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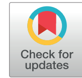
Development of a nematode offspring counting assay for rapid and simple soil toxicity assessment☆



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Since the introduction of standardized nematode toxicity assays by the American Society for Testing and Materials (ASTM) and International Organization for Standardization (ISO), many studies have reported their use. Given that the currently used standardized nematode toxicity assays have certain limitations, in this study, we examined the use of a novel nematode offspring counting assay for evaluating soil ecotoxicity based on a previous soil-agar isolation method used to recover live adult nematodes. In this new assay, adult *Caenorhabditis elegans* were exposed to soil using a standardized toxicity assay procedure, and the resulting offspring in test soils attracted by a microbial food source in agar plates were counted. This method differs from previously used assays in terms of its endpoint, namely, the number of nematode offspring. The applicability of the bioassay was demonstrated using metal-spiked soils, which revealed metal concentration-dependent responses, and with 36 field soil samples characterized by different physicochemical properties and containing various metals. Principal component analysis revealed that texture fraction (clay, sand, and silt) and electrical conductivity values were the main factors influencing the nematode offspring counting assay, and these findings warrant further investigation. The nematode offspring counting assay is a rapid and simple process that can provide multi-directional toxicity assessment when used in conjunction with other standard methods.

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1. Introduction

Nematodes play an important role in environmental trophic systems and nutrient cycles, and are widely distributed in soil and microbe-rich habitats (Félix and Braendle, 2010). *Caenorhabditis elegans* (*C. elegans*), a free-living nematode, has standardized developmental stages with a short lifespan, and it is the only animal with an entirely known cell lineage and nervous system (Sulston and Horvitz, 1977; Wood, 1988). Therefore, *C. elegans* is generally known as a key species for use in toxicity assays. Williams and Dusenbery (1990) first suggested an aquatic test method using *C. elegans*, since then several studies have used this bioassay (Cressman and Williams, 1997; Donkin and Williams, 1995; Hitchcock et al., 1997). Nematode toxicity assays have also been conducted on soil medium (Donkin and Dusenbery, 1993, 1994), and various studies have reported the development of this type of bioassay using whole-sediment and soil samples from natural sites (Höss et al., 1999, 2001, 2009, 2012; Traunspurger et al., 1997). Since the establishment of standardized nematode toxicity assays by the American Society for Testing and Materials (ASTM) and the International Organization for Standardization (ISO) (ASTM, 2001; ISO, 2010), studies have routinely used these standard methods. Recently, *C. elegans* has been widely used to evaluate toxic mechanisms of various xenobiotics, including endocrine-disrupting chemicals and metal-based nanomaterials (Starnes et al., 2015; Zhou et al., 2016), and bioassays also have been used to estimate complex exposure scenarios, such as multi-generational or trophic transfer effects (Kim et al., 2016; Moon et al., 2017). In these previous studies, however, agar and liquid were chosen as test media, but the soil matrix was not used.

In standard toxicity tests using nematodes, *C. elegans* individuals are exposed to a contaminated soil and sediment system, and are extracted from the medium by a colloidal silica flotation method using LUDOX solution (ASTM, 2001; ISO, 2010). The ISO (2010) standard method includes the rose Bengal staining process for

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improving the recovery rate. Although these methods are internationally standardized, there are limited reports regarding the derivation of toxicity values. Previous studies using the ISO standard have focused on interpreting the relationship between media properties and toxicity, methods of application on natural samples, and method standardization (Höss et al., 1999, 2001; 2009, 2012; ISO, 2010; Traunspurger et al., 1997). Studies using the ASTM standard are designed for the assessment of the lethal or sublethal effects of several metal toxicants on nematodes in soil media (ASTM, 2001; Black and Williams, 2001; Boyd et al., 2001; Boyd and Williams, 2003; Donkin and Dusenbery, 1993, 1994; Freeman et al., 1999; Graves et al., 2005; Ma et al., 2009; Perendney and Williams, 2000a, 2000b; Power and De Pomerai, 1999). To determine the protection level for ecological receptors in the soil environment, a probabilistic ecological risk assessment (PERA) is conducted with species sensitivity distribution (SSD) to qualify and quantify the toxicity data (ECB, 2003; NEPC, 2011). For this step, the use of various ecotoxicity data from a battery of bioassays is very important, and calculations should be carefully and reliably performed using the standardized process. Although the ISO and ASTM standard methods are already published and provide reliable data, there is a further need for the derivation of toxicity values, rapid processes, and accessibility to researchers.

In the present study, we examined the use of a nematode offspring counting assay for soil ecotoxicity evaluation. The bioassay was performed according to Kim et al. (2014) with some modifications. The nematode *C. elegans* individuals were exposed following a standardized toxicity method, and the offspring in the test soil were attracted by a microbial food source in an agar plate. This method is similar to that used by Kim et al. (2014), who counted the number of surviving adults, but differs in term of its endpoint, that is, the "number of offspring." To determine the optimal conditions for our bioassay, we performed a sufficient number of replications, and metal-spiked soils and various field soils were used to assess its applicability as a toxicity assay.

2. Materials and methods

2.1. Test organism

C. elegans (wild type, Bristol strain N2) was obtained from the Animal Genomics Laboratory, Konkuk University (Seoul, South Korea). All worms were cultured on nematode growth medium (NGM: NaCl 3 g/L, peptone 2.5 g/L, agar 17 g/L, 1 M potassium phosphate 25 mL/L, 1 M $CaCl_2.2H_2O$ 1 mL/L, 1 M $MgSO_4 \cdot 7H_2O$ 1 mL/L, cholesterol 1 mL/L) with the *Escherichia coli* strain OP50 as the food source (Brenner, 1974). The *E. coli* strains were obtained from the Korean Agricultural Culture Collection (KACC, Seoul, Korea). The cultures were maintained at 20 ± 2 (SE) °C under darkness. To obtain age-synchronized worms, at least 3-d-old *C. elegans* were treated with Clorox solution for 10 min, and the solution was centrifuged at $400 \times g$ for 2 min. The treated samples were washed with K-medium (0.032 M KCl, 0.051 M NaCl) (Williams and Dusenbery, 1990) 3 times, and pellets of eggs were applied to NGM plates with *E. coli* OP50. The plates were cultured in an incubator until the early-adult stage (54–58-h synchronization after Clorox solution treatment) under darkness at 20 °C. All ingredients in the media were purchased from BD (Maryland, USA), except for sodium chloride and potassium chloride (Duksan, Asan, Korea).

2.2. Nematode offspring counting assay

Landwirtschaftliche Untersuchungs-und Forschungsanstalt (LUFA) 2.2 (LUFA Speyer, Germany) was selected as a test soil for assessing the utility of the nematode offspring counting assay. According to the supplier, the pH, organic matter (OM) content, organic carbon content, and the texture class are 5.5 ± 0.2, 3.82%, 1.77%, and loamy sand, respectively. The LUFA 2.2 soil was sifted through a 2-mm sieve and air-dried for 7 days. K-medium (0.2 mL) was added to each well of a 24-well plate containing 0.3 g of LUFA 2.2 soil, followed by the addition of 10 age-synchronized adults. After 24 h, the soil containing worms were spread on soil-agar isolation plates (Kim et al., 2014). To prepare the soil-agar isolation plates, *Escherichia coli* stain OP50 was cultured in Luria-Bertani medium (25 g/L) at 37 °C for 16 h, and 150 μL of the cell suspension (optical density 1.1–1.2, $8.0–9.6 \times 10^8$ cells/ml) was smeared onto an NGM agar plate (Fig. S1a). The test soils were arranged linearly in the central area of the soil-agar isolation plates, and the offspring emerged from soils were counted (Fig. S1b). The arrangement of test soils should be performed with utmost care and precision, and the contact between the food area and the boundary of soil should be minimal. First, the soil mass, which can be scooped using a laboratory spatula, was transferred onto a soil-agar isolation plate, and the remaining water containing soil particles and worms was moved using water surface tension between the well plate and laboratory spatula (Fig. S1c; Supplementary Video). Thereafter, 50 μL of K-medium was added to prevent loss of moisture. The offspring that emerged from the test soil after 0.5, 1, 2, 3, 4, and 5 h were counted under a microscope ($n = 3$). To evaluate whether the absence of food affects offspring recovery, soil-agar isolation plates without a food source were also prepared ($n = 4$). The optimal observation point (h) was determined, and the mean value of offspring was calculated based on 50 replications.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.envpol.2018.01.037.

2.3. Applicability of the nematode offspring counting assay

To evaluate the applicability of the nematode offspring counting assay for toxicity assessment, we investigated its utility by testing on metal-amended soils. Arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn) soils were prepared at concentrations of 0, 200, 400, 600, 800, and 1000 mg/kg in LUFA 2.2. Test chemicals $NaAsO_2$, $CdCl_2$, $CuCl_2 \cdot 2H_2O$, $NiCl_2$, $PbCl_2$, and $ZnCl_2$ were obtained from Sigma, and a stock solution was prepared in K-medium to adjust the ionic concentration of each metal (As, Cd, Cu, Ni, Pb, and Zn) in soil. Soil samples (0.3 g) were placed in each well of the 24-well plates ($n = 3$), and 0.2 mL of stock solution containing metals. The final concentration in soil was determined as each of the aforementioned concentrations (0, 200, 400, 600, 800, and 1000 mg/kg). Ten worms were added to each well and exposed for 24 h at 20 °C under darkness. We used the nematode counting assay at the end of test, and the number of offspring emerged from test soil were counted after 3 h.

2.4. Application of the nematode offspring counting assay using field soil

Thirty-six field soils (S1–S36) were collected from 3 metalliferous sites in South Korea. These sites are located on the perimeter of an arsenic smelter, and have been contaminated by dust emitted from the smelter chimney. High-grade arsenic (As), cadmium (Cd), and lead (Pb) were detected in soils at nearby sites (2 km) that have chimneys located at their center (Kim et al., 2017). Soils were hand-sorted, air-dried, and sieved to <2 mm. The aggregated stability (AS) and bulk density (BD) of the soils were analyzed using the methods of Friedman et al. (2001) and Tan (2005), respectively. Cation-exchange capacity (CEC) was assessed by extraction using ammonium acetate and potassium

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