Application of Bio-Toxicity Test on Investigation and Assessment of Incident Caused Contaminated Sites

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Abstract. Earthworm acute toxicity, plant seed germination bio-toxicity tests were conducted to evaluate the contaminated site polluted by a pharmaceutical intermediates accident. In the earthworm acute toxicity test, the mortality rate of earthworms in the control group was 0, while the earthworms mortality rate in the two groups of contaminated soil both reached 100% within 24h and three days respectively. The plant seed germination rates of four species except ryegrass were all less than 20% in the treatment dose (contaminated soil: reference soil, 25%). The test results indicated that the soil samples showed high biological toxicity. Eco-toxicity tests can effectively characterize the ecotoxicity and be used in quick identification of accident hazard and risk assessment in environmental emergency response. In addition, it can provide government departments with the basis for the environmental management and provide techniques of detailed site survey and risk assessment for relevant scientific research departments and enterprises as well.

1. Introduction
The long-term industrialization and urbanization process has caused a large number of contaminated sites in China. The extensive economic growth and lack of system has led the organic pollution in the Yangtze River Delta from point-like and local to regional. The industrial site pollution has been more and more serious. In addition, the Yangtze River Delta region has always been an important chemical industry base in China, with a large number and wide distribution of chemical, pesticide, printing and dyeing companies. Nowadays, many cities have been implementing the “suppress the second industry and develop the third industry” strategy to adjust the industrial structure, remaining serious soil and groundwater pollution and bringing environmental risks and health hazards. With the promotion of soil environmental protection work in China, the incidents caused contaminated sites have also attracted people’s attention. Contamination incidents, for instance, chemical pipeline rupture, transport vehicle leakage, solid and hazardous wastes dumping and burial (such as the Jingjiang Houhe River incident), informal landfill, production safety accidents (such as the Tianjin Port explosion accident), etc., have got its impact increased and aroused high concern from the whole society. It is particularly necessary to properly conduct site investigation, evaluation and control. At present, most of the studies focus on emergency monitoring and control of sites polluted by sudden accidents, and there are few studies on investigation, evaluation and control of such sites. Since China has not yet established pollution site identification standards, the current soil environmental quality standard pollutants project is extremely limited, and most organic materials...
have no standards to refer to. The screening values in the pollution site risk assessment guidelines released so far are also basically referenced to domestic and foreign standards. In the absence of corresponding standards, the environmental hazard significance of chemically measured pollutant concentration information cannot be determined. In addition, the background information of many contaminated sites is unclear. After several times of conversion of owners and business projects, the production history is difficult to find out and the characteristic pollutants are unknown. The pollution sources and pollutants are various and have complex composition, involving heavy metals, a variety of volatile organic compounds, semi-volatile organic compounds, and intermediates, including “three causes matter”, highly toxic and persistent organic pollutants. The pollution information in the site environment is also unclear. So, it is difficult to accurately and comprehensively select monitoring indicators in site survey. Moreover, the long-term accumulation of chemical pollutants can cause low-dose combined pollution and joint toxic effect. So, biotoxicity test is a necessarily supplementary means to diagnose and display relevant hazard information.

For the requirements of initial and fast identification of site pollution ecological hazards and combined with the characteristics of the pollutants, the acute and short-term toxicity tests are mainly used in the bio-toxicity test system. Representative and sensitive organisms are selected as test organisms, such as luminescent bacteria, alfalfa and rapeseed. Through bio-toxicity test, the toxic effect of contaminated soil and water at different sampling points can be identified. In addition, it can serve as an assistant means to quickly determine the contaminated area, identify pollution characteristics and ecological risk.

In this study, a site contaminated by a pharmaceutical intermediates accident was used as an example. According to quick chemical analysis, the pollutants in this site were mainly halogenated aromatic hydrocarbons, halogenated anilines and nitroaromatics. To assess the hazard of the “unconventional” pollution factors and comprehensively reflect the pollution situation, earthworms and terrestrial plant seeds were used as test organisms in bio-toxicity test.

2. Materials and Method

2.1. Experimental organisms

In this test, earthworms (Eisenia foetida) and five species of terrestrial plant seeds were used as experimental organism.

2.1.1. Earthworms. The earthworms were purchased from a breeding base in Jurong. Worms should be adult and health (more than 2 months old with clitellum and weighed about 300-600 mg) and originate from the same population. Before the test, earthworms must be cultured for at least 7 days in uncontaminated soil at the test temperature. When one of the followed situations takes place, the test will be ineffective: food is insufficient or excessive; humidity is too high or too low; temperature is not suitable; pH range is not appropriate; the worms have been hurt.

2.1.2. Plant seeds. Five species were selected in this test including corn (Zea mays L.), wheat (Triticum aestivum L.), ryegrass (Lolium perenne Linn.), soybeans (Glycine max (Linn.) Merr.) and rape (Brassica campestris L.), which were purchased from the seed company of Nanjing agricultural university. The seeds should have basically the same size and be intact with the same level of fullness. They were grouped by standard seed sieves according to the particle size. The seeds used in the test should all come from the dominant particle size group. They should be germinated in the artificial climate chamber 24 hours before the test.

2.2. Soil samples

Soils samples were obtained from an Incident Caused contaminated site near the Chemical Industry Park in Guannan County, Lianyungang City, Jiangsu Province, located close to the Guanhe River which is connected to the Yellow Sea. The site is about 600 square meters with hydrops inside. The
color of the soil is yellowish-brown, and some soil samples are darker and smelly. Samples were air-dried, ground and mixed in a cool condition.

2.3. Experimental equipment
Mettler electronic balance (model PL602), soil temperature and humidity moisture meter (model Takeme), Mettler portable pH meter (model S8), 1L specimen bottle, cling film, tweezers, Merck pH test paper, white porcelain plate, Artificial climate chamber, etc.

2.4. Earthworm toxicity test
The experimental methods referred to “OECD Guidelines for the Testing of Chemicals Test No. 207: Earthworm, Acute Toxicity Tests”.

2.4.1. Sample preparation. Soil samples collected from the contaminated site were used as test medium and placed in a glass beaker. The soil samples should to be air-dried, then screened with 4 mm sieve and mixed. Physical and chemical parameters such as soil pH, texture, water content, saturated water holding capacity, and cation exchange amount were tested. Unpolluted Soil collected close to the contaminated site was chosen as reference soil. According to the Pre-tests, direct exposure to the contaminated soil can cause worms to die. Therefore, the test soil was mixed with reference soil in different dilution ratios in the formal test. The mixing ratios (contaminated soil: reference soil, dry soil weight) were 1:9, 1:4, 3:7, 2:3, 1:1, 3:2, 7:3 and 4:1, three replicates were set for each concentration and a control group was set as well.

2.4.2. Earthworm inoculation and culturing. Worms were kept on moist filter paper before formal test for 24h to void their gut contents and then weighed their initial body weight. For each treatment, ten earthworms were placed into 500g of soil in a glass beaker sealed with plastic film. The film was punched with holes by disposable needles. Then placed the glass beakers in the culture chamber. The test temperature was 20±2℃ and humidity was about 80%. A small amount of water was sprayed periodically to maintain the humidity of the substrate. Tests were done for a period of 7 days.

2.4.3. Observation of test results. The survival rate and state of the worms were checked regularly. Experimenters gently poured the test medium into a white porcelain dish, spread it out and picked out the worms, then counted the number of live worms, observed the reflection of the front tail on mechanical stimulation, and recorded abnormal behavior or symptoms.

2.5. Plant seeds toxicity test
The experimental methods referred to “OECD Guidelines for the Testing of Chemicals Test No. 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test”.

2.5.1. Seeds cleaning and sterilization. All glassware and plant seeds were cleaned before tests. Before the selected seeds being placed (about 100 seeds each species), the culture dishes should be sterilized by Hydrogen peroxide, alcohol and sodium hypochlorite. First, the dishes were sterilized with 3% H₂O₂ for 10min and rinsed with tap water. Then, in the same way, 70% alcohol and 30% NaClO solution were used in turn for 1-2min and 20min respectively. At last, the culture dishes were soaked in water for 30 minutes. After the seeds had absorbed moisture, they were moved to the clean culture dishes.

2.5.2. Plant seed germination / growth experiment
Pre-test was accomplished to determine the test period of each tested plant before the formal test. When the selected seeds being placed (about 100 seeds each species), the culture dishes should be sterilized by Hydrogen peroxide, alcohol and sodium hypochlorite. First, the dishes were sterilized with 3% H₂O₂ for 10min and rinsed with tap water. Then, in the same way, 70% alcohol and 30% NaClO solution were used in turn for 1-2min and 20min respectively. At last, the culture dishes were soaked in water for 30 minutes. After the seeds had absorbed moisture, they were moved to the clean culture dishes.
In the formal test, contaminated soil was mixed with control soil in proportion (dry weight ratio) and divided into 5 different concentration groups (including control group): 15:95, 25:75, 15:85, 5:95, 0:100. The test soil and control soil were weighed (50g) and put on glass culture dishes with a diameter of 9cm and a depth of 11cm. Then the proper amount of deionized water was added with the water-soil ratio of 1:5 to reach 50% of the maximum water holding capacity. After that, the experimenters selected 10 seeds with uniform size to grow and cover the glass culture dishes. It is then germinated in an artificial incubator at a natural light temperature of 21±2°C. The root length referred to the length between the budding joint to the root tip, and the test was considered to be successful when the root length and the bud length were both more than 3mm. Three soil samples were repeated for each concentration group, and 10 seeds are repeated for each group. When the seed germination rate of the control group reached more than 65% and the root elongation length was 20 mm, the test could be completed. The average number of germinated seeds and average root length of each treated and control seed were determined at the end of the test.

Analysis and measurement: Numbers of germinated seeds of each species were counted and their root length were measured. The next test plant should be measured after measuring all root lengths of one test plant in sequence. Root length should be measured from the transition point of the hypocotyl and root to the end of the root tip.

2.6. Quality control

2.6.1. Earthworm toxicity test. The mortality rate of the control group should not exceed 10% at the end of the test. The Experimental method and operations referred to GB/T 21809-2008 (Chemicals—Test method of earthworm acute toxicity test), ISO 11269-1 (2012) and OECD Guidelines for the Testing of Chemicals 207 (2006) “Earthworm, Acute Toxicity Test”.

2.6.2. Plant seeds toxicity test. The following conditions shall be met. (1) Only the untreated seeds from the same batch and the same year or season can be used in the test, without the fungicide or insect repellent. The same particle size and fullness is required. The tested seeds should have a moisture content less than 10% and are stored at 5 °C. (2) The germination rate of the control groups should be greater than 60%. (3) Control groups should be set for each test. (4) All glassware used in the test should be clean and unpolluted.

2.7. Data processing

In the earthworm toxicity test, the content of the contaminated sample in the test medium (g/kg) was taken as the treatment dose. Mortality, LC50, and 95% confidence limits were calculated based on the earthworms’ survival state. In the plant seeds toxicity test, the seed germination rate of each treatment and control group was counted. All statistics were conducted with the SPSS 16.0 software.

3. Results

3.1. Earthworm toxicity test

3.1.1. Effect of the treatment dose of contaminated soil. During the test, earthworms in the control group had normal activities and no one died, while the test group’s worms all hided and fled, refused to penetrate the test medium, their body gradually appearing swollen, blackened and suppurated, and all died in 24h (table 1). It indicated that soil samples in this area were highly bio-toxic and should be paid more attention to.
Table 1. Earthworm mortality in different treatment dose (24h).

| Treatment dose (contaminated/reference) | Earthworm mortality (24h) |
|----------------------------------------|---------------------------|
| 0%                                     | 100%±0                    |
| 70%                                    | 100%±0                    |
| 60%                                    | 100%±0                    |
| 50%                                    | 100%±0                    |
| 40%                                    | 100%±0                    |
| 30%                                    | 100%±0                    |
| 20%                                    | 100%±0                    |
| 10%                                    | 100%±0                    |
| CK                                     | 0±0                       |

3.1.2. Effect of the depth of contaminated soil. The two soil samples of the contaminated site (0-40cm, 40-80cm) had extremely lethal effect to earthworms while all the worms in the control group survived. Referring to the biotoxicity levels classification method recommended by the International Standards Organization (ISO) based on earthworm mortality (%) in acute toxic test, the contaminated soil of this site was indicated highly toxic to earthworms and should be listed as a key survey site.

Table 2. Earthworm mortality in soil samples of different depth

| Sampling sites | Mortality (1d) | Mortality (3d) |
|----------------|----------------|----------------|
| CK             | 0              | 0±5%           |
| 0-40cm         | 55%±7%         | 100%±0         |
| 40-80cm        | 100%±0         | 100%±0         |

3.2. Plant seed germination test
There were some wrong with soybean (Glycine max (Linn.) Merr.) seeds group. Therefore, four plant species are planted in pots to test the contaminated soil. Seedling emergence and seedling growth of the different species and control groups were significantly different (P<0.05).

Table 3. Seedling emergence in soil samples of different seeds

| Treatment dose (contaminated soil) | germination rate of corn (%) | germination rate of rape (%) | germination rate of wheat (%) | germination rate of ryegrass (%) |
|------------------------------------|------------------------------|------------------------------|--------------------------------|---------------------------------|
| CK                                 | 90±0                         | 90±0                         | 86.67±5.77                    | 86.68±5.77                      |
| 5%                                 | 76.67±5.77                  | 73.33±5.77                  | 80±0                          | 83.33±5.77                     |
| 25%                                | 11.67±5.77                  | 11.67±2.89                 | 18.33±7.64                    | 53.33±5.77                     |
| 50%                                | 0                            | 0                            | 0                             | 0                               |
| 75%                                | 0                            | 0                            | 0                             | 13.32±5.77                     |
| 100%                               | 0                            | 0                            | 0                             | 3.33±5.77                      |

Table 4. Effects on root length of different seeds in treatment dose

| Treatment dose (contaminated soil) | root length of corn (%) | root length of rape (%) | root length of wheat (%) | root length of ryegrass (%) |
|------------------------------------|-------------------------|-------------------------|--------------------------|----------------------------|
| CK                                 | 1.74±0.14               | 0.92±0.07               | 0.47±0.06                | 1.38±0.20                   |
| 5%                                 | 0.85±0.06               | 0.72±0.05               | 0.39±0.03                | 1.19±0.12                   |
| 25%                                | 0.29±0.07               | 0.34±0.03               | 0.12±0.07                | 0.79±0.06                   |
| 50%                                | 0                       | 0                       | 0                         | 0.42±0.03                   |
| 75%                                | 0                       | 0                       | 0                         | 0.21±0.05                   |
| 100%                               | 0                       | 0                       | 0                         | 0.12±0.07                   |
4. Discussion and Conclusions
In this study, the ecotoxicity of an incident caused contaminated site was concerned. In the earthworm acute toxicity test, the mortality rate of earthworms in the control group was 0, while the earthworms mortality rate in the two groups of contaminated soil both reached 100% within 24h and three days respectively. The plant seed germination rates of four species were all less than 20% except ryegrass in the treatment dose of contaminated soil (25%). The test results indicated that the soil samples had high biological toxicity. However, the eco-toxicity of incidents caused contaminated sites has not been studied systematically and the toxic grading method is lacking. Although some studies have found out the LC_{50} or LD_{50} of test organisms for specific pollutants, such as some heavy metals and organic pollutants, but they are not applicative to the assessment of combined pollution sites. Li (2015) has established a comprehensive weighted evaluation and ecotoxic grading method but it has not been generally accepted and further study is needed.

Despite the shortcomings, the bio-toxicity test of site pollution has good usability in site pollution detection in terms of toxicity characterization, test conditions and test results, and can quickly characterize the comprehensive pollution effect with reliable results. The method has good extension and application value. It can provide government departments with the basis for the environmental management such as preliminary screening and file building of contaminated sites, screening priority management site, and setting site remediation target (standard). It also provided technics of detailed site survey, risk assessment and site remediation effect test for relevant scientific research departments and enterprises. It can also be used in quick identification of accident hazard and risk assessment in environmental emergency response.

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References
[1] Brombal D, Wang H, Pizzol L, et al. (2015) Soil environmental management systems for contaminated sites in China and the EU Common challenges and perspectives for lesson drawing. Land Use Policy, 48: 286-298.
[2] Zhao H. (2015) Causes of oil and gas pipeline incidents in China and environmental precautions. Oil & Gas Storage & transportation, 34(4): 368-372.
[3] Bai Y, Wang H. (2006) Preliminary discussion on the disposal of road hazardous chemical tanker leakage incident on Road. Fire Science and Technology, (s1):88-89.
[4] Chen M, Sun Z, Wang J. (2011) Strengthening environmental supervision to ensure environmental safety - the enlightenment of incidents caused by illegal off-site dumping of hazardous wastes. Environmental Protection, (21): 52-54.
[5] Li X, Zhou J. (2016) Secrets under the pig farm. China Weekly, (7): 48-52.
[6] Han H, Li S, Yu Y. (2011) Preliminary survey and assessment of fill sites. Journal of Engineering Geology, 19(5): 771-777.
[7] Cao X. (2016) After the Tianjin explosion accident… Hidden dangers of surrounding groundwater and soil pollution spread remained. The Earth, 0(7): 39-41.
[8] Critto A, Torresan S, Semenzin E, et al. (2007) Development of a site-specific ecological risk assessment for contaminated sites; Part I. A multi-criteria based system for the selection of eco toxicological tests and ecological observation. Science of the total environment, 379(1): 16-33.
[9] Semenzin E, Critto A, Carlon C, et al. (2007) Development of a site-specific ecological risk assessment for contaminated sites; Part II. A multi-criteria based system for the
selection of bioavailability assessment tools. Science of the total environment, 379(1): 34-45.
[10] Hernández A J, Bartolomé C, Pérez-Leblic M I, et al. (2012) Ecotoxicological diagnosis of a sealed municipal landfill. Journal of Environmental Management, 95(2): S50-S54.
[11] Song Y, Zhou Q, Song X, et al. (2005) Evaluation of eco-toxicity of integral quality of soils. Environmental Science, 26(1): 130-134.
[12] Shen C. (2008) Eco-toxicological diagnosis of contaminated soil from an e-waste recycling area. Zhejiang University, Hangzhou.
[13] Li J, Gao C, Li H, et al. (2017) Screening of soil eco-toxicity diagnosis methods of contaminated sites. Environmental Pollution & Control, 1(39): 106-110.
[14] Li J, Liu Z, Li H, et al. (2015) Evaluation method of soil ecological toxicity diagnosis results in contaminated sites. In: International Symposium on Environmental Risk Assessment and Standards/Standards for Chemical Substances.