebrafish. Therefore, chemical analytical data and screening values are inadequate to assess the potential ecological impact of contaminated soils. Ecotoxicological tests could complement the ecological risk estimation of a complex mixture of contaminants in soils. The different sensitiveness of bioassays calls for the simultaneous use of several ecotoxicological tests to offer a more integrative perspective on environmental risk of soil contamination and its rehabilitation.

The water extracts were used to simulate the natural process of soil percolation by rainwater or flood event, and the leachates were toxic to test species, which demonstrated the existence of mobile and bioavailable toxicants. To obtain a closer characterization of the potential biological impact of the contaminated soils, future studies should include different extraction methods besides water extraction and distinct organisms of different trophic levels. The extraction with organic solvents could increase the solubility of organic contaminated soils. The challenge has been to develop a risk-based correction action approach to integrate ecological assessment methods to make management decisions efficiently and for minimal cost.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15234061/s1. Table S1: Ecotoxicological tests of the soils. Refs. [97–107] are cited in supplementary materials.

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