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DEVELOPMENTS OF BIOASSAY PROTOCOLS FOR TOXICANTS IN SOIL

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DEVELOPMENTS OF BIOASSAY PROTOCOLS FOR TOXICANTS IN SOIL

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ABSTRACT

The report describes the development of three whole soil bioassay protocols (*Brassica rapa*, earthworms, and microarthropods) and gives the details of the optimal methods of their practical use. It also describes the results of the application of these bioassays to determine the effects of two contaminants; zinc and mercury. Detrimental effects were observed on Brassica rapa at 10 - 20 mg Hg/kg of soil in sand, at 220 mg Hg/kg in clay, and at 50 mg Zn/kg soil in sand and 600 mg Zn/kg in clay. Earthworms were more sensitive than Brassica rapa to Hg in garden soil, but less so in sand. Also, a novel method of conducting whole soil bioassays on arthropods was developed. The results emphasize the need for whole soil bioassays on different species to properly assess terrestrial toxicity of contaminants.

"Acknowledgement and Disclaimer"

This report was prepared for the Ontario Ministry of the Environment as part of a Ministry funded project. The views and ideas expressed in this report are those of the author and do not necessarily reflect the views and policies of the Ministry of the Environment, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The Ministry, however, encourages the distribution of information and strongly supports technology transfer and diffusion.

Executive Summary

Bioassays of soils have not been extensively developed. This is especially true of tests that use the whole soil as opposed to soil extracts. Bioassays are important to assess toxicity impacts for contaminated soils where the contaminants may or may not be known and where they may be a mixture of toxic materials. In addition, bioassays are useful in setting generic cleanup and assessment guidelines where specific contaminants are added to selected soils to measure effect levels. The objective of this study was to extend the development of a discrete set of bioassays. The bioassays examined in detail were a life-cycle plant test, an earthworm survival test, and a novel test involving microarthropods. In each case, a series of experiments investigated the response of the assays to general soil and cultural variables. Their sensitivity to selected toxicants was then investigated, including comparisons to other established tests. The results are reported here and in three papers prepared for the open literature. The Abstracts or summary paragraphs of the papers follow.

Bioassays using rapid-cycling plants allow measurement of multiple endpoints and assessment of impacts on both growth and reproduction. Selections of *Brassica rapa* develop rapidly in a broad range of soils and are very consistent in production of flower and seed. Their sensitivity to variation in growth conditions was investigated to define the variables that most affect performance. Yield differences between soils were substantial, indicating the need for careful selection and use of control treatments. The sensitivity to contaminants was investigated with applications of mercury (Hg) and zinc (Zn) to three soils. In a sand soil, bloom initiation was slowed by <10 mg Hg kg^{-1} soil and <50 mg Zn kg^{-1} soil. In contrast, lettuce emergence and earthworm survival were less sensitive to these metals in this soil. Survival of *Daphnia magna* and the Microtox assay in soil extracts were more sensitive to Hg than bloom initiation, but less sensitive to Zn. A similar relationship among the bioassays was observed for two finer-textured soils, although for these, effects were usually apparent only at soil metal concentrations >200 mg kg^{-1}. Enzyme assays were included for comparison, but were not sensitive to Hg contamination. Rapid-cycling *B. rapa* selections are suitable for routine bioassays, and are representative of several widely distributed and utilized species.

Survival tests in soil using *L. terrestris* have been reported in the literature, but many of the reports involve few replicates, and we found no reports that investigated the optimal experimental design. Optimal designs should maximize the statistical power of comparisons and minimize the numbers of worms and experimental units required, and the costs. Our first experiment involved experimental units with five worms each, and we observed that 5 to 10 replicates were required to give reasonable precision. It was also apparent that cascade deaths were a problem, so that most units had either all alive or all dead. An obvious solution to the problem of cascade deaths is to use units with one worm each. The method we developed was convenient and low-cost, it allowed us to weigh individuals before and after exposure to the soil, and it allowed survival scoring at various times without disturbance. To obtain good statistical precision, it required 30 to 50 replicates. In contrast, experiments with units that contain 5 to

10 worms needed somewhat fewer replicates, but markedly more worms. Through our experiments and using simulated data to compare statistical power, we conclude that the optimal design involves large numbers of replicates of units with one worm each.

A novel bioassay based on microarthropods was developed. It entails placing contaminated soil in modified petri dishes that are then placed in a mesocosm that is an ideal habitat for microarthropods. The contaminated soil is amended with ground alfalfa, which moulds and becomes bait to attract the microarthropods. After a short exposure time, the petri dishes are removed and the microarthropods are extracted and counted. The counts of microarthropods in this system are a holistic measure of the ecology of the treated soil, encompassing effects on fungal proliferation on the alfalfa substrate, invasion of fungivore microarthropods from the mesocosm, and then survival of those microarthropods in the contaminated soil. There may also be effects on or from other related trophic levels. The relative sensitivity of this bioassay was compared with three others, and sensitivity varied among the three soils and two contaminants used. Clearly, the microarthropod counts were as sensitive, and in some cases more sensitive, than the other bioassays. The costs of operation of the microarthropod bioassay is about one fifth that of the *B. rapa* bioassay, and about equal to that of the earthworm survival and lettuce seed emergence bioassays. We conclude that the simplicity and sensitivity of microarthropod bioassay is good and that this method deserves further development.

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1. Introduction

1.1 The need for bioassays and bioassay development

"How clean should soil be" is a persistent question that regulators must answer[1] . The need is urgent and the answer will impact many land development plans. To date, guidelines such as the OME Decommissioning Guidelines[2] have been based largely on information compiled from the literature and refer to total-soil concentrations of contaminants. To support this and to expand guidelines to include other contaminants, standard test protocols are required. There is also a need to address characteristics other than total contaminant concentration. For this, bioassays are essential, because the objective is to regulate the impact on biota. The literature on bioassays is very diverse: there are seemingly infinite variations on methods, and some of the more established methods such as summarized by Keddy and Greene (1992 [3]) are not founded in the scientific literature. The present project set out to develop test protocols that are sensitive, yet robust enough to be transferable to commercial labs. We especially sought methods that could be immediately implemented in the OME Phytotoxicology laboratory with minimal extra equipment or training requirements. The methods used previously on behalf of the OME (Sheppard et al. 1992[4]) were candidates for consideration.

1.2 The approach used in this study

The approach used in our study consisted of three components. The first was to update our review of the literature on bioassay protocols to determine if further development of the methods used by Sheppard et al. (1992 [4]) would be fruitful. These methods had been selected based on a review of the literature. In the interim, the report by Keddy and Greene (1992) was

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1. Sheppard, S.C., Gaudet, C., Sheppard, M.I., Cureton, P.M. and Wong, M.P. 1992. The development of assessment and remediation guidelines for contaminated soils, a review of the science. (Invited review) Can. J. Soil Sci. 72:(in press).

2. OME 1989. Guidelines for the decommissioning and cleanup of sites in Ontario. Ontario Ministry Environ. Queen's Printer for Ontario, Toronto, 19 pp.
   OME 1991. Soil clean-up guidelines for decommissioning of industrial lands: background and rationale for development. Ontario Ministry Environ., Queen's Printer for Ontario, Toronto, 33 pp.

3. Keddy, C. and Greene, J.C. 1992. A review of whole organisms bioassays for assessing the quality of soil, freshwater sediment and fresh water in Canada. Draft report to Canadian Council of Ministers of the Environment, Winnipeg, Man., May 1992, 224 pages.

4. Sheppard, S.C., Evenden, W.G. and Anderson, A.J. 1992. Multiple assays of uranium toxicity in soil. Environ. Toxicol. Water. Qual. 7:275-294.

prepared. The latter dealt entirely with protocols formally promulgated by various jurisdictions, and put major emphasis on aquatic tests. This meant that few tests appropriate for whole soils were described in detail. In several cases, the details for the whole-soil tests, as described (Keddy and Greene 1992), appear to have been adopted by precedent rather than proof of optimization. With little doubt, there is an urgent need to demonstrate protocols for whole soils, with considerable effort devoted to optimization of test parameters for the purpose of bioassay.

Once we confirmed the choice of bioassays, we conducted a series of experiments to investigate the sensitivity of the bioassays to laboratory variables other than the presence of toxicants. In each case, the variables investigated were chosen based on hypothesized importance and our experience.

The final component was an investigation of the sensitivity to selected contaminants. This also involved comparison to other bioassays. In practice, the three components were not separated in time; improvements in the details of the protocols were implemented throughout.

1.3 The choice of bioassays

The protocols promulgated by various agencies and compiled by Keddy and Greene (1992) are listed in tabular form in Appendix A. Following that are a list of protocols found in the literature, many of which are not yet endorsed by an agency, but that we think show promise. This list cannot be considered exhaustive, because virtually any method used to measure the effect of any environmental variable on the performance of biota is a potential bioassay. The list did form the basis for our choice of which bioassays to develop.

The list in Appendix A illustrates the enormous scope for bioassay development. Any organism that interacts with soil or water is a candidate, and any function of those organisms from biochemical through to behavioural is a potential bioassay endpoint. There is also argument in favour of multispecies bioassays (e.g., Sugiura 1992). We set about selecting bioassays with the following criteria:

Whole-soil bioassay - There are many bioassays for water, most of which can be adapted for use with soil elutriates and pore waters. There is a deficiency in bioassays that use the whole soil with realistic moisture contents, temperatures and related terrestrial conditions. These bioassays are critical if for no other reason than to calibrate and validate the use of aquatic bioassays on soil waters. *We* emphasized development of whole-soil bioassays and used aquatic bioassays on soil elutriates for comparison.

Functional representation - The soil functions that are critical to the health of the environment include support of plant life, decomposition of organic residues, cycling of nutrients and habitat for soil-dwelling animals. There is general agreement that no one bioassay is sufficient, and it follows therefore that the selected suite of bioassays should

address some or all of the critical soil functions. We used plants, earthworms and microarthropods, and did some preliminary work with enzymes important to nutrient cycles.

Realism - There is a tendency in bioassay development to emphasis laboratory efficiency rather than the relationship to field situations. In contrast, there is greater credibility for bioassays that use common organisms in realistic settings. *We used full life cycle growth of a selected Brassica rapa that is genetically related to many field and garden crops. We used the common Lumbricus terrestris worm, which has much broader geographic and edaphological distribution than other earthworm species used in bioassays. We used native microarthropod communities.*

Unambiguous endpoints - Many bioassays for whole soils, most notably the enzyme bioassays, have somewhat ambiguous endpoints because the ecological significance of the test system is not well known. For example, decreased urease activity in soil is probably undesirable, but the ecological significance in all settings is not clear. *We selected simple and relatively unambiguous endpoints of survival, reproduction (seed production) and growth.*

Sensitivity - Despite the importance of sensitivity, there is no reliable way to estimate sensitivity prior to development of a bioassay. There is a good prospect of developing a sensitive bioassay by further development of current methods. However, marked technical and philosophical improvement is unlikely. There is a greater risk in development of a novel bioassay, but here a marked improvement is possible. *We balanced these strategies. The earthworm bioassay is well established and our development was of a experimental design change that will improved precision. The life cycle plant bioassay with Brassica rapa had had little development and, although the endpoints are simple, the optimal laboratory conditions needed to be defined. The microarthropod bioassay is novel.*

Technology transfer - Many bioassays are technologically complex or specialized, and require special skills (e.g., biochemistry, entomology) for valid interpretation. These are not easily transferred to commercial or government laboratories. Some bioassays are sufficiently complex or have been specified in such a way that the technology is proprietary. Here, transfer is restricted and perhaps costly. Our objective was to develop bioassays that can be done in simple laboratories with no special technical skills, and that can be transferred easily and freely.

Turnkey operation - Many bioassays require the maintenance of a culture of the test organism. This helps ensure uniformity in the test populations and represents no logistic constraint to a laboratory routinely conducting bioassays. However, many laboratories need bioassays on a discontinuous basis, and here the culture may become a problematic

overhead. *We selected bioassays that were nearly turnkey in operation; the test organisms are readily available. The Crucifer Genetics Cooperative has the mandate to maintain uniform stocks of the Brassica rapa. There is a large bait worm business in Ontario, and although there may be a sacrifice in precision because of inhomogeneity among the worms, this is easily and economically balanced by an increase in animal numbers (replication). Native microarthropods are very abundant, but we cannot yet comment on the reproducibility of results from different populations.*

1.4 The format of this report

The central parts of this report are three papers prepared for the open literature. These are the reports most accessible to the global public. However, the formats imposed by open literature journals require very concise reporting. Detail is inevitably lost. In addition, in any research there are hypotheses tested where the results are not considered of sufficient interest for the readership of open journals. Yet this information is important to those who want to follow the research in its entirety. Because of this need to communicate to two quite different audiences, our approach in this report has been to present the formal report as prepared for the open literature, and to augment it with following sections that describe everything in more detail. The text of the open literature reports has been slightly amended to give reference to the detail sections that follow.

Following these sections is a specification of the recommended protocol details. These are intended as the notes a laboratory would need to implement the bioassays. These details reflect our practice in conducting the bioassays. The important aspects of the bioassays have been optimized through the developmental investigations described, whereas the other aspects are based on informed judgement.

2. Plant life-cycle bioassay

2.1 Overall report

A Plant Life-cycle Bioassay for Contaminated Soil, with Comparison to other Bioassays: Mercury and Zinc

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Abstract. Bioassays using rapid-cycling plants allow measurement of multiple endpoints and assessment of impacts on both growth and reproduction. Selections of *Brassica rapa* develop rapidly in a broad range of soils and are very consistent in production of flower and seed. Their sensitivity to variation in growth conditions was investigated to define the variables that most affect performance. Yield differences between soils were substantial, indicating the need for careful selection and use of control treatments. The sensitivity to contaminants was investigated with applications of mercury (Hg) and zinc (Zn) to three soils. In a sand soil, bloom initiation was slowed by <10 mg Hg kg^{-1} soil and <50 mg Zn kg^{-1} soil. In contrast, lettuce emergence and earthworm survival were less sensitive to these metals in this soil. Survival of *Daphnia magna* and the Microtox™ assay in soil extracts were more sensitive to Hg than bloom initiation, but less sensitive to Zn. A similar relationship among the bioassays was observed for two finer-textured soils, although for these, effects were usually apparent only at soil metal concentrations >200 mg kg^{-1}. Enzyme assays were included for comparison, but were not sensitive to Hg contamination. Rapid-cycling *B. rapa* selections are suitable for routine bioassays, and are representative of several widely distributed and utilized species.

Bioassays to determine the impact of contaminants in soil have not been extensively developed, especially when compared with those for aquatic systems (van Straalen and Denneman 1989). Standards associations and government agencies have detailed or are developing a few bioassays for soils (e.g., OECD 1984a, 1984b; Greene et al. 1989; Hortensius et al. 1989; Wang et al. 1990; Gorsuch et al. 1991; Hortensius and Nortcliff 1991), but many aspects of toxicity assessment have yet to be formally addressed. Many plant bioassays deal solely with seed germination or root elongation. In contrast, life-cycle tests with plants are less common, although they offer multiple endpoints including both vegetative growth and reproductive

potential (Ratsch and Johndro 1987). Shimabuku et al. (1991) outlined the advantages of toxicity tests using rapid cycling selections of *Brassica rapa.* These selections have been developed and maintained by the Crucifer Genetics Cooperative (1985). They are grown under lighting levels typical of controlled growth chambers, they germinate in 12-36 h, flower in 12 d, and produce mature seed in as little as 24 d. This rapid development allowed Sheppard et al. (1992) to look for toxicity effects in second generation seed germination, with both parent crop and second generation germination tests done within about 60 d.

Shimabuku *et al.* (1991) considered these *B. rapa* selections superior to *Arabidopsis,* another candidate for plant life-cycle assays (Shirazi *et al.* 1992), with the major benefits being more applicability to commercial crops and a more manageable seed size. Pestemer and Auspurg (1986) previously had noted that *B. rapa* and *Avena sativa* were the most suitable of 15 candidate plants for soil bioassays.

Among rapid cycling B. rapa selections, various attributes are available that may be of use in routine bioassays. For example, certain selections are more uniform than others, and some are self-fertile (Crucifer Genetics Cooperative 1985). Sheppard *et al.* (1992) used the base population selection, numbered Aaa 1-1, for a toxicity study. It was as sensitive as beans *(Phaseolus vulgaris)* grown to maturity in field lysimeters and as sensitive as seed germination tests with several species. They did note considerable plant-to-plant variability and recommended that this needed attention.

The objective of the present study was to investigate ways to decrease the variability in a soil bioassay using the rapid cycling *B. rapa* selections, to determine their sensitivity to laboratory variables such as light and nutrient, and to compare their sensitivity to that of several other whole-soil bioassays and two soil-extract bioassays. Mercury (Hg) and zinc (Zn) were used for the intercomparison of the bioassays.

Methods and Materials

Basic Methodology for B. rapa

The basic cultural methods are adapted from the manual supplied by the Crucifer Genetics Cooperative (1985). The soil was prepared by air-drying and sieving to pass a 2-mm mesh. The moisture holding capacity (MHC) was determined for each soil and container size by filling the container with soil, wetting to excess and allowing to drain for 24 h. Nutrient additions and other soil amendments such as liming were not used routinely, to avoid soil chemical interference with the contaminants. A 400-cm^3 aliquot of dry soil was placed into a 500-mL plastic container and gently tamped. Ten seeds were distributed evenly on the soil surface, at least 1 cm from the edge of the container, and pressed 5 mm into the soil using a marked rod. The soil was then moistened to the MHC, covered, and placed in the growth chamber. The growth chamber provided day/night temperatures of 25°C/22°C, with an accuracy of ±0.5°C, and a light flux

density at the top of the container or plant of about 250 µE m^{-2} s^{-1}. Lighting was provided by wide spectrum fluorescent tubes.

The cover was removed when germination began. When germination was judged to be complete, the number of seedlings was counted and thinned to five per container. With these few seeds, the emergence counts were not intended as a reliable toxicity endpoint, but they occasionally proved useful. The containers were removed, randomized and replaced in the growth chamber at least every other day to minimize location effects. When about half the plants were in bloom, defined as stage 4.1 by Harper and Berkenkamp (1975), a count was made of the number of plants blooming in each container. As the flowers opened, manual pollination was done daily until the blooming ended. A small cluster of feathers was used for pollination.

In initial experiments, watering was done on a weight basis: weighing and adding water to reach the MHC in each container daily. However, the MHC of the soils varied markedly, so that some dried out rapidly relative to others in the same study. To achieve more uniformity, holes were cut in the bottoms of all containers and, after germination, they were placed on a capillary-action mat (Metro Products, Lee Valley Tools, Ottawa) that kept all soils constantly at a more uniform soil moisture tension.

The *B. rapa* selections are not determinant and terminalization is recommended to avoid increased variability associated with secondary growth. The timing of this is a subjective decision. Although the current recommendations for production of seed suggest removal of buds and apices after a defined stage, these methods directly confound potential toxicity endpoints. We terminalized by withholding water for 2-3 d, and did this when seeds from the first blooming phase were beginning to mature, yet before extensive second blooming or axial bud development began (about stage 5.3 of Harper and Berkenkamp 1975). This is about 20 d after pollination. In some cases, terminalization was not needed, whereas in others secondary growth was vigorous. We present results of an experiment where the timing of terminalization was varied ±7 d, and the effects were not too critical from the perspective of a routine bioassay.

The timing of harvest was based on the maturation of seed, which become darker as the seed pods dry (stage 5.4 to 5.5). At harvest, the height of individual plants was recorded and the pods were removed and counted. The stems and pods were dried at 50°C and weighed separately. The seeds were separated from the pods and cleaned by hand, and the clean seed was weighed.

Sources of Variability

Based on the observations of previous studies (Shimabuku *et al.* 1991, Sheppard *et al.* 1992), two experiments were initiated to investigate sources of variation. The first addressed three variables that could enhance variation within an experiment. One variable was seed

characteristics, and the seeds were sorted into three classes based on colour. Colour was the most apparent variation among the seeds, and is related to seed maturity when the parent plant was terminalized. Another variable was watering regimes, which were controlled by watering by weight to achieve 75%, 100% or 110% of the soil MHC. The third variable was seeding depths, which were 1, 5 or 10 mm. The experimental design was a partial factorial (there were insufficient seed in two of the colour classes for a full factorial design) with three replicates.

The second experiment, which was concurrent with the first, addressed three variables that could cause variation from experiment to experiment. One variable was nutrient supply, which was augmented by additions of soluble fertilizer in the irrigation water at zero, recommended or three-fold above recommended levels. The recommended level was 0.25 g L^{-1} of soluble 20-20-20 fertilizer in the irrigation water. Another variable was light levels, which were set at about 100, 250 (the recommended level) or 500 µE m^{-2} s^{-1}. Proximity of the plants to the lights and shade cloth were used to adjust the light levels, and these were confirmed by measurement (Quantum Light Sensor, Licor, Lincoln, Nebraska). The third variable was timing of the terminalization, where water was withheld 7 d before, on or 7 d after the recommended timing. The experimental design was a full factorial with three replicates.

In these experiments, the Aaa 1-1 selection (Crucifer Genetics Cooperative 1985) was used. A commercial potting soil that was 48% clay and pH 7.2 with an organic matter content (OMC) of 230 g kg^{-1} and a cation exchange capacity (CEC) of 59 cmol (NH_4) kg^{-1} was used. Watering was to weight and nutrient was added at the recommended level in both experiments, except where specified otherwise.

Sensitivity to Soil Types and Amendments

Growth in three soils and with several mixtures and amendments were investigated. The commercial potting soil, used previously, was included. A silty-clay soil, known to be of poor physical structure and difficult to use in the laboratory, was selected as a challenge to the bioassay. It was 43% clay and pH 7.9 with an OMC of 27 g kg^{-1} and a CEC of 18 cmol (NH_4) kg^{-1} This soil shrank when dry to about 80% of its volume when moist. The third soil was a sand, representing another extreme in properties, and was 3% clay and pH 6.3 with negligible OMC and a CEC of 1 cmol (NH_4) kg^{-1}.

The clay soil was processed in two ways. One aliquot was aggressively prepared (AP) by drying at 105°C and grinding with a hammer so that all but stones passed a 2-mm sieve. Another aliquot was conservatively prepared (CP) (after Didden *et al.* 1991) by partial air drying followed by a freeze/thaw cycle to break up clods, and then by gentle sieving to pass a 5-mm mesh. The processing was gentle enough that during sieving, about 30% of the soil remained unbroken and was retained on the sieve and discarded.

Twelve treatments were established to included the whole soils, 1:1 mixtures of the soils,

and selected amendments. They were 1) the potting soil, 2) the clay aggressively prepared (clay/AP), 3) the clay conservatively prepared (clay/CP), 4) the clay/AP amended with 20% v/v Perlite, 5) the clay/AP amended with 50% v/v Perlite, 6) the clay/AP amended with 50% v/v potting soil, 7) the clay/AP amended with 50% v/v of the sand, 8) the sand, 9) the sand limed to pH 6.5, 10) the sand amended with 50% v/v Perlite, 11) the sand limed and amended with 50% v/v Perlite, and 12) the sand amended with 50% v/v potting soil. Liming of the sand was based on previous trials and involved addition of 0.5 g $CaCO_3$ kg^{-1}. There were four replicates of the 12 treatments in a fully randomized design. The Aaa 1-33 selection of *B. rapa* was used because of its better uniformity than the base 1-1 population (personal communication, Crucifer Genetics Cooperative). No nutrient was added and watering was by capillary mat.

Sensitivity to Mercury and Zinc

To investigate the relative sensitivity of *B. rapa,* experiments were established with soils treated to a range of Hg concentrations. Mercury has been regulated largely on its effects on human health, and sometimes under very conservative assumptions about the fraction of soil Hg that is methylmercury (Bevis *et al.* 1990). Typical regulatory guideline levels, as mg Hg kg^{-1} soil, are 0.8-10 in Canada (CCME 1991), 0.1-1 in China (Wu *et al.* 1991) and 0.5 in Sweden (Johansson *et al.* 1991). There is relatively little information on the effects of Hg on soil biota (Crowder 1991; Lindqvist 1991), and Hg was chosen here to both assess the sensitivity of the bioassay and to provide basic ecotoxicity data. Siegel *et al.* (1984), Cappon (1987) and Lenka *et al.* (1992) all indicate that *Brassica spp.* accumulate more Hg than several other crops. Based on the scant literature available on toxicity in soil (Table 2.1), the concentrations of Hg chosen to study were 0, 10, 22, 46, 100, 220, 460, and 1000 mg Hg kg^{-1} soil, applied as $HgCl_2$. The clay and sand were used, as well as a humus-rich garden soil. The garden soil was classified a clay, with 46% clay and pH 7.3 with an OMC of 89 g kg^{-1} and a CEC of 40 cmol (NH_4) kg^{-1}.

To diminish effects of the attendant Cl addition with the Hg treatment, the Cl added to each soil was made uniform across the treatments by adding a mixture of $CaCl_2$, $MgCl_2$, NaCl and KCl. The ratio of these salts was determined from the mean ratios of Ca, Mg, Na and K in pore-water extracts of the three soils (Thibault and Sheppard 1992). *Further details are given in the Expansion of Details, Part P1.* The Hg and other salts were added, as solutions, in stages to large batches of the soils, with thorough mixing at each stage. The large batches were then subdivided into the experimental containers for *B. rapa* and for the tests described below. For the *B. rapa,* there were three replicates of the Hg-treated soils and five replicates of controls.

An unknown contaminated soil was also included. This soil was a loam with 24% clay and pH 7.0 with an OMC of 113 g kg^{-1} and a CEC of 31 cmol (NH_4) kg^{-1}. It was provided, unidentified, by a field agent, and was later identified as from the grounds of a plumbing porcelain manufacturer. No control soil was available, so a surrogate control was concocted. This was done based on measurements of soil texture, MHC, pH, and CEC of the unknown soil and several other soils, including the potting, clay, sand, and garden soils. A weighted linear mixing

algorithm was used to select mixing ratios of other soils to create a blend most like the unknown soil. Primary weighting in the algorithm was given to measurements of MHC. *Further details on the soil blend are given in the Expansion of Details, Part P2.* Treatments for the unknown soil were based **on** mixtures with the blended control soil - the ratios of control:unknown were 1:0, 16:1, 8:1, 4:1, 2:1, 1:1, 1:2 and 0:l. Replication was as for the Hg-treated soils.

Following the approaches of Cheung *et al.* (1989) and Sheppard *et al.* (1992), a series of Zn concentrations were included as a relative toxicity benchmark. Duplicate treatments with 50, 100, 300, 600 and 1000 mg Zn kg^{-1}, with the Zn applied as solutions of $ZnSO_4.7H_2O$, were established in batches of the clay, sand and garden soils. Controls were shared in common with the Hg treatments.

The Aaa 1-33 selection of *B. rapa* was used. There was no nutrient added and watering was by capillary mat.

Comparison with Lettuce Germination

After the *B. rapa* was harvested, the soils in the containers were partially dried and broken up by hand. Fifty seeds of leaf lettuce *(Lactuca saliva* cv Green Ice) were planted and the soil rewetted. Emergence counts were made on several days, and final emergence counts corresponded to when no further plants emerged. The numbers of treatments and replicates were identical to the *B. rapa* experiment.

Comparison with Earthworm Survival

Earthworm survival in the same soils and treatments was determined following the methods of Sheppard and Evenden (1992). Briefly, the treated soils were subdivided into 40 cm^3 aliquots and placed in small plastic bags. The bags had been perforated with a needle to allow air passage. There were 30 replicates of the contaminated soils and 50 replicates of controls. One mature earthworm *(Lumbricus terrestris),* from an allotment recently obtained from a commercial bait supplier, was placed in each bag. The bags were sealed and incubated at 15°C for 30 d. Earthworm survival in the sand was poor, and dead worms were counted and removed at 12 d in this soil only. After 30 d, the worms in all remaining bags were scored dead or alive.

Comparison with Enzyme Assays

Three enzyme assays were done on some of the soil treatments. Alkaline phosphatase, dehydrogenase and urease activity were determined using the methods of Tabatabai (1982).

Comparison with Daphnia magna

Survival of *Daphnia magna* has become a very common bioassay for aqueous samples

(Parkhurst *et al.* 1992), and has been applied to soil extracts. The methods follow Poirier *et al.* (1988). Extraction of the three soils treated with 46, 100 and 1000 mg Hg kg^{-1} and 10O, 600 and 10O0 mg Zn kg^{-1} was done using 125 g of soil in 625 mL of distilled water. These were shaken for 30 min, centrifuged and decanted. The decantates were used as obtained, and also were diluted to 0.5, 0.26, 0.14 and 0.06 of the original concentrations. Dilutions of the extracts from the higher soil concentrations overlapped the concentrations expected from the lower soil concentrations. Spring water from a sand aquifer was used for dilution and controls. For each extract and diluted sample, neonatal *D. magna* were selected from the stock colonies and two were placed in each of five 20-mL test tubes with 30-mL of the soil extract. After 48 h the number of living and dead was determined.

Comparison with Microtox

The commercial Microtox assay has been widely used for waters and liquid effluents, and has been modified for analysis of soil extracts (Microbics 1992). Extraction of the three soils treated with 46, 10O and 1000 mg Hg kg^{-1} and 100, 600 and 1000 mg Zn kg^{-1} was done using a 1:4 soil to water suspension shaken for 22 h (Mathews and Hastings 1987). The suspensions were centrifuged and the decantates analysed within 4 h by the Microtox procedure detailed by Microbics (1992). The soil concentration that reduced bioluminesence to half of that of the control soil was determined from the data, based on observations at both 5 and 15 min. The responses did not differ significantly between the observation times and results for the 5 min observations time are presented.

Chemical Analysis

Soil solids were analysed for Hg by digesting 0.2 g dry soil in 5 mL water, 3.75 mL HCl and 1.25 mL HNO_3, with heating for 5 min at 95°C. The digest was cooled, 15 mL of 5% $KMnO_4$ was added, the solution was heated 60 min at 95°C and then sealed and stored for analysis (<3 d). Just prior to analysis, the excess $KMnO_4$ was reduced, and solution analysed by inductively coupled plasma (ICP) spectroscopy. These analyses were done on selected concentrations of the spiked stock soils, and on the same concentrations of the soils after the *B. rapa* and lettuce experiments. Extractions of organically-bound Hg, using toluene, yielded nothing detectable, implying that the fraction of soil Hg that was methyl-Hg was <0.3% at the end of the experiments.

The Hg and Zn concentrations in the soil extracts were measured by ICP. Solid/liquid partition coefficients (K_d) were computed as the concentration on the soil solids divided by that in the extracts.

Statistical Interpretation

The general linear model of SAS (1985) was used throughout. Significance was assigned

to the 0.05 probability level. Statistical contrasts with controls or with standard-condition treatments were by single-degree-of-freedom, two-tailed tests. The soil concentrations that reduced performance to 75%, 50% and 25% of that of the controls were estimated graphically. The 50% level is termed the Effect Concentration to 50% (EC_{50}) or the Lethal Dose to 50% (LD_{50}).

Results and Discussion

Sources of Variability

Seed sorting had been proposed by Sheppard *et al.* (1992) as a means to lessen plant-to-plant variation. Sorting was based on seed colour, which varies because of the stage of seed maturation during terminalization of the parent plant. There were no significant effects of seed colour on the parameters measured. Results are shown in the Expansion of Details, Part P3.

In the same experiment, the effects of moisture supply and depth of planting were investigated. *Results are shown in the Expansion of Details, Part P4.* Excess water was detrimental when in combination with deep planting: relative to results at the standard condition, total shoot dry weights were significantly reduced to 44% and *seed* dry weights to 54%. Although these effects are notable, we conclude that nominal care in planting and watering will eliminate unwanted variation from these variables. In this and later experiments, we did find that germination can be markedly decreased if the soil is too wet. For this reason, we allow germination to be complete before placing the containers on the capillary mat.

B. rapa was sensitive to nutrient supply and light levels, with lesser effects due to the timing of terminalization (Table 2.2). Low light levels were particularly detrimental: bloom initiation was delayed and diffuse, and seed set was especially poor (Table 2.2). Under low light, the plant habit was not erect and the plants were difficult to handle in daily movements of the containers. These B. *rapa* selections were developed under light levels of about 250 $\mu E\ m^{-2}\ s^{-1}$. At higher light levels, the plants were more compact in early growth; however, it was difficult to maintain an adequate moisture supply even though high air flows ensured good temperature control.

B. rapa responded to nutrient levels, although at low and recommended light levels the seed yield did not differ significantly with nutrient supply (Table 2.2). The other parameters measured, *(the other parameters are shown in the Expansion of Details, Part P5),* reflected the trends we discuss for total and seed yield. There was a strong interaction between light level and nutrient supply so that, at high levels of both, the total yield was increased 2.6 fold and the seed yield 1.9 fold (Table 2.2). This is indicative of the plastic developmental characteristics of *B. rapa:* it can readily utilize increased resources through lateral shoot growth and sustained blooming.

The timing of terminalization had little effect, and the only significant interaction was with nutrient supply where, combined, they affected total yield (Table 2.2). *B. rapa* grew more in response to the combination of longer growth period and increased nutrient supply, again illustrating its plastic developmental nature.

These experiments indicated that light level and nutrient supply are key variables to control. Variations in these, typical of normal laboratory practice, will have marked effects. In contrast, variations in seed characteristics, moisture supply, seeding depth and timing of terminalization were not as critical. The standard conditions are appropriate for use in bioassay, except that consideration should be given to nutrient supply. For bioassays involving an artificial contamination series, where the control soil differs from the treated soil only in contaminant load, we opt to not apply nutrient. This avoids effects of the nutrient material, especially micronutrient chelates, on the chemistry of the contaminant. However, in bioassays where the control is less well matched, or where the addition of the contaminant involves the addition of nutrient element, it is very important to equalize the nutrient supply across the treatments.

With the help of the Crucifer Genetics Cooperative, a more uniform *B. rapa* selection was identified. The Aaa 1-33 selection is considered an idiotype, with more uniform developmental characteristics. This selection was used in all further experiments.

Sensitivity to Soil Types and Amendments

The soils chosen represented the extremes in soil properties likely to be encountered in bioassays. The potting soil was ideally suited to plant growth in containers. The clay had very poor structure, became very hard when dry and shrank sufficiently to lose contact with the capillary watering mats. The sand packed very tightly when watered and gave significant resistance to root penetration. The effects on *B. rapa* were even more marked than expected (Figure 2. 1) *. The data are presented in tabular form in the Expansion of Details, Part P6.* Seed yield in the sand was 40-fold lower than in the potting soil, and was consistently lower than the clay. Although this emphasizes that matching control and contaminated soil proprieties is crucial, it also illustrates the sensitivity and plasticity of *B. rapa.* Plants in all soils bloomed and produced seed, allowing measurement of reproductive success despite a very broad range in height and total dry weight.

Mixtures of the soils gave intermediate results (Figure 2.1). Clearly, the effects were profound enough that, for example, amending the sand with 50% potting soil did not eliminate all of the growth inhibition presented by the sand. Similarly modest results were found for the other amendments with Perlite and lime (data not shown). None of these amendments improved growth significantly.

Two modes of preparation of the clay were investigated. The aggressively prepared clay gave consistently better plant growth than the conservatively prepared clay, and this difference

was statistically significant for total yield. We expected the opposite, on the basis that the inherent poor structure of the clay would be especially susceptible to further deterioration after drying and grinding. The aggressively prepared clay may have had better capillary continuity within the container and between the container and the watering mat than the more-coarsely-aggregated, conservatively prepared clay.

We conclude that soil type has a profound effect, and that amendments are not useful. This means that B. *rapa* may be well suited to detect subtle differences between contaminated and control soils, but that these soils must be well matched in other properties. Perhaps bioassays with *B. rapa* should be limited to studies with artificial contamination, where the contaminated and control soils are initially the same.

Sensitivity to Mercury and Zinc

In our previous refinements of the method, counts of early bloom initiation had not been made. It was observed that total shoot growth was often as sensitive as seed yield to treatments, which suggested that indices of reproduction offered no advantage over the simpler indices of vegetative growth. In the experiments with Hg and Zn, bloom counts were made and these proved to be relatively sensitive (Figures 2.2, 2.3 and 2.4). Timing of bloom initiation has several advantages as a bioassay endpoint: it is a reproductive indicator, it occurs early in the life cycle, and it is simple to measure.

There were no detrimental effects observed using any of the bioassays in the unknown soil (data not shown). Growth of *B. rapa* was slightly better in the unknown soil than in the corresponding control soil, emphasizing again the need to have well-matched control and contaminated soils. The notable boron and metal concentrations (mg kg^{-1} dry soil) in the unknown soil, based on extraction with HNO_3 heated to 75°C for 6 h, were B:148, Cr:26, Cu:35, Ni:305, Pb : 43, and Zn:510. *More complete elemental analyses are given in the Expansion of Details, Part P7.*

Mercury was historically used as a fungicide for seed, and this function opens the possibility of positive plant response to Hg additions. Seed yield and a few other attributes were significantly increased in the clay and garden soils by Hg concentrations of 46 to 460 mg kg^{-1}. There were no symptoms of fungal damage on any of the plants; it is not clear why this growth stimulation occurred. Siegel *et al.* (1984) also noted stimulation in growth of certain plant species following exposure to Hg.

Overall, detrimental effects of Hg were statistically significant at 10 to 22 mg kg^{-1} in the sand, and at 220 mg kg^{-1} in the clay and garden soils (Figures 2.2 and 2.3). Detrimental effects of Zn were statistically significant at 50 mg kg^{-1} in the sand and garden soil, and at 600 mg kg^{-1} in the clay. Timing of bloom initiation was generally the most sensitive parameter.

Emergence of lettuce was less sensitive than *B. rapa* to Hg or Zn in the sand (Figures 2.3 and 2.4). In the other soils, Hg decreased lettuce emergence to below 25% of controls at 220 to 46O mg kg^{-1}, whereas the effects on *B. rapa* were not as marked. However, lettuce was unaffected by Zn concentrations that were detrimental to B. *rapa.*

The earthworm survival assay was less sensitive than the plant assays in the sand, and was perhaps more sensitive to Zn in the other soils (Figures 2.3 and 2.4). This illustrates what is well recognized: several bioassays representing a variety of life forms, trophic levels and endpoints must be used to fully assess impacts of soil contamination.

The enzyme assays were quite insensitive to Hg (data not shown). Significant effects were observed when the sand was treated with Hg concentrations above 1000 mg kg^{-1}. Because of this, the enzyme assays were not investigated further.

The survival of *D. magna* was very sensitive to Hg, with effects at concentrations well below those observed with the whole-soil assays. The detrimental soil Hg concentrations, corresponding to the diluted soil extracts, were 2 mg kg^{-1} in the sand, and 14 to 26 mg kg^{-1} in the other soils. The Microtox assay was sensitive to Hg in the sand, but relatively insensitive in the other soils (Figure 2.3). Interpretation of these results as indicators of soil quality is not as direct as for the whole-soil bioassays, but could suggest that leaching of soils at these concentrations to groundwater or surface waters would cause detriment to other parts of the environment.

In contrast to the results for Hg, the *D. magna* assay was less sensitive than the whole-soil bioassays to Zn. Zinc is less soluble than Hg in soil; Sheppard *et al.* (1992) cite K_d values of 1300 and 10 L kg^{-1} respectively. In this study, Zn and Hg were only detectable in water extracts for the sand, and the corresponding mean K_d values were 2600 and 18 L kg^{-1}. This difference in relative amount of Zn and Hg in solution may be the reason that the *D. magna* assay on the soil extracts was not the most sensitive assay for Zn, whereas it was the most sensitive for Hg. The role of soil in attenuating the toxicity of Zn is well illustrated by comparison with the results of Miller *et al.* (1985). They found *D. magna* sensitive to 0.54 mg Zn L^{-1} water compared to comparable effects on earthworms at >600 mg Zn kg^{-1} soil.

Gaseous Losses of Mercury

Analyses of stock soils, stored dry after spiking, indicated that the concentrations after 150 d were 94±15 % (the t is the standard deviation, n=11) of the target spike concentrations. In contrast, after the same time interval, but in the soils kept moist and with growing plants, there was substantial loss of Hg in the sand. Recovery of Hg in these moist soils, relative to the dry-stored soils, was 34 ±22 % for the sand, 83 ±19 % for the clay, and 99 ±13 % for the garden soil. These recoveries correlate with the soil organic matter contents, as might be expected. The loss in the sand indicates that the actual concentrations affecting the bioassays

may have been slightly lower than when spiked. The apparent half-time of Hg in the sand was 96 d, so that at the mid-point of the *B. rapa* and earthworm experiments, the Hg concentrations were probably decreased by 10-20%.

Conclusions

Life-cycle bioassays are important because they reflect several toxicity endpoints, including effects on growth and reproduction. With rapid cycling *B. rapa,* it is possible to measure many endpoints, and we used counts of bloom initiation, stem height, total-shoot dry weight, number of pods and seed dry weight. The most useful attribute of *B. rapa is* its rapid and reliable development; plants as short as 5 cm will produce mature seed. This means that even under relatively severe stress, it is possible to measure impacts on reproductive endpoints.

Several genetic attributes are available among the family of rapid cycling *B. rapa.* We opted for an idiotype that is more uniform than the base population. Self-fertile selections are also available and may lessen the effort and potential variability resulting from manual pollination.

Growth of *B. rapa* is very sensitive to soil physical and chemical properties. This is a desirable attribute for a bioassay. However, considerable care is required to ensure that controls and contaminated soils differ only by the presence of the contaminant. It is especially important to provide uniform nutrient supply and to avoid excess water during germination.

The sensitivity of *B. rapa* to Hg and Zn was comparable with other bioassays and the literature (Table 2.1). Effects on bloom initiation were especially sensitive, and this endpoint was more sensitive than the other whole-soil bioassays in several cases. The results confirmed again the need for a battery of bioassays, because no one species or endpoint was most sensitive to all contaminants in all soils. *B. rapa* is well suited as a representative of terrestrial plants in a battery of bioassays. Results for D. *magna* and Microtox in soil extracts showed marked sensitivity, and the concept of using *aquatic* organisms to assess contaminants in soils deserves more attention.

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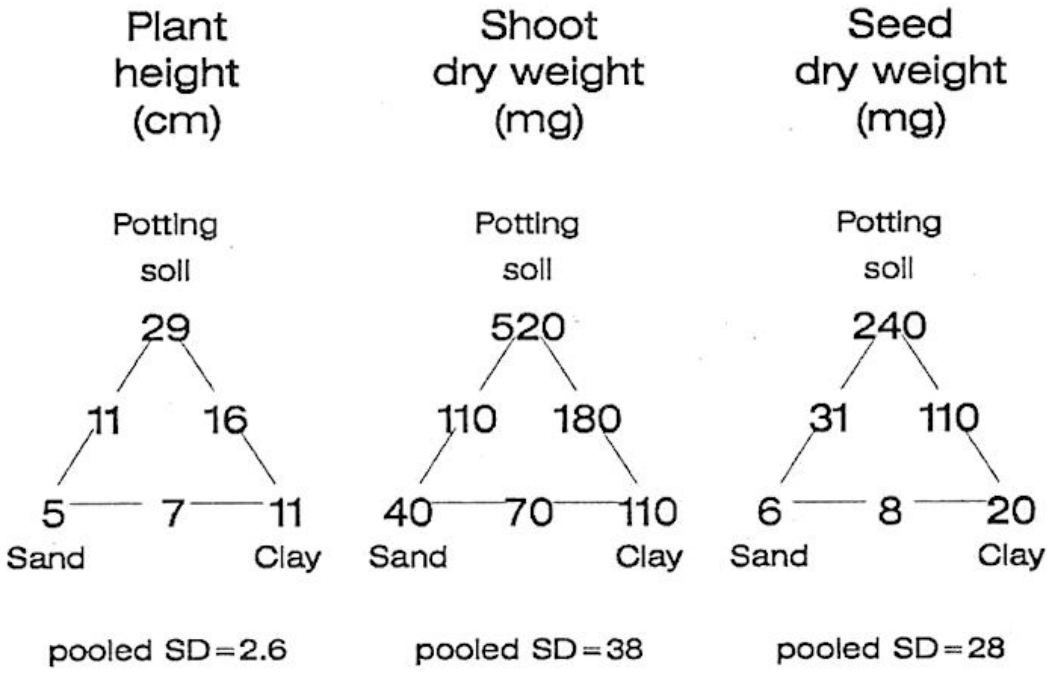

Figure 2.1 Effect of soil type on plant height and individual-plant shoot and seed dry weights at harvest. At the apices of the triangles are the results of the potting, clay and sand soils, and at the midpoints are the results of 1:1 mixtures of the three soils. For example, the 1:1 mixture of potting soil and sand had plants 11 cm tall.

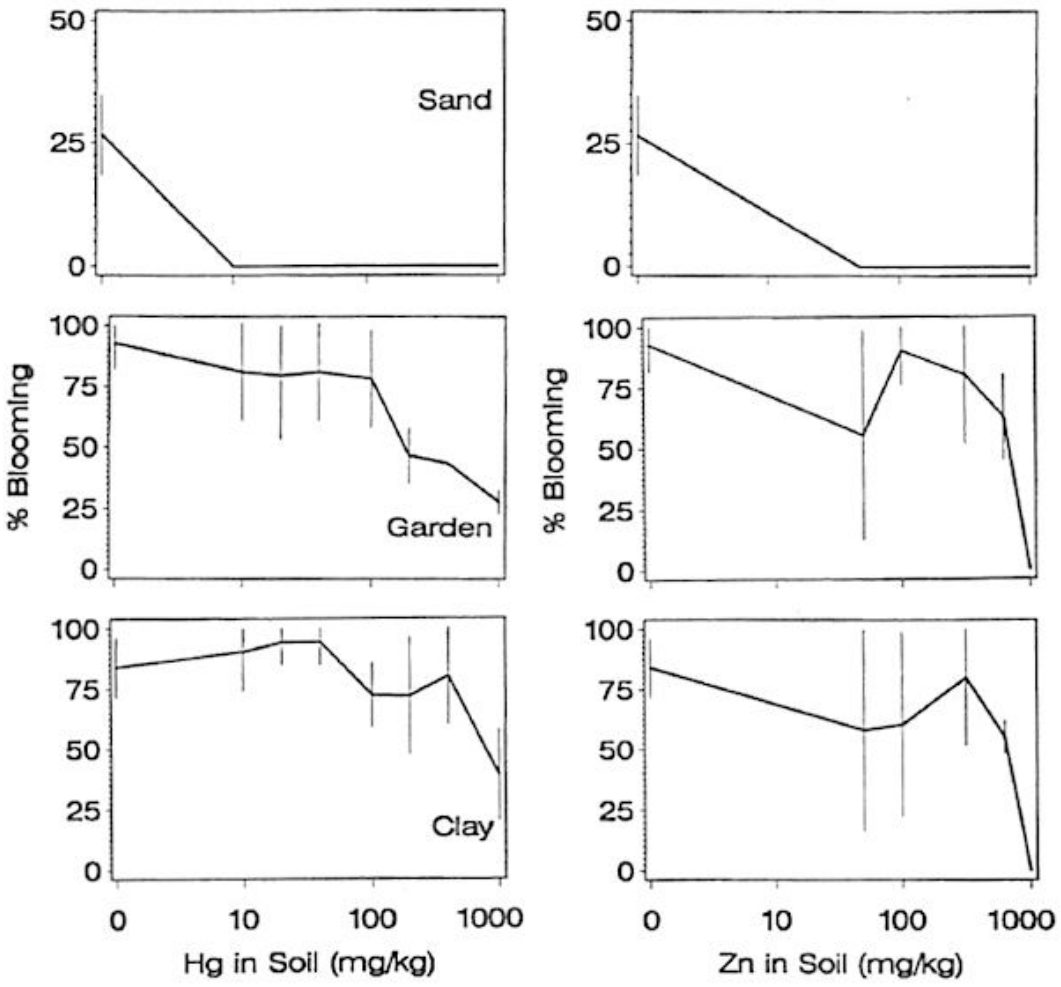

Figure 2.2 Percentage of *B. rapa* plants with early bloom, in response to concentrations of Hg and Zn in three soils.

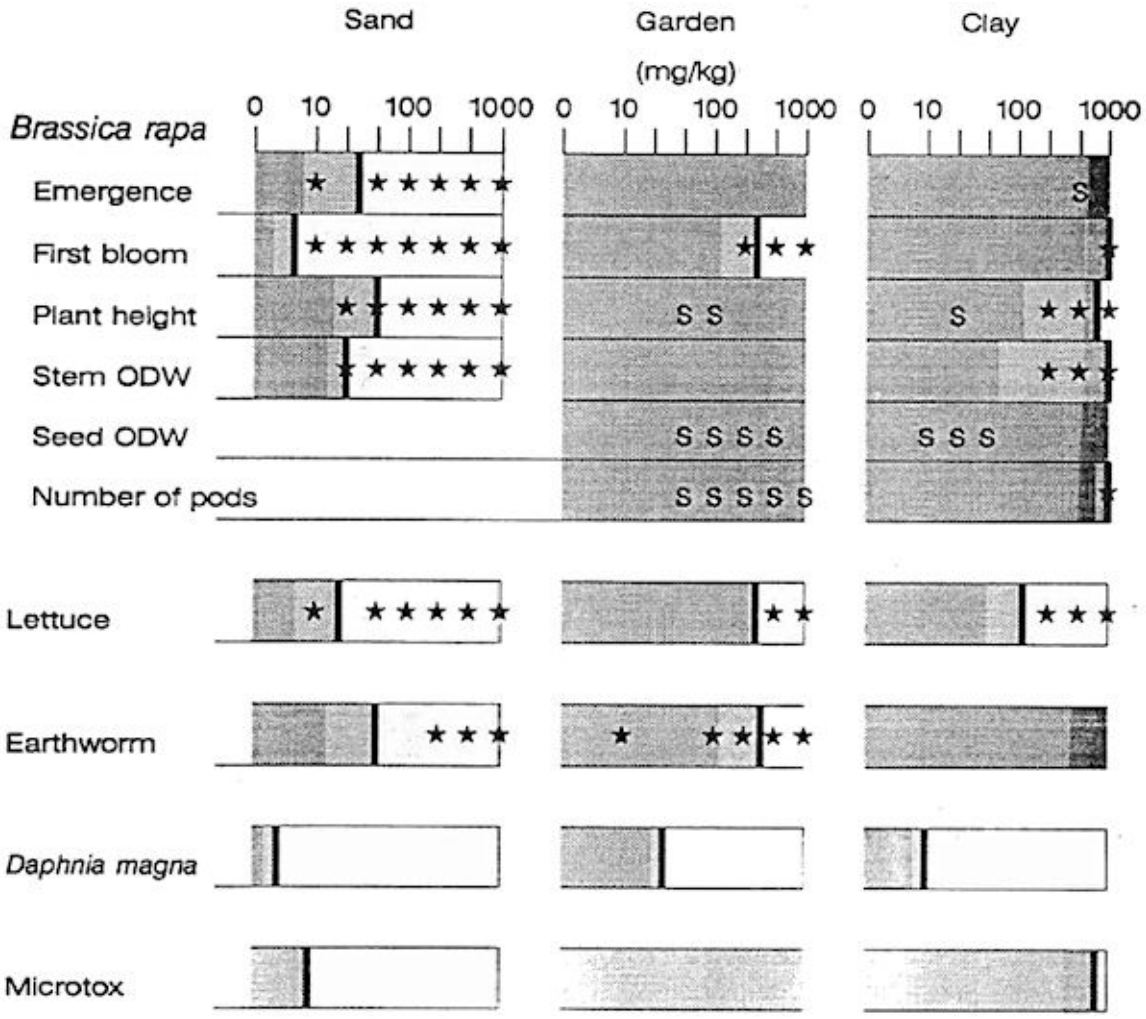

Figure 2.3 Relative sensitivity of several bioassay endpoints to soil Hg concentrations. The stars indicate Hg treatments that were significantly lower than the controls by single-degree-of-freedom contrasts, and the S's indicate those that were significantly higher than the controls. The shading indicates response relative to the controls, as >75% (darkest shade), 75-50%, (middle shade), 50-25% (light shade) and <25% (white). The EC_{50} or LD_{50} is shown by the vertical line separating middle and light shading.

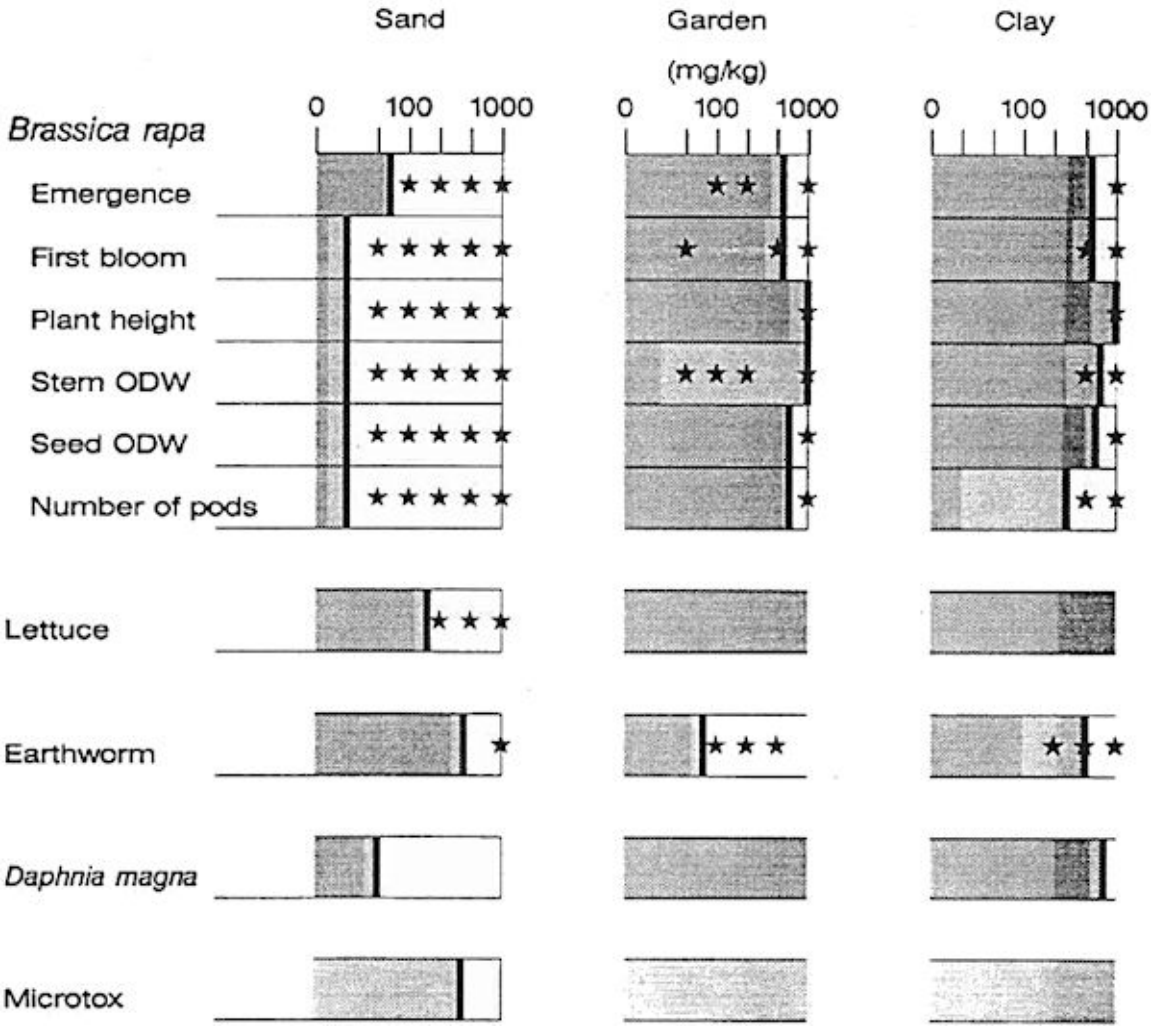

Figure 2.4 Relative sensitivity of several bioassay endpoints to soil Zn concentrations. The stars indicate Zn treatments that were significantly lower than the controls by single-degree-of-freedom contrasts. The shading indicates response relative to the controls, as >75% (darkest shade), 75-50%, (middle shade), 50-25% (light shade) and <25% (white). The EC_{50} or LD_{50} is shown by the vertical line separating middle and light shading.

Table 2.1 Summary of reports in the literature on toxicity of inorganic mercury (Hg) in soils. Only studies that reported results for whole soils are included.

Organism/endpoint	Soil Hg conc. (mg kg^{-1})	Effect observed	Reference
Bermudagrass, growth	8-50	decrease	Weaver *et al.*(1984)
Turf grass	455	no effect	Estes *et al.*(1973)
Native plants	557	no effect	Lenka *et al.*(1992)
Insect, emergence	1	24% lower ($P<0.05$)	Schmidt(1986)
Earthworm, survival	1.5	50% at 30 d	Abbasi and Soni (1983)
Microbial, respiration	0.1	10% decrease,7-28d	Landa and Fang (1978)
Microbial, respiration with added glucose, recently applied Hg	1.3	20% decrease	von Stadelmann and Santschi-Fuhrimann (1987)
Several microbial indicators	2-20	up to 50% decrease	Hund *et al.*(1988) Zelles *et al.*(1986) Zelles *et al.*(1991)
Microbial, respiration with added glucose, aged application of Hg	50	25% decrease	Wilke (1988)
Amylase activity	70	75% decrease at 1 d	Tu (1988)
Invertase activity	70	62% decrease at 1 d	Tu (1988)
Ammonification	100	15-24% decrease	van Faassen (1973)
Nitrification	100	40-95% decrease	van Faassen (1973)
Microbial, respiration	100	0-86% decrease, 28 d	Landa and Fang (1978)
Microbial, respiration	140	28% decrease	Tu (1988)
Microbial, respiration	200	2-41% decrease at 6h	Landa and Fang (1978)
Various enzymes	200	20-54% decrease	Wilke (1988)
Urease activity	1000	75-98% decrease	Tabatabai(1977)

Table 2.2 Relative effect of fertility and light levels on growth of *B. rapa,* using the Aaa 1-1 selection and potting soil. At the standard condition, where fertility, light and terminalization were as recommended [1], the plant height was 47 cm, the total shoot dry weight was 0.53 g per plant and the seed dry weight was 0.15 g per plant. The corresponding, pooled standard deviations are 5 cm, 0.09 g and 0.05 g respectively.

Fertility level	Light level (µE m^{-2} s^{-1})			Timing of terminalization		
	100	250	500	-7 d	Recomm.	+7 d
	Plant height (% of mean at standard condition)					
None	59 *[2]	94	89	84 *	94	97
Recommended	70 *	100	106	90	100	89
3-fold higher	65 *	86	95	78 *	86	92
	Total shoot dry weight (% of mean at standard condition)					
None	65	45 *	54 *	50 *	45 *	53 *
Recommended	79	100	165 *	102	100	131
3-fold higher	78	159 *	258 *	126	159 *	209 *
	Seed dry weight (% of mean at standard condition)					
None	12 *	104	94	84	104	74
Recommended	10 *	100	125	66	100	86
3-fold higher	16 *	118	187 *	90	118	95

[1] The recommended fertility level was 0.25 g L^{-1} of soluble 20-20-20 fertilizer in the irrigation water, the recommended light level was 250 µE m^{-2} s^{-1}, and terminalization by temporary drought stress was recommended when seeds from the first blooming phase were beginning to mature, yet before extensive second blooming or axial bud development began.

[2] Means followed by * were significantly different from the mean at the standard condition.

Part P1 ... Balancing the anion additions

The addition of any cation or anion to soil to give a range of concentrations necessarily results in a range in concentrations of the companion ion. The companion ion potentially confounds the study because it increases linearly with the contaminant ion, In this case, we added Hg as $HgCl_2$. Because the atomic weight of Hg is large relative to Cl and Cl is relatively nontoxic, the companion ion in this case is not a serious problem. However, we devised an approach to minimizing the effect of the companion ion that will be useful in other studies.

The difficulty in making the concentration of the companion ion constant across all concentrations of the contaminant ion is that whatever form of the companion ion that is added will also carry another companion ion. In cases where the contaminant can be added as a nitrate salt, then ammonium nitrate can be used to make the N levels uniform across all treatments[5]. Here, we used Cl additions from several salts of cations common in soil solution. The ratio of salts was chosen so that the cations were added at ratios close to those measured in the various soil solutions[6] . The cation ratios for Ca:Mg:Na:K by hydrogen equivalent weights were 5:4:4:1. The calculations are as follows, showing the specific volumes and weights used in the present study.

- The Hg treatments were: 0, 10, 22, 46, 100, 220, 460, and 1000 mg Hg kg^{-1} soil, applied as $HgCl_2$.
- The amount of soil to be treated at each concentration was 4.00 kg dry weight.
- The volume of solution used to treat each 4 kg batch of soil was 200 mL.
- The total volume of the stock solution, allowing for excess for standards, was 250 mL.
- The atomic weight of $HgCl_2$ is 271.50 g/mol, the molecular weight of Hg is 200.59 g/mol.
- The balance solution used to make the concentration of Cl uniform across

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5. Sheppard, S.C., Evenden, W.G. and Anderson, A.J. 1992. Multiple assays of uranium toxicity in soil. Environ. Toxicol. Water Qual. 7:275-294.
Sheppard, S.C., Evenden, W.G. and Pollock, R.W. 1989. Uptake of natural radionuclides by field and garden crops. Can. J. Soil Sci. 69:751-767.

6. Thibault, D.H. and Sheppard, M.I. 1992. A disposable system for soil pore-water extraction by centrifugation. Commun. Soil Sci. Plant Anal. (in press)

treatments contained 17.5 g Cl/L, composed of 12.0 g $CaCl_2$, 8.0 g $MgCl_2$, 5.0 g NaCl and 2.0 g KCl per L. A 20-mL volume of this solution will give approximately the same Cl amount as in 1000 mg Hg as $HgCl_2$, and approximately the cation equivalent ratio 5:4:4:1, respectively.

Calculation table showing the additions of $HgCl_2$ to provide the required concentrations, and the additions of the balance solution to result in uniform Cl concentrations.

Hg treatment applied to the soil (mg/kg soil)	Concentration of Hg in stock solution (mg/200 mL)	Weight of $HgCl_2$ in stock solution (g/250 mL)	Resulting amount of Cl from the $HgCl_2$ (g/250 mL)	Amount of balance solution to add to the stock solution (mL/250 mL)
0	0	0.0	0	100
10	40	0.06768	0.0175	99
22	88	0.14889	0.0386	98
46	184	0.3113	0.0807	95
100	400	0.6768	0.175	90
220	880	1.4889	0.3860	78
460	1840	3.113	0.807	54
1000	4000	6.768	1.75	0
Totals		12.5 g $HgCl_2$		614 mL

Part P2 ... Control soil blend for the unknown soil

The unknown soil in this study was collected from a contaminated site without a corresponding control soil. This is likely a common, though undesirable situation. Even when a control soil has been collected, there remains some question of how appropriate it is, because soil properties vary over relatively small distances. To provide a soil as a control for the unknown soil, we developed a soil blending algorithm. Five reference soils; the sand, clay, potting and garden soils of this study, plus another reference soil we retain; and the unknown soil were analysed by the Manitoba Soil Testing Laboratory for sand, silt and clay contents, cation exchange capacity (CEC), organic matter content (OMC), pH, and concentrations of extractable NO_3-N, P, K, SO_4-S, Cu, Fe, Mn, and Zn. In addition, we measured the amount of water held at saturation and the amount of water held against gravity after draining for 24 h. Mixing ratios of the five soils were compared by computer in order to achieve a blend that matched the properties of the unknown soil. The following restraints and assumptions were used:

- Only 20% fractions were considered, so that each of the five soils could have made up 0, 20, 40, 60, 80, or 100% of the blended soil.
- Linearity was assumed so that, for example, adding equal amounts of one soil of pH 5 with another soil of pH 7 was assumed to yield a blend with pH 6. This is not a good assumption for chemical parameters such as pH, but should be accurate for textural properties.
- The sum of the sand, silt and clay fractions was unity.
- The relative importance of the various soil properties in the context of our bioassays was estimated and then set with weighting factors. The weighting factors were five for the indices of pore space and water holding capacity, three for the single-grain textural properties, two for CEC and one for pH and OMC.

The algorithm ranked all possible blends for closeness of match to the unknown soil, and the best 20 were listed for consideration. The blend used three of the soils from this study and one other. It was two parts of the clay, one part of the garden soil, one part of the potting soil and one part of the other soil. The other soil was 11% clay with a pH of 8.0, an organic matter content of 37 g kg^{-1}, and a cation exchange capacity of 15 cmol (NH_4) kg^{-1} (the Almassippi soil of Sheppard *et al.*[7]).

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7. Sheppard, M.I., Sheppard, S.C. and Amiro, B.D. 1991. Mobility and plant uptake of inorganic ^{14}C and ''C-labelled PCB in soils of high and low retention. Health Physics 61:481-492.

Part P3 . .. Effects of seed colour

Relative effect of three classes of seed colour in *B. rapa,* expressed as percent of results for brown seeds, the most common seed colour. Standard conditions were used.

Measurement	Mean with brown seed	Relative effect (%)	
		Black seed	Yellow seed
Plant height	47 cm	85 [1]	93
Total shoot dry weight	0.53 g	81	81
Seed dry weight	0.15 g	124	123
Number of pods	17	75	100
Pod dry weight	0.31 g	114	116
Plant dry matter content	17%	87	88

[1] No means were significantly different from the mean of the brown seed treatments.

Part P4 ... Effects of water regime and seeding depth

Relative effect of water regime and *seeding* depth on B. *rapa,* expressed as percent of results at standard conditions. Seeding depths were 1, 5 and 10 mm.

Measurement and mean at standard condition		Drought			Standard			Excessive		
		1	5	10	1	5	10	1	5	10
Plant height	47 cm	83	83	89	87	100	97	102	89	74 *[1]
Total shoot dry weight	0.53 g	80	85	87	81	100	91	78	75	44 *
Seed dry weight	0.15 g	85	62 *	69	115	100	96	101	88	54 *
Number of pods	17	80	80	67	96	100	73	97	78	46 *
Pod dry weight	0.31*g*	79	67	65 *	97	100	81	96	72	81
Plant dry matter content	17%	104	101	110	92	100	102	93	91	75 *

[1] Means followed by * were significantly different from the mean at the standard condition.

Part P5 ... Further data related to Table 2.2

Relative effect of fertility and light levels on *B. rapa* parameters other than those given in Table 2.2 of the Overall report. Results are expressed as a percent of those at standard conditions. At the standard condition, where fertility, light and terminalization were as recommended[1], there were 17 pods per plant, the pod dry weight was 0.31 g per plant and the plant dry matter content was 17%. The corresponding, pooled standard deviations are 4.5 pods, 0.08 g and *2%* respectively.

Fertility	Light level (µE $m^{-2}s^{-1}$)			Timing of terminalization		
level	100	250	500	-7 d	Recomm.	+7 d
Number of pods per plant (% of mean at standard condition)						
None	14 *	99	106	63	99	77
Recommended	22 *	100	146	72	100	78
3-fold higher	24 *	108	210	86	108	80
Pod dry weight (% of mean at standard condition)						
None	13 *	99	118	84	99	75
Recommended	13 *	100	154	68	100	82
3-fold higher	11*	126	220 *	97	126	98
Plant dry matter content (% of mean at standard condition)						
None	68 *	98	148 *	103	98	97
Recommended	65 *	100	161*	94	100	71*
3-fold higher	73 *	89	147 *	121*	89	94

[1] The recommended fertility level was 0.25 g L^{-1} of soluble 20-20-20 fertilizer in the irrigation water, the recommended light level was 250 µE m^{-2} s^{-1}, and terminalization by temporary drought stress was recommended when seeds from the first blooming phase were beginning to mature, yet before extensive second blooming or axial bud development began.

[2] Means followed by * were significantly different from the mean at the standard condition.

Part P6 ... Further data related to Figure 2.1

The effect of soil type and mixtures of soils on the performance *of B. rapa.* Counts and weights are expressed per plant. Shown are the results for unmixed potting, clay and sand soils and for 50:50 mixtures involving the potting soil. The clay soil in these treatments was all aggressively prepared, as described in the text. The results of the ANOVA which included all treatments is indicated.

Treatment	Germ. (%)	Height (cm)	Num. of leaves	Dry Wt. of stem (g)	Num. of pods	Dry Wt. of pods (g)	Dry Wt. of seed (g)	Dry matter content (%)
Potting soil	93	29	41	0.52	30	0.64	0.24	23
Clay, aggres.	85	11	21	0.11	6	0.07	0.021	18
Clay/potting	50	16	16	0.18	16	0.28	0.11	19
Sand	100	5	19	0.04	3	0.02	0.006	19
Sand/potting	98	11	25	0.11	7	0.09	0.031	19
Overall F [1]	***	***	***	***	***	***	***	***

[1] The *** indicate an overall F ratio $P<0.001$, no means comparisons are reported because of the inhomogeneity of variance among treatments (the means span a broad range)

The effect of soil type and mixtures of soils on the performance of *B. rapa.* Counts and weights are expressed per plant. Shown are the results for clay and sand soils only. The clay soil was aggressively prepared except where indicated as conservatively prepared. The dilutions with Perlite are by volume. The results of ANOVA for these treatments, excluding the potting soil treatments, which had much higher means, is indicated.

Treatment	Germ. (%)	Height (cm)	Num. of leaves	Dry Wt. of stem (g)	Num. of pods	Dry Wt. of pods (g)	Dry Wt. of seed (g)	Dry matter content (%)
Clay soil								
Aggressive	85	11	21	0.11	6	0.07	0.021	18
Conservative	75	8[1]*	16	0.07*	6	0.05	0.019	18
20% Perlite	88	9	18	0.09	5	0.05	0.018	17
50% Perlite	98	7*	21	0.08*	5	0.04*	0.010	17
50% sand	90	7*	21	0.07*	4*	0.03*	0.008*	19
Sand soil								
Unlimed	100	5	19	0.04	3	0.02	0.006	19
Limed	98	4	20	0.03	2	0.01	0.004	22[2]*
Unlimed 50% Perlite	95	6	15	0.04	3	0.02	0.006	15*
Limed 50% Perlite	98	5	17	0.03	3	0.02	0.003	20
50% clay	90	7	21	0.07*	4	0.03	0.008	19*
Overall F[3]	NS	***	NS	***	*	*	NS	**
Clay vs sand[4]	*	*	NS	*	*	*	NS	NS
Coefficient of variation	18[5]	24	23	29	38	51	73	9

[1] An * indicates means among the clay soil treatments that were significantly different ($P<0.05$) from the aggressively prepared clay.

[2] An * indicates means among the sand soil treatments that were significantly different ($P<0.05$) from the unlimed sand soil.

[3] The overall ANOVA F ratio was not significant (NS, $P>0.05$) or significant at $P<0.05$ (*) or $P<0.001$ (***)

[4] The aggressively prepared clay and the unlimed sand are contrasted, with * indicating $P<0.05$.

[5] The coefficient of variation is expressed as a percent.

Part P7 ... Elemental analyses of the unknown soil

Elemental analyses (mg kg^{-1}) of the unknown soil[1], subsequently identified as from the grounds of a plumbing porcelain manufacturer. Concentrations of B, Co, Ni and Zn are notably high.

Al	12000	±400	Ni	305	±15
As	<5		P	745	±15
B	148	±30	Pb	43	±3
Ba	680	±20	Ru	<3	
Bi	<3		S	740	±10
Ca	8590	±610	Sb	<3	
Cd	<0.3		Se	<6	
Ce	<6		Si	68	±2
Co	65	±5	Sn	<3	
Cr	26	±2	Sr	114	±4
Cu	35	±2	Te	<20	
Fe	18900	± 900	Ti	475	±25
Gd	<3		U	<30	
Hg	0.55		V	30	±5
Mg	3830	±270	Zn	510	±10
Mn	551	±12	Zr	88	±5
Mo	<0.6				

[1] Analysis was by inductively coupled plasma spectroscopy, following acid extraction. Extraction was of 2 g of soil in 10 mL 1:1 HNO_3 refluxed at 75°C for 6 h except for Hg. Extraction of Hg followed method described in Overall Report.

2. Plant life-cycle bioassay

2.3 Detailed protocol

The detailed protocol expands upon the *Basic Methodology for B. rapa* subsection in the **Methods and Materials** of the literature paper (Section 2.1 of this report). The detailed protocol is described under the following headings.

- Source and storage of *B. rapa* seed
- Basic bioassay plan
- Preparation and characterization of soil
- Preparation of treatments or dilution series
- Preparation of pots, including filling with soil
- Planting, watering and special care during emergence
- Maintenance, pollination and midterm observations
- Final sampling and observation
- Test acceptance criteria
- Disposal of materials
- Statistical summation of data

Source and storage of *B. rapa* seed

Seed is obtained from the Crucifer Genetics Cooperative (CrCG), Department of Plant Pathology, University of Wisconsin - Madison, 495 Russel Labs, 1630 Linden Drive, Madison, WI, 53706, U.S.A., telephone (608) 262-8638, FAX (608) 263-2626. This organisation provides many kinds of seeds well suited to research and teaching. There is a modest membership fee which includes some free seed orders, and then a nominal cost for further seeds. The CrGC is a small organisation within the University, but is widely known among crucifer plant breeders. It has proven to be very reliable in shipping seed and ready to answer specific questions.

The CrGC supplies a manual for the use of Rapid Cycling plants, and the methods described here conform to many of the CrGC recommendations. Seed storage requires care: the seed will germinate in moist air. It is imperative that the seed be stored with desiccant in sealed containers in a refrigerator. If possible, do not use seed stored longer than one year.

Basic bioassay plan

Bioassays will likely be applied in either of two modes. One is the experimental investigation of contaminants, where a soil of specific properties is chosen and is artificially contaminated to meet the requirements of an experimental plan. The second mode is the application to a contaminated soil collected in the field.

The use of artificially contaminated soil is the most appropriate mode for a whole-soil bioassay because an uncontaminated control soil is extremely important in interpretation of results. *B. rapa* is very sensitive to soil physical and chemical conditions, so that small differences between the control and contaminated soil can have marked effects (this sensitivity to environment is a dilemma for all sensitive bioassays). In practice, an uncontaminated soil with specified properties is collected. Control and treatment aliquots are handled uniformly, and the treatment aliquots are contaminated with a range of at least five concentrations typically spanning at least an order of magnitude. The specific concentrations and ranges are determined by reports in the literature. Use a geometric scaling of the concentrations and consider placing more levels near the expected-effect concentration. At the same time, it is highly recommended to include a treatment at a concentration well above the expected-effect level, so that trends and symptoms observed at the lower concentrations can be clearly related to the effects at the extreme concentration. Great care can be taken to counterbalance companion ion and solvent additions so that the only significant difference among the aliquots is the presence of the contaminant. Use a replicated design, with extra replicates assigned to the control.

In the second mode, the extent and identity of the contaminant(s) may or may not be known. It is very important to seek a control soil to correspond to the contaminated soil. The field agent must estimate the extent of the contamination and try to collect a soil that appears to have properties comparable to the contaminated soil yet be outside the influence of the contamination. It would be desirable to thoroughly characterise both soils to ensure similarity in non-contaminant properties and dissimilarity in contamination. When an unambiguous control soil cannot be found in the field, it is necessary to identify one in the laboratory. For this, the laboratory should keep a supply of several soils (that can be renewed with further collections) that represent a range of key properties such as pH, texture and organic matter content. In the Expansion of Details, Part P2, efforts to create a control soil by matching a blend of laboratory reference soils to a contaminated field soil are described. This is a difficult task to accomplish.

For both modes of operation, include a series of concentrations of a reference contaminant in a similar soil. Zinc is useful as the reference contaminant, applied at concentrations of 50, 100, 300, 600 and 1000 mg Zn kg^{-1} with solutions of $ZnSO_4.7H_2O$. It is acceptable to use only two replicates of these soils. The soil should be a laboratory reference soil or an additional aliquot of the control soil.

Preparation and characterization of soil

About 20 L of soil is required, varying somewhat with the mode of the bioassay and the amount of processing needed to render a homogenous, finely-divided soil for use. Storage and initial preparation of the soil for the plant bioassay can likely follow protocols established for other bioassays. Spread the soil on a large flat, plastic-covered surface where lumps can be broken and the sample homogenized, and where drying to a workable moisture content is allowed. This operation would have to be modified for volatile contaminants.

A workable moisture content is one where the soil will no longer adhere in clumps when handled. It is undesirable to fully dry the soil, as this affects both contaminant behaviour and soil microbial condition. The soil is then passed through a 4- to 10-cm mesh. Material retained on the mesh is normally discarded. The operator must decide what amount of force to use to grind the soil through the mesh, bearing in mind that the goal is a chemically and physically homogenous sample. The processed soil is then stored in sealed pails. Each time a sample is removed for analysis, an additional subsample is taken to measure the moisture content. Soil moisture content is defined as the weight loss on drying at 105°C for 24 h. All soil concentrations must be expressed on a soil dry weight basis.

It is likely that the bioassay of a soil will be accompanied by chemical and physical characterization of the soil to meet other needs. However, to facilitate interpretation of the plant bioassay results, quantitative soil pH and at least qualitative texture and organic matter content are required. Routine extractable nutrient analyses by a soil fertility testing laboratory would be desirable. In addition, the plant bioassay requires a measure of the soil moisture holding capacity. This is not interpreted as a basic property of the soil - it is an application-specific measurement. Measure by weight the amount of water retained after 24 h draining of a thoroughly wetted aliquot of the soil held in the same physical container as used in the application. In this case, the container is the individual plant pot. This measurement of moisture holding capacity will give higher values than field moisture retention or third-bar tension measurements. It may also be above the optimal moisture content for seed germination in a given soil because of the effect of water on oxygen diffusion. Wet the soils in the bioassay to 90Z of the measured moisture holding capacity.

Preparation of treatments or dilution series

The principles applied here are outlined in the Basic Bioassay Plan section. For artificial contamination, five replicates of the control soil and three replicates of each contaminant concentration are recommended. If more than about seven concentrations are used, then the numbers of replicates could be reduced. Each pot will hold about 400 mL of soil, so that 1.5 to 2.5 L of soil for each treatment should be prepared. Care should be taken to counterbalance companion ion and solvent additions across the treatments. This requires judgement and compromise, approaches are described in the Overall Report sections here, in the Detail section 2.2, and elsewhere [8].

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8. Sheppard, S.C., Evenden, W.G. and Anderson, A.J. 1992. Multiple assays of uranium toxicity in soil. Environ. Toxicol. Water Qual. 7:275-294.

Sheppard, S.C., Evenden, W.G. and Pollock, R.W. 1989. Uptake of natural radionuclides by field and garden crops. Can. J. Soil Sci. 69:751-767.

Sheppard, S.C. and Evenden, W.G. 1988. The assumption of linearity in soil and plant concentration ratios: An experimental evaluation. J. Environ. Radioactivity 7:221-247.

After addition of the contaminant, it is desirable to moisten the soil to near the moisture holding capacity and incubate it for some time. The length of time is dependent on the volatility of the contaminant and the expected reaction kinetics in the soil. Elevated temperature to 30°C may be appropriate to speed reactions for non-volatile contaminants. The incubation should last at least 7 to 14 d to pass the burst in soil respiration that follows rewetting of a dry soil.

For field contaminated soil, the objective is to provide a dilution series of the contaminated soil diluted with a control soil. As described, the choice of the control soil is very important. In the absence of a field control soil, then a laboratory reference soil or blend can be used. A broad dilution series is ideal, but unlike water samples, dilution of whole soils is limited by the physical scale of the soil particles. It is inappropriate to dilute the contaminated soil to the extent that the probability of a plant root encountering contaminated particles is affected. Dilution ratios, control:contaminated, of 1:0, 16:1, 8:1, 4:1, 2:1, 1:1, 1:2 and 0:1 are usually appropriate. Mixing should be very thorough, and it is appropriate to moisten the mixtures and allow them to incubate as described above.

Preparation of pots, including filling with soil

The pots are commercial, 500-ml, plastic food containers with diameter about twice the height. Six slots are cut into the bottom of the containers using a band saw. The slots perpendicular to the bottom edges of the pot and extend 1 cm from the edge into the bottom and up the side of the pot. These allow capillary contact with the watering mats.

The soil is added to the pots ensuring equal, 400 mL volume measures of soil for each pot. The soil may be tamped with gentle tapping of the pot on a hard surface, but do not apply pressure on the surface.

Planting, watering and special care during emergence

The seeds are randomly selected for planting from the stock received from the CrGC. Seed stocks are not mixed and there should be sufficient new seed from a single shipment for each bioassay. Ten seeds are distributed evenly on the soil surface, at least 1 cm from the edge of the container, and pressed 5 mm into the soil using a marked rod. The soil surface is smoothed. These operations should be done by replicate, so that all of the first replicate is planted, then the second and so on.

After all pots have been planted, the soil is moistened to 90% of the MHC using distilled water. This must be done with care to avoid uncovering seed or creating an unlevel surface. One technique is to place a blotter paper on the soil surface during wetting to help distribute the water. This blotter paper is removed after the water has penetrated the soil.

The wetted pots are placed on dry capillary watering mats in a growth chamber. The CrGC also use watering mats in their seed production. The mats must have a water reservoir just below

grade level. For germination, it is appropriate to cover the pots to reduce evaporation. This can be done with a weighted sheet of polyethylene film placed over all the pots in the growth chamber.

The growth chamber is set to provide a day/night temperature and light cycle. Temperatures of 25°C/22°C, with an accuracy of ±0.5°C, are appropriate. The light flux density at the top of the pot, or plant once they are growing, should be about 250 µE $m^{-2}s^{-1}$. Lower light levels must be avoided, and higher light levels may cause difficulties because of rapid evaporation of water. A light meter should be used to record and adjust the level, and the wide spectrum fluorescent tubes should be changed on a routine basis.

After about 12 h, some emergence will likely be evident and the cover is removed. Germination is judged to be complete when no newly emerged seedlings are observed for at least 24 h, and usually within 60 h of seeding and wetting. The number of seedlings emerged is counted. With these few seeds, the emergence counts are not intended as a reliable toxicity endpoint, but they occasionally prove useful. In some cases, emerged seedlings may die because of latent toxicity effects. In especially dense soils, the root tip may not penetrate and as a result root elongation pushes the rest of the seedling out of the soil. Score emergence as those seeds that produce seedlings, and do not change this count to account for seedlings that die after emergence.

The capillary mats are wetted and the reservoirs filled at this time. It is important to press each pot into the wet mat to ensure capillary contact. Shrinking soils can be problematic, and these on occasion need additional water added to the soil surface to keep the soils moist. Decisions to do this based on visual inspection of the soils are sufficient, with care to add a small enough amount of water that leaching does not appear. The capillary mats should also be watered from above every day to ensure good capillary connection.

During the emergence counting, the seedlings are thinned to five per pot. Seedlings that have died are removed, but thinning among the remaining seedlings is done without regard for apparent vigour. Where possible, the thinning is done to leave seedlings that are evenly spaced in the pot.

Maintenance, pollination and midterm observations

The pots are removed from the bench in the growth chamber, randomized and replaced in the growth chamber at least every other day, to minimize location effects. Formalized randomization techniques are not needed here because the procedure is repeated. Do ensure that handling sequence and other ordering is not repeated.

When about half the plants are in bloom, defined as stage 4.1[9] (see Figure 2.5), a count is made

9. Harper, F.R. and Berkenkamp, B. 1975. Revised growth-stage key for *Brassica campestris* and *B. napus.* Can. J. Plant Sci. 55:657-658.

of the number of plants blooming in each container. Blooming is defined to occur when the first flowers on the plant have opened their petals.

Manual pollination is required to ensure that the seed set reflects soil conditions rather than random pollination. Self fertile selections of the rapid cycling plants are available to eliminate this requirement. A small cluster of feathers is useful for pollination, using the same cluster on all pots in the trial. This is done daily until the blooming ends.

The B. rapa selections are not determinant and terminalization is recommended to avoid increased variability associated with secondary growth. The timing of this is a subjective decision. Although the current recommendations for production of seed suggest removal of buds and apices after a defined stage, these methods directly confound potential toxicity endpoints. Therefore, terminalize by withholding water for 2-3 d, and do this when seeds from the first blooming phase are beginning to mature, yet before extensive second blooming or axial bud development begins (about stage 5.3). This is about 20 d after pollination. In some cases, terminalization is not needed, whereas in others secondary growth is vigorous.

Final sampling and observation

The timing of the harvest is determined largely by the terminalization. At harvest, most of the seed will be mature; the seed become darker as the seed pods dry (stage 5.4 to 5.5). The plant will have lost most of its green colour. It is appropriate to make notes describing the plants, especially if there are apparent maturity differences among the treatments.

At harvest, the plants are cut at the soil surface and the height of individual plants is recorded, to an accuracy of about ±2%. The arithmetic mean height for each pot is the variable ultimately analysed: individual plants cannot be considered replicates. The height measurements implicitly indicate the number of plants, which may be less than five in some treatments.

The fresh weight of the plants may be recorded. This can be a useful parameter when the contaminant has caused some variation in the maturity of the plants.

The pods are removed and the total number of pods from each pot counted. The pods are placed in metal boxes and dried at 50°C for 24 h. Metal boxes that have tight fitting lids are useful because they facilitate the later removal of the seed. The stems are placed in paper bags and dried at 50°C for 24 h. After drying, pods and stems are weighed separately. Do this without tare by transferring the plant materials directly onto the pan of a pan balance.

The seeds are separated from the pods by first breaking the pods. This is done by placing two ⅜-inch steel nuts in the metal boxes, fitting on the lid and vigorously shaking for a few seconds. Visual inspection is required to judge the amount of this action needed: the pods should be broken open and the seed released, but the pod residue should remain in long pieces. The

material is then passed once or twice through a 2-mm mesh which will retain the pod residue. Some pods may have to be broken by hand. Some pod residue will pass through the screen with the seed: operator judgement is required to decide on further screening. The most important criteria must be uniformity of handling among all samples. Simplicity and minimal handling help ensure uniformity. The cleaned seed is weighed.

Test acceptance criteria

There must be uniform and sufficient emergence among the control pots. Soil conditions apart from the contamination can reduce emergence. Acceptable trials have enough emerged plants to thin to five good plants in each control and low-contaminant pot. If emergence in the control pots is poor, record the final emergence count, dry and rework the soil, and start the trial again.

This bioassay is lengthy and there is a chance of accidental deviation in growth conditions. This includes drought and failure of the growth chamber. Do not use data from trials where plants have been stressed by deviations in the growth conditions. Deviations that do not markedly stress the plants, such as failure of lights to come on for a few hours, are undesirable but probably acceptable.

On occasion, and usually related to some deviation in growth condition, the plants will be markedly indeterminant. For example, if pollination has not been effective, the plant will produce many pods with few seed in each. Similarly, vigorous secondary growth may occur, especially if terminalization was ineffective. These occurrences may not jeopardize the trial, but are worthy of note and may lead the operator to decide to repeat the trial.

A monotonic response to the contaminant is considered an important criteria. Tests that show multi-modal response curves should be repeated with care to address possible sources of variation.

Disposal of materials

The plant material and containers should be disposed of in the same manner used for the contaminated soil.

Statistical summation of data

Statistical summation can follow several approaches. A replicated design allows either regression or analyses of variance (ANOVA), or a combination. The SAS programs [10] are ideal, they are broadly accepted and very flexible.

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10. SAS (1985) SAS User's Guide, Statistics, Version 5 Edition, SAS Institute Inc., Cary, NC.

The life-cycle plant bioassays allow interpretation of a number of endpoints and variables. The following set of variables is recommended:

- the number of plants in bloom for each pot on the date when they were counted.
- the height of plants at harvest, as the mean of five plants in each pot (cm).
- the total dry weight per plant, computed as the sum of the stem and pod dry weights divided by the number of plants in the pot (g per plant).
- the seed dry weight per plant, computed as the measured seed dry weight divided by the number of plants (g per plant).
- the number of seed pods per plant, computed as the pod count divided by the number of plants (pods per plant).

Additional variables can be computed from the data obtained. In general, they are less directly related to plant performance. Those that are products of raw data are often more variable and hence less sensitive than the recommended set of variables. However, they may provide a more complete interpretation of the trial:

- whole plant dry matter content, computed as the ratio of the dry/fresh weight (%). Lower values indicate the plant contains more water and therefore may have been less mature at harvest.
- harvest index, computed as the ratio of the seed/total dry weights (%). Low values indicate poor reproductive success, the plants have not been able to channel resources into seed production.
- seed/pod weight ratio, computed as indicated (%). Low values indicate few seeds maturing in each pod. If pollination was impaired, the plants will produce many pods with few seeds each.

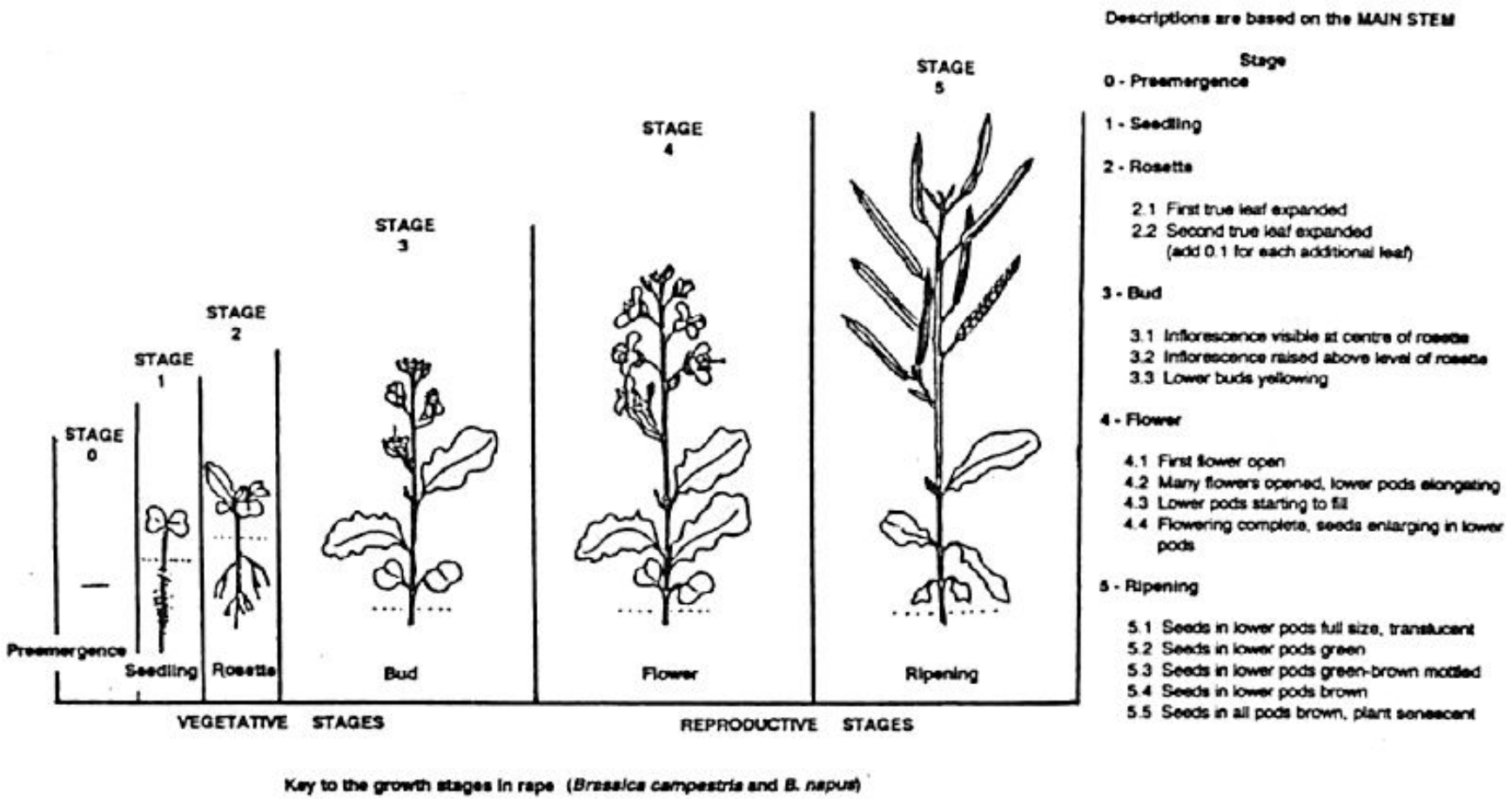
The recommended variables tend to be normally distributed. However, as with all statistical interpretations of data, the nature of the distribution should be considered. For example, in the experiment to evaluate the effect of soil properties (Figure 1 of the Overall Report and Part P6 of the Expansion of Details), very large ranges in the values were obtained. This resulted in uneven variances among the treatments. It would have been inappropriate to use the overall measure of variance, which was dominated by the treatments with large plants, to compare differences among treatments with small plants. Log-transformation is a common approach to lessening this problem. The Overall Report Figure 1, the data were divided into subsets and the statistical tests conducted on each subset.

Ratio data, such as those indicated as optional variables, are more likely to be log normally distributed. This is because of an extension of the Theorem of Central Tendency. For these, log-transformation of the data may be considered. In general, statistical tests on log-transformed data will indicate fewer significant differences among large data values and more among small data values when compared to tests on the untransformed data. This may be theoretically more correct, but may not be conservative for identifying toxicity thresholds.

As indicated by the example, reserve log transformation in toxicity tests for severe cases of inhomogeneity variance.

The first step is to plot the data, with error bars, versus the contaminant concentration or dilution ratio. Log-scaled treatments should be plotted on log scale. The second step is an overall ANOVA, where the F ratio for the effect of the contaminant is calculated. If the F ratio is not significant at $P<0.05$, then further interpretation must be considered with caution. There is some controversy about the next step. Individual statistical tests, equivalent in results to t tests, on each treatment versus the control can be used. These result from the PDIFF option of the LSMEANS statement in the GLM procedure of SAS. If you can assume no possibility of enhanced performance as a result of the contaminant, a one-tailed test is appropriate. In the sense of a bioassay to detect negative environmental impact, a one-tailed test is also conservative. The multiple comparisons to the control are not an orthogonal decomposition of the overall treatment sum of squares. They are *a priori* and are conservative in the sense of a bioassay.

The statistical indications require interpretation supported by the plots. The no-observable-effect-level (NOEL) is associated with the highest-concentration position on the curve where there is no continuous trend toward poorer performance than the control. This point will never be significantly different from the control. The lowest-observable-effect-level (LOEL) is associated with the lowest concentration that had performance significantly different from the control. The EC_{10} and EC_{50} can be interpolated visually from the graph, but only when within the range of the data.



Key to the growth stages in rape (*Brassica campestris* and *B. napus*)

Figure 2.5 Diagram of stage definitions for *Brassica rapa* adapted from Harper and Berkenkamp (1975, Can. J. Plant Sci. 55:657-658).

3. Earthworm survival bioassay

3.1 Overall report

Optimized Design for Earthworm Survival Tests in Soil

Stephen C. Sheppard and William G. Evenden

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Toxicity tests for soils have not been as extensively developed as those for aquatic systems (van Straalen and Denneman 1989); yet there is an urgent need to assess the biological impact of soils contaminated with unknown or mixed contaminants (van Leeuwen 1990). A dilemma is caused by the profound effect that soil properties, quite apart from the presence of a contaminant, can have on test organisms. For this reason, many protocols call for the use of liquid soil extracts rather than the whole soil. Unfortunately, organisms also respond to particle-bound contaminants and can play a role in the release of these contaminants. Therefore, there is a need to assess toxicity in whole soils. Assays with earthworms have been developed, although many of them involve artificially contaminated soils (e.g., Pizl 1988) and often artificial soils as well (e.g., Neuhauser *et al.* 1986). Some modification is required to use these assays for whole soils from contaminated sites where the contaminants may or may not be known.

Several researchers have sought to use the growth and reproduction of earthworms in toxicity tests (Lofs-Holmin 1980; van Gestel *et al.* 1988, 1989). Certain species are well suited to laboratory rearing. Others have longer life cycles, so that measures of growth and reproduction are time consuming and often impractical (Butt 1991; Iglisch and Kriegerowski 1986; Lofs-Holmin 1980). *Lumbricus* species are among these; they would otherwise be well suited to toxicity assays because of their broad ecological and geographical distributions and importance as decomposers. Large numbers of *L. terrestris* are available as commercial angling bait; 10^9 worms worth $50 million are picked annually in Ontario, Canada (Tomlin and Protz 1990). Although these sources may not provide strictly the one species, the uniformity and continuity of supply are distinct advantages, and these worms have been used for toxicity assessment in Germany (Heimbach 1985) and on Superfund sites in the U.S. (Callahan *et al.* 1991).

The objective of this paper is to present the results of a study on the optimal experimental designs for survival tests using bait worms. We found no reports in the literature addressing this subject for earthworms. The most common experimental design with earthworms uses 1 to 10 worms per container and 3 to 5 replicates (e.g., Pizl 1988; Heimbach 1985; Neuhauser *et al.* 1986). Experience in our laboratory indicated this arrangement was not satisfactory (Sheppard *et al.* 1992), and this study was initiated to investigate the alternatives.

A preliminary experiment, not reported in the journal paper, was conducted to investigate several variables we thought could be important. It was also our first use of the single-worm packaging method. The objectives, methods and results are given in the following Expansion of Details, Part W1.

Materials and Methods

The objective of Experiment A was to determine the number of replicates per soil needed to indicate significant differences in survival across a series of treatments, in this case soil types. Eleven soils were chosen to represent a broad range of soil properties (Table 3.1). *For notes about the experiment not included here, see Expansion of Details, Part W2.* The soils were partially dried to a workable moisture content, sieved to pass a 0.5-cm mesh, and placed in 500-mL plastic containers with soil surface areas of 90 cm^2. Sixteen containers of each soil were used. The soils were moistened to field capacity, and five mature worms *(L. terrestris),* selected for uniformity of size, were added. The worms had been freshly obtained from a commercial angling-bait supplier in Ontario and were relatively uniform in size and apparent maturity. After 54 d at 15°C, the remaining live worms were extracted by hand and counted. To assess the optimal number of replicates, the observations from Experiment A were randomly sorted six times. Each time an analysis of variance (ANOVA) was calculated using subsets with 3, 5, 8, 10 or 16 observations for each soil.

The objective of Experiment B was to compare means of improving the precision of survival tests, with the underlying hypothesis being that increased numbers of earthworms or experimental units would be useful. Preliminary trials indicated that placing earthworms with soil in plastic bags was expedient, and that packaging worms singly in this manner was practical *(see Expansion of Details, Part W1).* The bags were made of polyethylene tubing, 9-cm or 25-cm diameter when flat, heat-sealed to form small bags. The size of the bag allowed the soil to be spread to a 2-cm-thick layer. Each bag was punctured with about ten holes, using a hypodermic needle, to ensure sufficient air exchange.

The treatments (Table 3.2) included a series at a constant density of 80 cm^3 of soil per worm, with 1, 5, 10 and 50 worms per container. Replication of these units was 100, 20, 10 and 2, respectively. The 4000 cm^3/50 worm treatment was held in plastic pails, rather than bags, to reduce handling injuries to the worms. Another series of a decreased soil-to-worm ratio using the same volumes of soil and numbers of replicates, ranging from 40- to 20-cm^3 of soil per worm. A commercial potting soil moistened to field capacity was used. Earthworms were obtained and selected for use as before. For the treatments that involved one or two worms per container, the worms were weighed as they were placed into the soil, and again after they were removed. After the worms were added to the soil, the containers were incubated at 15°C for 28-30 d. Hoist absorbent paper was placed over the bags to reduce moisture loss through the aeration holes. After 14 d, counts of live worms in the bags were done by observing them through the plastic. At the end of the incubation, live worms in all containers were counted.

An ANOVA was done on the full Experiment B to determine the significance of the treatments on survival. The data for each treatment had different expected frequency distributions. The data were transformed to compute non-parametric tests by calculating the surviving fraction for each treatment, then assigning median scores, where survival fractions above the experiment median were assigned the score 1, and fractions below the median were assigned the score 0. The scores were used to compute the ANOVA, and treatments were compared by single degree of freedom contrasts.

To investigate the optimal number of replicates, different numbers of observations for each treatment in Experiment B were randomly selected, the means calculated, and the results compared to the overall treatment means. This was repeated in six independent cases for each level of replication, and the optimal number of replicates was estimated as the number where all six cases had means within five percentage points of the overall treatment mean.

Simulated data, based on the frequency distributions observed in Experiment B, were used to compare the statistical power of various experimental design options. The options considered were experimental units that contained one worm, where the data are binary survival scores, and experimental units that contained ten worms, where the data may approximate a normal or bimodal frequency distribution. It was anticipated that these approaches would require markedly different numbers of units and worms to achieve the same level of statistical precision. The issue of parametric versus non-parametric statistical methods also arises. We used non-parametric methods based on median scores, unless otherwise specified.

Simulated control data were compared with simulated treatment data that had half the number of survivors of the controls, equivalent to the concentration lethal to half the sample (LC_{50}) frequently sought in toxicity trials. Two control survival regimes were used. One used the survival rate observed in Experiment B with one-worm units (88%). This was simulated by randomly assigning values of 0 or 1 to a set of 1000 observations, with the target of 120 observations set to 0 (dead) and 880 set to 1 (live). The corresponding simulated LC_{50} observations had a target of 44% survival. The other control survival regime simulated the mean survival and survival count frequency distribution observed in Experiment B with ten-worm units. Here the mean survival was 75%, with 20% of the units with no survivors, 10% with 5 survivors, and 70% with 10 survivors. The corresponding simulated LC_{50} observations had a target of 37.5% survival. The frequency distribution of the simulated LC_{50} observations was designed to mimic that of the controls, and therefore was bimodal.

Each simulation case was reproduced five times, each with independent, randomized generation of observations. Conclusions were based on the calculated probability levels for a difference between the control and LC_{50} treatments, based on ANOVA. The analysis of binary data by ANOVA is equivalent to non-parametric analysis of median scores. The bimodal data from the ten-worm units was analysed both as parametric and as non-parametric using median scores.

Results and Discussion

Survival was significantly different among the soils in Experiment A (Table 3.1), with the poorest survival in acidic sandy soils. Neutral sands and slightly acidic heavier textured soils did not markedly reduce survival. Soils that had poor survival would be difficult to use in toxicity tests, because the impact of the contaminant is assessed on the numbers of live worms in uncontaminated controls.

Perhaps the most important observation in this experiment is the occurrence of cascade deaths. The data were clearly bimodal, with most containers having either all dead or all living worms. This is reflected in the fractions of units with all dead (Table 3.1), which almost fully account for the survival data. Evidently, when worms are stressed by soil conditions, soil-borne disease or toxicants, it is likely that all the worms in the container will suffer and, if one worm dies, it is likely to precipitate the deaths of others. For this reason, individual packaging has apparent advantages.

There was a four-fold range in survival among soils in Experiment A (Table 3.1). The number of replicates required to consistently show significant differences among the soils was 5 to 8 (Figure 3.1). About 10 replicates gave as much consistency among the means and as much statistical precision in ANOVA as the full 16 replicates. These ANOVA involved 11 soils, so that there were 44 error degrees of freedom (edf, Figure 3.1) when 5 replicates were considered. For a test between only two treatments, it would require 23 replicates to obtain the same number of error degrees of freedom. These numbers of replicates and implied numbers of worms are well above those commonly used, and may present a logistic constraint in laboratory use. Because of this, we sought more efficient experimental designs.

There were marked effects of grouping worms and decreasing the soil-to-worm ratio in Experiment B (Table 3.2). Survival was optimal in containers that held one or two worms. The proportion of cascade deaths increased as more worms were used per container. Evidently, using multiple worms per container decreased survival enough to jeopardize the estimation of effects such as LC_{50}. These data strongly support the use of containers with only one or two worms. However, the implications for statistical power are not clear, and this led to our simulation investigations.

We also used the data from Experiment B to determine the optimal number of replicates to obtain consistent results for each treatment (Table 3.2). For the one- and two-worm treatments, about 50 to 56 replicates gave consistency comparable to 100 replicates. After 30 replicates, the improvements in consistency were small. In contrast, about 9 replicates were optimal for treatments with 10 to 30 worms per unit, implying the need for 90 to 270 worms.

The worms lost about 13% of their original live weight in the one- and two-worm treatments of this experiment. In a preliminary experiment, also using one worm per bag, they gained about

20%. The weight gains and losses within each trial were fairly consistent, with standard deviations of about 7% and 16% of the means. However, a method is needed to allow correction for the weight of soil in the gut. We did preliminary investigations contacting worms with varying concentrations of phenolphthalein (a common purgative for mammals), ethanol and methanol, but were unable to accelerate depuration of soil *(see Expansion of Details, Part W3).*

Using the simulated data, we investigated the number of replicates required to be able to assign statistical significance to the simulated LC_{50} treatment *(see Expansion of Details, Part W4 for the computer programs used).* In simulations with an 88% control survival, LC_{50} was significant at the 0.O1 probability level when there were 5 replicates with 10 worms per unit, or when there were 27 replicates with 1 worm per unit (Figure 3.2). The former is efficient to minimize numbers of units, the latter to minimize numbers of worms. However, our experimental results indicate that control survival decreases when 10 worms are grouped together (Table 3.2), and when the observed control survival for 10 worms per unit was simulated, the number of replicates to obtain the 0.01 probability level rose to 29 (33 if analysed as parametric data). This is inefficient both for numbers of units and numbers of worms when compared to the one worm per unit case. We conclude that to assign significance to LC_{50} effects, the one worm per unit design is superior.

Survival tests in soil using *L. terrestris* have been reported in the literature, but many of the reports involve few replicates, and we found no reports that investigated the optimal experimental design. Optimal designs should maximize the statistical power of comparisons and minimize the numbers of worms and experimental units required, and the costs. Our first experiment involved experimental units with five worms each, and we observed that 5 to 10 replicates were required to give reasonable precision. It was also apparent that cascade deaths were a problem, so that most units had either all alive or all dead. An obvious solution to the problem of cascade deaths is to use units with one worm each. The method we developed was convenient and low-cost, it allowed us to weigh individuals before and after exposure to the soil, and it allowed survival scoring at various times without disturbance. To obtain good statistical precision, it required 30 to 50 replicates. In contrast, experiments with units that contain 5 to 10 worms needed somewhat fewer replicates, but markedly more worms. Through our experiments and using simulated data to compare statistical power, we conclude that the optimal design involves large numbers of replicates of units with one worm each.

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Table 3.1: Properties of the soils used in Experiment A, ordered by clay content, and showing mean (± standard deviation) bait worm survival (5 worms per container, n = 16) and fraction of units with all worms dead.

Soil [a]	Texture [b]	Clay (%)	pH [c]	Organic matter content (%)	Survival [d] (%)	Fraction of units with all dead (%)
A1	CL	33	6.3	3.1	88 ± 34	13
A2	L	24	7.4	2.2	98 ± 5	0
G	fine SL	18	7.1	18.4	50 ± 47 *	44
A3	fine SL	15	7.5	2.6	56 ± 51 *	44
A4	fine SL	13	6.4	5.7	100 ±7	0
AS	fine SL	12	7.8	4.2	82 ± 38	13
B1	LS	6	5.8	3.5	74 ± 44	25
A6	L fine S	4	6.2	0.8	68 ± 48	31
lB2	fine S	2	5.9	1.0	24 ± 43 *	75
uB2	fine S	2	5.1	0.7	26 ± 42 *	69
O	0	<1	5.4	41.5	34 ± 46 *	60

[a] The symbols refer to agricultural soils (A1-A6), humus enriched garden soil (G), boreal forest podzolic sands (B1, lB2-limed, and uB2-unlimed) and organic (O).

[b] Symbols denoting texture are C (clay), L (loam), O (organic) and S (sand).

[c] pH measurements shown were taken after the experiment.

[d] The overall F ratio from analysis of variance for the effect of soil was significant at $P \leq 0.0001$ when all 16 replicates were included, and means indicated by * were significantly below 100%.

Table 3.2: Worm survival at 14 and 30 d in Experiment 8, which evaluated the most effective means to increase the number of worms used in an assay.

Treatment		n	Soil per worm (cm^3)	Worms per unit	Type of container	Survival at 14 d		Survival at 30 d		Fraction o units with many dead [c] (%
						mean (%)	score [a]	mean (%)	score	
High	1.	100	80	1	bag	88	0.88[b]	88	0.88	12
ratio	2.	20	80	5	bag	90	0.80	87	0.80	10
	3.	10	80	10	bag+tray	75	0.70	75	0.70	20
	4.	2	80	50	bucket	---	---	75	0.50	---
Low	5.	100	40	2	bag	93	0.92	92	0.92	8
ratio	6.	20	33	12	bag	88	0.70 *[b]	85	0.70 *	15
	7.	10	27	30	bag+tray	68	0.60 *	59	0.50 *	40
	8.	1	20	200	bucket	---	---	10	---	---

[a] Means are arithmetic mean survival fractions, scores are the median scores used in non-parametric tests of significance where the scores were 1 for survival fractions above the treatment median, and 0 for fractions below the treatment median.

[b] Tests of significance contrasted the score of each treatment versus the score of treatment #1, with $P < 0.05$. The score for treatment 8 was meaningless because $n = 1$.

[c] The fraction of units with many worms (>80%) dead indicates the incidence of cascade deaths.

[d] The number of units and worms to obtain consistent results was determined by randomly selecting different-sized subsets of data from each treatment. Results were considered consistent when the survival in the subsets was no more than five percentage points different from the survival mean of all replicates.

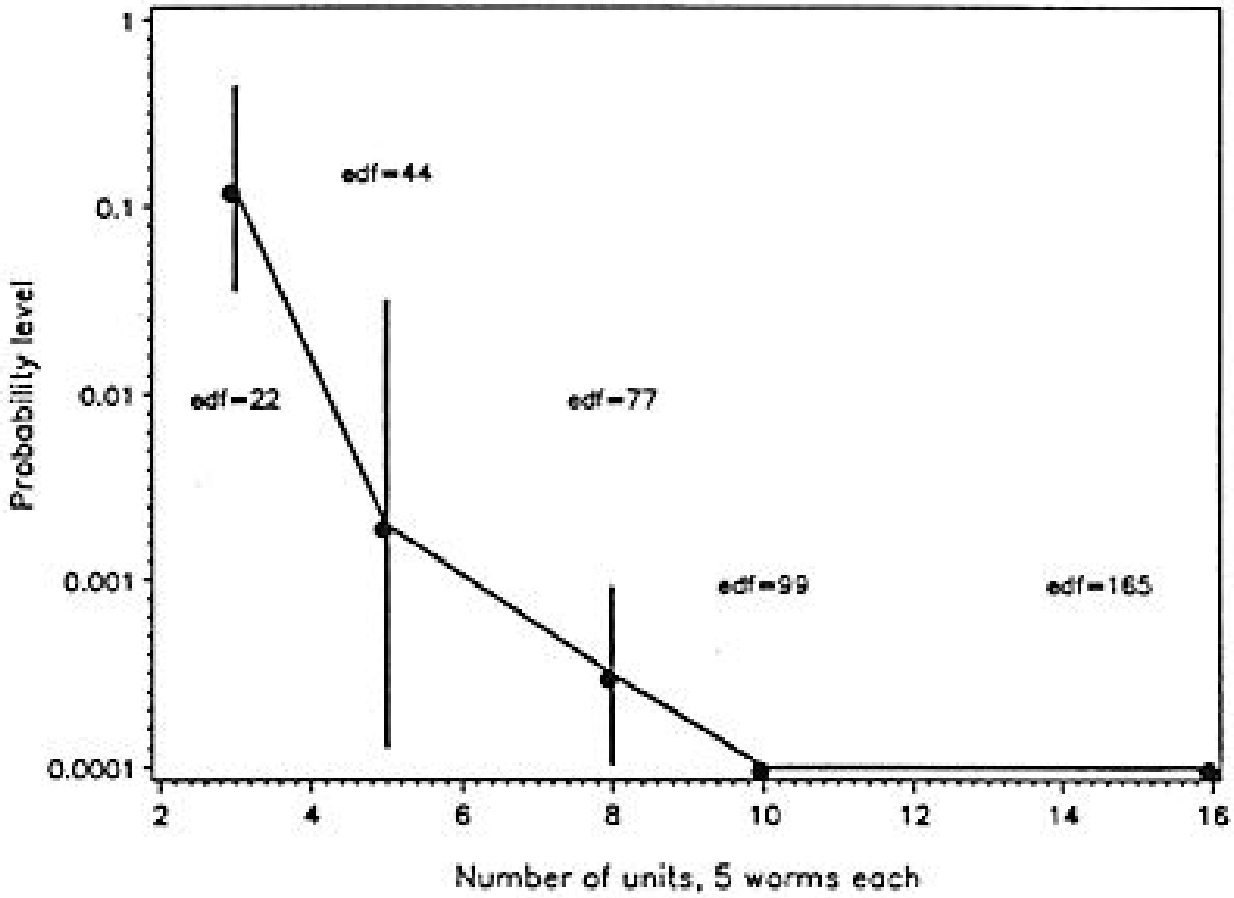

Figure 3.1: Probability (P) for an effect on earthworm survival of soil type, among 11 soils in Experiment A, as influenced by the number of replicates considered. Error bars are ± one standard deviation for six random selections of replicates from the full 16 replicates of the experiment. P was based on one-way ANOVAs, and the corresponding number of error degrees of freedom (edf) are indicated.

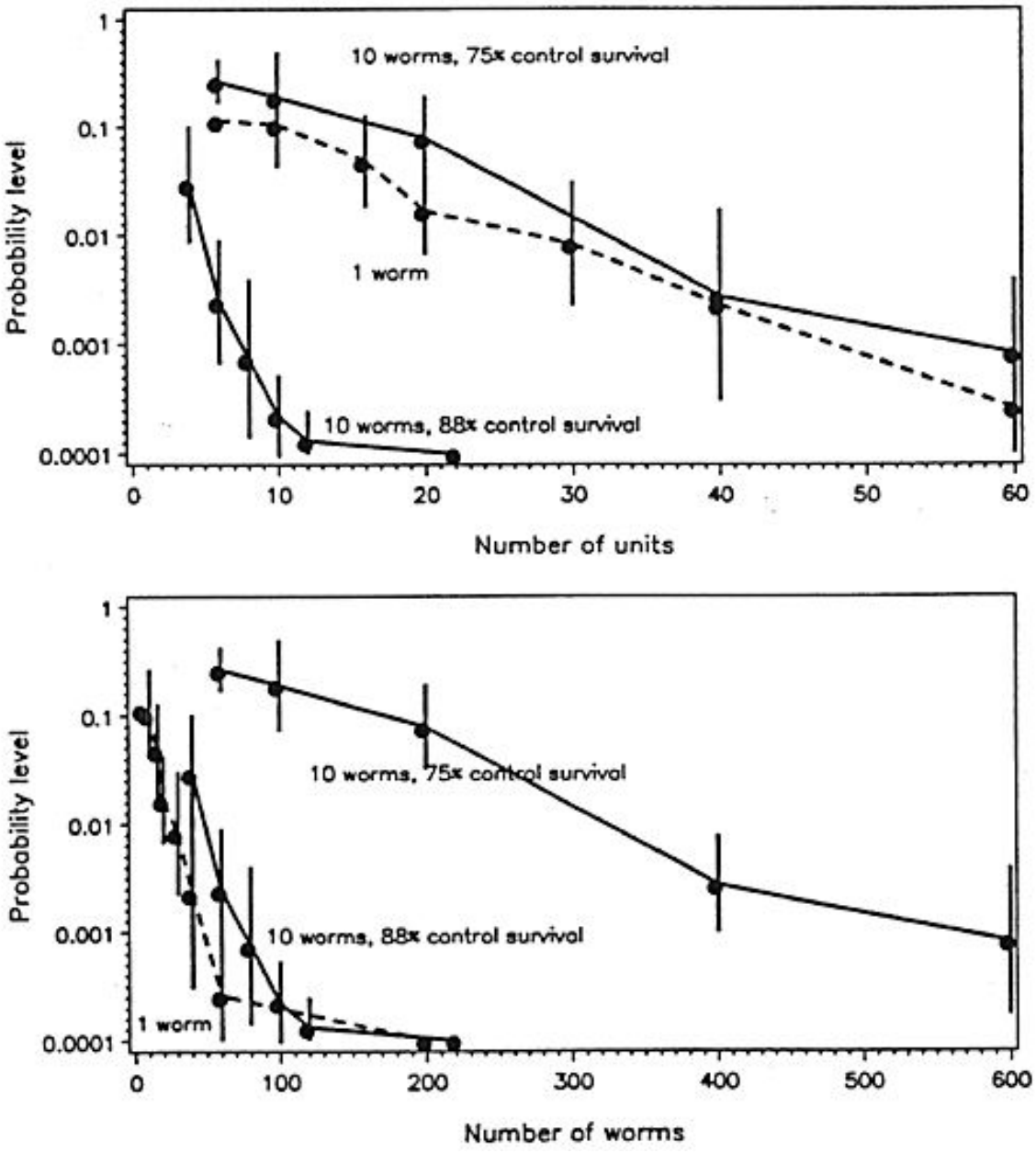

Figure 3.2: Probability (P) for a significant difference between controls and a treatment where half the earthworms died (equivalent to an LC_{50} treatment), plotted versus the number of experimental units (a) and versus the number of worms (b). Shown are the results for the one-worm units, which had 88% control survival, ten-worm units with an 88% control survival, and ten-worm units with a 75% control survival. Error bars are ± one standard deviation for five sets of simulated data, as described in the text.

3. Earthworm survival bioassay

3.2 Expansion of developmental details

Part W1 . Preliminary trial with containers

Sheppard *et al.* (1992 [11]) found the results of earthworm survival tests to be erratic, although sensitivity was similar to that found with plants. The sources of variation were too few animals per sample given the variation in the source population, and deaths from other causes. Sheppard *et al.* (1992) used animal numbers found in the literature, with 10 worms per 1-L pot and up to 4 replicate pots. The animals in the controls did not survive well, indicating possible disease problems. In general care, it is usual to remove dead worms found on the soil surface because they may harbour diseases and precipitate deaths not related to toxicity.

The overall objective was to improve upon the precision and sensitivity of earthworm tests based on bait worms, concentrating on means to increase animal numbers yet control disease problems. A preliminary experiment was conducted to optimize container shape, food supply and temperature.

The treatments were as follows:

1. 15°C, 500 mL food can, corn meal on surface
2. 20°C, 500 mL food can, corn meal on surface
3. 25°C, 500 mL food can, corn meal on surface
4. 30°C, 500 mL food can, corn meal on surface
5. 15°C, 500 mL food can, 5 x corn meal on surface
6. 15°C, 500 mL food can, corn meal mixed
7. 15°C, 500 mL food can, 5 x corn meal mixed
8. 15°C, oversized plastic bag, corn meal mixed
9. 15°C, 1 worm per 40 ml soil per smaller bag, corn meal mixed, 30 replicates

Ten bait worms were used with 400 mL of moist potting soil in each container, except treatment 19. The tests were done in triplicate, except treatment #9. The worms were checked visually throughout, and at 14 d they were dumped from the containers and the living were counted. The normal amount of corn meal is approximately 0.3 g. For treatment #9, the worms were washed and weighed as they are added to the soil and at the end of the 14 d.

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11. Sheppard, S.C., Evenden, W.G. and Anderson, A.J. 1992. Multiple assays of uranium toxicity in soil. Environ. Toxicol. Water Qual. 7:275-294.

By 7 d, all worms at 30°C were dead, some at 20°C were dead in each container, and all others seemed healthy. The corn meal was moulding, but even the surface application did not leave the soil surface heavily covered with fungi, as had caused problems before. The individually bagged worms appeared healthy, although there is a problem of the bags drying. Placing them between layers of soaked blotter paper seems appropriate, and in future experiments they were also covered with plastic.

The worms in treatment 19 were weighed, and by 14 d they had gained 20% of body fresh weight, but this could be the effect of soil in the gut.

This experiment gave confidence that the individual bagging of worms was expedient, and that 15°C was an appropriate temperature. Certainly temperatures above 25°C could be detrimental. Although feeding with corn meal did not cause heavy fungal growth that we previously thought had killed worms, we did not feed worms in further experiments on the philosophical basis that we wanted them to actively ingest soil and not survive on uncontaminated feed materials. Our experience is that healthy worms can survive at 15°C in soil without supplemental feeding for several months.

Part W2 ....Background of study with 11 soils

Experiment A involved part of the data from a larger study (Sheppard and Evenden 1992[12]). The larger study looked at the accumulation by plants and earthworms of uranium (U) from eleven contaminated soils. The data used in the present study were of soils that had no more than 50 mg U kg-1. Sheppard *et al.* (1992[13]) found that there was no toxic effect of U at concentrations below 300 mg U kg^{-1}. The soils used in the present study were from treatments that had received 0, 3, 10, 20, 30 and 50 mg U kg^{-1}. There were 4, 1, 2, 3, 3 and 3 replicates, respectively, leading to 16 pots in total. These 16 pots were considered replicates for the present study. Five worms were placed in each of the 16 pots per soil. The soils were moistened. Inadvertently, because of small variations in the amount of soil in each pot, some containers were drier and some wetter than intended.

The important results are discussed in the paper. There were some other observations of use. The variations in soil moisture content were instructive: certain containers that were wet enough to be puddled had full survival, similarly others that were quite dry also had full survival. The worms showed an impressive ability to survive ranges in soil moisture content.

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12. Sheppard, S.C. and Evenden, W.G. 1992. Bioavailability indices for uranium: Effect of concentration in eleven soils. Arch. Environ. Contam. Toxicol. 23:117-124.

13. Sheppard, S.C., Evenden, W.G. and Anderson, A.J. 1992. Multiple assays of uranium toxicity in soil. Environ. Toxicol. Water Qual. 7:275-294.

Part W3 ... *Trials with phenolphthalein*

Several small experiments were conducted to investigate ways to obtain weight change or growth data for earthworms. Survival may be a relatively insensitive measurement compared to reproduction or growth: a sick worm could live a long time. Bait worms live too long naturally to be able to wait for eggs during a test run; for this the manure worm is more useful. It may be possible to buy or culture eggs of bait worms, but a supplier was not found and culturing was considered inappropriate for the type of laboratory envisaged as the user of our protocols. Growth of worms could be measured if juveniles were available, but again this is complicated.

Loss of weight among mature worms can occur and may be a useful indicator of stress. Worm weight is ambiguous because of the presence of soil in the gut. Soil can be purged by starving (keeping worms in an empty container for up to 10 d), manual stripping, injection of water into the alimentary canal, dissection, or transfer to an organic soil (where the organic soil retained in the gut is a small fraction of the total dry weight).

We had the idea to use phenolphthalein (PHN), which is the most common active ingredient in human purgatives. We did a literature search to see if it has ever been applied to worms, and found nothing directly applicable. It is soluble in alcohol (6%), is used at 1% as a pH indicator, and its action as a purgative is to enhance bowel peristaltic action. In humans, its application is in the mg/kg range.

Our first experiment was to determine toxicity and efficacy of PHN on worms, in a system applicable to worm weight measurements. A 1% solution of PHN in methanol was prepared. Ten worms per unit, removed directly from the bulk storage soil, were washed and placed in tared, 100-mL food containers with 25 mL distilled water. The PHN was added to give treatments of 0, 50, 200, 500, 2000 and 5000 4g/container. Average worm fresh weights are 5 g, so that is a range of 1 to 100 mg PHN kg^{-1}. This trial was done in triplicate, and the containers were left at room temperature and observed at 30 min, and then at hourly intervals.

The worms had an immediate avoidance reaction, especially to the higher concentrations, but this lasted only minutes. There was no visible purge effect. They were left overnight and there was still no apparent effect. There were no mortalities related to PHN. The use of 10 worms per container made observation difficult.

The next step was to increase the concentration of PHN, and to facilitate this we determined the lethal concentration using individual worms and successive dilution to the no-effect level. Because the PHN is soluble in alcohol, one set of blanks included alcohol and another distilled water. The PHN and alcohol was added to moist blotter paper in the bottom of 12-cm-diameter petri dishes. Individual worms were placed in the dishes and effects observed immediately and after 24 hr.

Higher concentrations of alcohol in the blanks were immediately lethal, and at no concentration did the PHN treatments differ from the controls. On exposure to alcohol, the worms often purged immediately. We speculated that alcohol alone may give the desired effect. We used methanol originally, and repeated the trials with ethanol. This was even more toxic than methanol. We concluded that worms on moist paper purged as readily as those on treated paper. Further efforts in this direction were ended.

Part W4 ... Computer codes for simulations

The simulations discussed in the paper were done using SAS software. The programs are included here to illustrate the method, with actual program statements in bold.

```
data t_75;
*** This is a simulation of one-worm versus ten-worm experimental units.

*** The first step is to create the simulation data base (n=1O0), here based on 75% control survival and actual count frequencies for the ten-worm units. The RANUNI random number generator is used to give uniform distribution from 0 to 1.

do ij=1 to 100;
unit=ij;
pickit=ranuni(0);
       if pickit le 0.2 then nlive=0;
       if pickit le 0.3 and pickit gt O.2 then nlive=5;
       if pickit gt 0.3 then nlive=10;
trmt='control';output;

       if pickit le 0.45 then nlive=0;
       if pickit le 0.8 and pickit gt 0.45 then nlive=5;
       if pickit gt 0.8 then nlive=10;
 trmt='LC_50';output;end;
```

*** By using the rank procedure, median scores are assigned. This procedure groups the data into two classes, one above and the other below the median.

```
proc rank groups=2 out=new;var nlive;ranks med_nl ;
```

*** The GLM general linear model procedure is used for ANOVA. Here various subsets are chosen to give different numbers of replication. The entire program is run several times to repeat the analysis on different data sets.

*** ANOVA of nlive gives the parametric analysis, and ANOVA of med_nl gives the median score non-parametric analysis.

```
proc glm;classes trmt;model nlive med_nl =trmt;
data part;set new;if unit le 60;
proc glm;classes trmt;model nlive med_nl =trmt;
data part;set new;if unit le 50;
proc glm;classes trmt;model alive med_nl =trmt;
data part;set new;if unit le 40;
proc glm;classes trmt;model nlive med_nl =trmt;
data part;set new;if unit le 30;
proc glm;classes trmt;model nlive med_nl =trmt;
data part;set new;if unit le 20;
proc glm;classes trmt;model nlive med_nl =trmt;
data part;set new;if unit le 10;
proc glm;classes trmt;model nlive med_nl =trmt;
data part;set new;if unit le 5;
proc glm;classes trmt;model nlive med_nl =trmt;
data part;set new;if unit le 3;
proc glm;classes trmt;model nlive med_nl =trmt;
data part;set new;if unit le 2;
proc glm;classes trmt;model nlive med_nl =trmt;
ends as;
```

*** An alternate run is based on 88% control survival and the same data set can be used to represent either single or ten-worm units.

```
data t_88:
do ij=1 to 1000;
       pickit=ranuni(0);
       worm=1;
       if pickit le 0.12 then worm--0;trmt='controlt;output;
       if pickit le 0.56 then worm=0;trmt='LC_50';output;
end;
```

*** The GLM general linear model procedure is used for ANOVA. Here various subsets are chosen to give different numbers of replication. The entire program is run several times to repeat the analysis on different data sets.

```
data one;set t_88;if ij le 500;
proc glm;classes trmt;model worm=trmt;
data one;set t_88;if ij le 400;
proc glm;classes trmt;model worm=trmt;
data one;set t_88;if ij le 300;
proc glm;classes trmt;model worm=trmt;
data one;set t_88;if ij le 200;
proc glm;classes trmt;model worm=trmt;
data one;set t_88;if ij le 100;
proc glm;classes trmt;model worm=trmt;
data one;set t_88;if ij le 50;
proc glm;classes trmt;model worm=trmt;
data one;set t_88;if ij le 25;
proc glm;classes trmt;model worm=trmt;
 data one;set t_88;if ij le 10;
proc glm;classes trmt;model worm=trmt;
```

*** The same data set from t_88 can be used to simulate the ten-worm units when the control survival is assumed to be the same. Here groups of ten observations are pooled to give a survival value for a ten-worm unit.

```
data ten;set t_88;
unit=floor((ij-1)/10);
proc sort;by unit trmt;
proc means noprint;by unit trmt;var worm;output out=new sum=nlive;
```

*** The GLM general linear model procedure is used for ANOVA. Here various subsets are chosen to give different numbers of replication. The entire program is run several times to repeat the analysis on different data sets.

```
proc glm;classes trmt;model nlive=trmt;
data part;set new;if unit le 80;
proc glm;classes trmt;model nlive=trmt;
data part;set new;if unit le 60;
proc glm;classes trmt;model nlive=trmt;
data part;set new;if unit le 40;
proc glm;classes trmt;model nlive=trmt;
data part;set new;if unit le 30;
proc glm;classes trmt;model nlive=trmt;
data part;set new;if unit le 20;
proc glm;classes trmt;model nlive=trmt;
data part;set new;if unit le 10;
proc glm;classes trmt;model nlive=trmt;
data part;set new;if unit le 5;
proc glm;classes trmt;model nlive=trmt;
data part;set new;if unit le 3;
proc glm;classes trmt;model nlive=trmt;
ends as;
```

The detailed protocol is described in sections under the following headings.

- Source of bait worms
- Holding of bait worms
- Selection and handling of individual worms for use
- Basic bioassay plan
- Preparation and characterization of soil
- Preparation of treatments or dilution series
- Preparation of bags, including filling with soil
- Introduction of worms to contaminated soil
- Incubation conditions and midterm observation of worms
- Final count and observation of live worms
- Test acceptance criteria
- Disposal of worms
- Statistical summation of data

Source of bait worms

Bait worms are available from a number of bait suppliers in southern Ontario. Addresses can be obtained through the Toronto telephone yellow-page directory. Laboratory animal suppliers seem to obtain their worms from the bait suppliers, and therefore do not provide greater uniformity and are substantially more expensive. It is possible to purchase on a commercial scale in boxes of 1000 worms. Require rapid (courier) delivery to ensure safe transport.

There is a ready supply of worms from early spring to late fall, and there may be year-round supply. Worms obtained at times of year when field picking by the suppliers would be underway may be better, whereas worms obtained mid-winter or early spring would likely have been stored since the previous season.

The bait worms are almost exclusively *Lumbricus terrestris* (Oligochaeta: Lumbricidae). Although periodic expert taxonomic confirmation is appropriate, it is not a routine requirement of the bioassay.

Holding of bait worms

At present, the use of earthworms in the laboratory does not require detailed approval by an Animal Care committee. However, humane care is expected to the extent possible.

On arrival, immediately place the worms in refrigeration at 2-5°C. Within a few days, dump the worms and place them into moist commercial potting soil. Whenever handling the worms, wear surgical rubber gloves. This may reduce disease problems, but is largely for operator comfort: inadvertent handling of a dead worm is very distasteful. Place about 200 worms in each holding box. Any low-profile plastic container of about 5 L will be adequate. The top must seal well: worms are very adept at penetrating small spaces. Worms may be fed small amounts of corn meal; large amounts that cause visible moulding may lead to worm deaths. Feeding is not necessary unless storage is long. The holding boxes are kept refrigerated at 2-5°C. A continuous low light source in the cooling chamber, for example the appliance light in a refrigerator, is kept illuminated. This markedly reduces worm escapes.

Worms can be kept in this manner for at least several months. Changes of soil may be beneficial for longer storage. The condition of the worms should be checked about weekly. Mould on the surface is extremely undesirable, and may result in substantial mortality. The presence of dead or unhealthy worms gives the cooling chamber a distinct and unpleasant odour, and this is the primary and most useful sign of problems. Cascade deaths are typical, so that once a few worms have died, there is a high probability that the others in the same container will also succumb. Early intervention by moving healthy worms to a new container with new soil can help.

Selection and handling of individual worms for use

The protocol calls for 30-50 worms per treatment or dilution, so that several hundred worms need to be available. Some selection based on visual appearance is appropriate. Dump a 200-worm holding box on a large tray and select individuals by hand. The operators wear surgical rubber gloves. Select for large worms with visible clitella (the band around the worm about one third the length from an end). Worms that appear to have a lot of adhering soil may be unhealthy and should be avoided. Although typically quite uniform in colour, uniform colour is an implicit criteria during selection.

When about half the worms have been removed from the tray, excess soil is discarded and another 200-worm box from the same original shipment is added to the tray. To pool all the worms prior to selection of individuals is impractical in large studies where each study may involve about 1000 worms. This method of partial pooling is a compromise, and in smaller studies could be avoided by pooling all the worms prior to selection.

Basic bioassay plan

Bioassays will likely be applied in either of two modes. One is the experimental investigation of contaminants, where a soil of specific properties is chosen and is artificially contaminated to meet the requirements of an experimental plan. The second mode is the application to a contaminated soil collected in the field.

The use of artificially contaminated soil is the most appropriate mode for a whole-soil bioassay because an uncontaminated control soil is extremely important in interpretation of results. For example, an uncontaminated acid soil may allow less than 50% survival, and the effects of contaminants are superimposed on this. The results would be most misleading if the survival in the control soil was unknown. In practice, an uncontaminated soil with specified properties is collected. Control and treatment aliquots are handled uniformly, and the treatment aliquots are contaminated with a range of at least five concentrations typically spanning at least an order of magnitude. The specific concentrations and ranges are determined by reports in the literature. Use a geometric scaling of the concentrations and consider placing more levels near the expected-effect concentration. At the same time, a treatment at a concentration well above the expected-effect level is highly recommended so that trends and symptoms observed at the lower concentrations can be clearly related to the effects at the extreme concentration. Great care can be taken to counterbalance companion ion and solvent additions so that the only significant difference among the aliquots is the presence of the contaminant. Use a replicated design, with extra replicates assigned to the control.

In the second mode, the extent and identity of the contaminant(s) may or may not be known. It is very important to seek a control soil to correspond to the contaminated soil. The field agent must estimate the extent of the contamination and try to collect a soil that appears to have properties comparable to the contaminated soil yet be outside the influence of the contamination. It would be desirable to thoroughly characterise both soils to ensure similarity in non-contaminant properties and dissimilarity in contamination. When an unambiguous control soil cannot be found in the field, it is necessary to identify one in the laboratory. For this, the laboratory should keep a supply of several soils (that can be renewed with further collections) that represent a range of key properties such as pH, texture and organic matter content. In the Expansion of Details, Part P2, efforts to create a control soil by matching a blend of laboratory reference soils to a contaminated field soil are described. This is a difficult task to accomplish.

For both modes of operation, include a series of concentrations of a reference contaminant in a similar soil. Zinc is useful as the reference contaminant, applied at concentrations of 50, 100, 300, 600 and 1000 mg Zn kg^{-1} with solutions of $ZnSO_4.7H_2O$. It is acceptable to use only 20 replicates of these soils. The soil should be a laboratory reference soil or an additional aliquot of the control soil.

Preparation and characterization of soil

About 20 L of soil is required, varying somewhat with the mode of the bioassay and the amount of processing needed to render a homogenous, finely-divided soil for use. Storage and initial preparation of the soil for the worm bioassay can likely follow protocols established for other bioassays. Spread the soil on a large flat, plastic-covered surface where lumps can be broken and the sample homogenized, and where drying to a workable moisture content is allowed. This operation would have to be modified for volatile contaminants. A workable moisture content is

one where the soil will no longer adhere in clumps when handled. It is undesirable to fully dry the soil, as this affects both contaminant behaviour and soil microbial condition. The soil is then passed through a 4- to 10-cm mesh. Material retained on the mesh is normally discarded. The operator must decide what amount of force to use to grind the soil through the mesh, bearing in mind that the goal is a chemically and physically homogenous sample. The processed soil is then stored in sealed pails. Each time a sample is removed for analysis, an additional subsample is taken to measure the moisture content. Soil moisture content is defined as the weight loss on drying at 105°C for 24 h. All soil concentrations must be expressed on a soil dry weight basis.

It is likely that the bioassay of a soil will be accompanied by chemical and physical characterization of the soil to meet other needs. However, to facilitate interpretation of the worm bioassay results, quantitative soil pH and at least qualitative texture and organic matter content are required. In addition, the worm bioassay requires a measure of the soil moisture holding capacity. This is not interpreted as a basic property of the soil - it is an application-specific measurement. Measure by weight the amount of water retained after 24 h draining of a thoroughly wetted aliquot of the soil held in the same physical container as used in the application. In this case, the container is the individual worm bag. This measurement of moisture holding capacity will give higher values than field moisture retention or third-bar tension measurements. It may also be above the optimal moisture content for worms in a given soil because of the effect of water on oxygen diffusion. Wet the soils in the bioassay to 90% of the measured moisture holding capacity.

Preparation of treatments or dilution series

The principles applied here are outlined in the Basic Bioassay Plan section. For artificial contamination, 50 replicates of the control soil and 30 replicates of each contaminant concentration are recommended. If more than about seven concentrations are used, then the numbers of replicates could be reduced. Each worm bag will hold about 40 mL of soil, so that 1.5 to 2.5 L of soil for each treatment should be prepared. Care should be taken to counterbalance companion ion and solvent additions across the treatments. This requires judgement and compromise, approaches are described in the Overall Report sections here, in the Detail section 2.2, and elsewhere [14]. After addition of the contaminant, it is desirable to moisten the soil to near the moisture holding capacity and incubate it for some time. The length of time is dependent on the volatility of the contaminant and the expected reaction kinetics in the soil. Elevated temperature to 30°C may be appropriate to speed reactions for non-volatile

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14. Sheppard, S.C., Evenden, W.G. and Anderson, A.J. 1992. Multiple assays of uranium toxicity in soil. Environ. Toxicol. Water Qual. 7:275-294.
Sheppard, S.C., Evenden, W.G. and Pollock, R.W. 1989. Uptake of natural radionuclides by field and garden crops. Can. J. Soil Sci. 69:751-767.
Sheppard, S.C. and Evenden, W.G. 1988. The assumption of linearity in soil and plant concentration ratios: An experimental evaluation. J. Environ. Radioactivity 7:221-247.

contaminants. The incubation should last at least 7 to 14 d to pass the burst in soil respiration that follows rewetting of a dry soil.

For field contaminated soil, the objective is to provide a dilution series of the contaminated soil diluted with a control soil. As described, the choice of the control soil is very important. In the absence of a field control soil, then a laboratory reference soil or blend can be used. A broad dilution series is ideal, but unlike water samples, dilution of whole soils is limited by the physical scale of the soil particles. It is inappropriate to dilute the contaminated soil to the extent that the probability of a worm encountering contaminated particles is affected. Dilution ratios, control:contaminated, of 1:0, 16:1, 8:1, 4:1, 2:1, 1:1, 1:2 and 0:1 are usually appropriate. Mixing should be very thorough, and it is appropriate to moisten the mixtures and allow them to incubate as described above.

Preparation of bags, including filling with soil

Polyethylene bags that hold 40 to 100 mL, with attached metal closure wires, are most expedient. The bags are labelled with indelible markers before filling. Use a numbering system such as #-# where the first # is the treatment number and the second #, the replicate number. The bags are perforated by placing them in stack of ten and piercing them ten times with a needle.

The soils are placed in the bags using a volume measure, so that each contains 40 cm^3. If required, water is added to each bag to bring the moisture content to 90% of the moisture holding capacity. All bags are prepared before any worms are added.

Introduction of worms to contaminated soil

The worms are selected as described above, always selecting successive worms from different areas on the tray. The bags should not be filled in any regular order, especially if it is not possible to pool all the worms initially. At least partially random ordering is required, where the ordering of the treatments is fully random and unknown to the operators. For this, fill half the replicates of each treatment first, re-randomize the treatment ordering, and then fill the second half of the replicates.

Worms frequently have an immediate reaction on contact with a contaminated or otherwise unfavourable soil. This does not necessarily indicate potential for survival, but is worthy of noting.

Considerable care is required to seal the bags to prevent escapes. With the small wire-top bags, it may only be possible to roll the top twice before bending the closure wires, and then it may be necessary to also staple the top closed with a normal desk-top paper stapler. A larger bag will allow more effective closure with the wires.

Although not part of the routine protocol, it is possible to wash and weigh worms as they are put in and when they are removed from the bags. Dipping or spraying with distilled water removes exterior soil. Worms can both gain and lose weight with time, and this data could support survival numbers in a detailed study. This ability to track the weight of individuals is a feature of the single worm packaging.

Incubation conditions and midterm observation of worms

The bags can be stacked on their edge in trays; 25x50 cm plastic horticultural trays are good. Moisture loss must be retarded and do this by covering the trays with a layer of damp paper towelling and placing each tray in a large plastic bag. The bag is not sealed, but the open end is folded under. Incubation is in a darkened, constant temperature chamber at 15 ± 1°C. A plant growth chamber suitable. Set the air exchange ports fully open.

Although the worms are individually bagged to reduce the possibility of cascade deaths, continue to inspect the worms at intervals and remove dead worms if they are numerous. Midterm survival counts may not improve upon the sensitivity of the bioassay. Use midterm counts for soils where control survival is likely to be poor, and especially where significant numbers of worms have died in a given bioassay. To do midterm counts, packages that very obviously contain dead worms are removed, counted and discarded. Generally, dead worms are partially decomposed, their presence is signalled by smell and visible deterioration of flesh. The tip of a dissection needle can be inserted through the bag wall to prompt the worm into movement. The bags can be discarded intact without opening.

Final count and observation of live worms

After incubation, ranging from 30 to 75 d, final counts are done. There seems to be no detriment to the longer times except the time delay in obtaining assay results. Counts at 14 d generally indicate the same sensitivity to contaminants as counts at 75 d. The recommendation is for 30 d incubations.

A live worm is defined as one that responds to tactile stimulus. Normally, touching the outside of the bag will prompt movement. A dissecting needle can be used, as described above, to touch the skin and prompt movement. In some cases, stressed worms may be darker in colour, contracted and narrow, and especially sluggish. These symptoms are worthy of note. Dead worms are often partially decomposed. On occasion, the worm may appear to be absent. Assuming no escapes occurred, the former presence of the worm can often be confirmed by a fungal mass associated with the site where the worm died. Smell is a last resort indicator.

Test acceptance criteria

The bioassay result may be judged as acceptable based on both survival in the control soil and

a monotonic response to the concentration series. Because the assay uses whole soils, and the control soils may not be ideal for survival of worms, survival in controls is considered a less demanding acceptance criteria than is typical of many, for example, aquatic bioassays. Survival in the controls must be sufficient that the counts have statistical meaning. Data with control survival as low as 25% may in some situations be acceptable. In this case, about 13 bags would have live worms after 30 d. Although this value is low and indicates the worms are under stress in this soil, if the response to the toxicant is monotonic the results may be useful. There is some argument in favour of bioassays with stressed organisms, as this may enhance sensitivity.

The monotonic response to the toxicant is considered a more important criteria. Tests that show multi-modal response curves should be repeated with care to address possible sources of variation.

Disposal of worms

Although it may be possible to recover worms from a bioassay for further trials, this may result in greater variation and is not recommended. Do not open the bags and dispose of the worms in the soil in a manner appropriate for the contamination present.

Do not store worms for use longer than six months. The preferred disposal is to release them to an outdoor lawn, or perhaps to anglers. If midwinter disposal is necessary, rapid freezing for euthanasia is recommended.

Statistical summation of data

Statistical summation can follow several approaches. A replicated design allows either regression or analyses of variance (ANOVA), or a combination. The survival scores are essentially binary data: code a 0 for dead and a 1 for live. Analyses of binary data can be done by normal ANOVA computer programs, and are analogous to certain types of non-parametric ANOVA. The SAS programs [15] are ideal, they are broadly accepted and very flexible.

The first step is a plot of the survival means, with error bars, versus the contaminant concentration or dilution ratio. Log-scaled treatments should be plotted on log scale. The second step is an overall ANOVA, where the F ratio for the effect of the contaminant is calculated. If the F ratio is not significant at $P<0.05$, then further interpretation must be considered with caution. There is some controversy about the next step. Individual statistical tests, equivalent in results to t tests, on each treatment versus the control can be used. These result from the PDIFF option of the LSMEANS statement in the GLM procedure of SAS. If you can assume no possibility of enhanced survival as a result of the contaminant, a one-tailed test is appropriate. In the sense of a bioassay to detect negative environmental impact, a one-tailed test is also conservative.

15. SAS (1985) SAS User's Guide, Statistics, Version 5 Edition, SAS Institute Inc., Cary, NC.

The multiple comparisons to the control are not an orthogonal decomposition of the overall treatment sum of squares. They are *a priori* and are conservative in the sense of a bioassay.

The statistical indications require interpretation supported by the survival plot. The no-observable-effect-level (NOEL) is associated with the highest-concentration position on the curve where there is no continuous trend toward poorer survival than the control. This point will never be significantly different from the control. The lowest-observable-effect-level (LOEL) is associated with the lowest concentration that had survival significantly different from the control. The LC_{10} and LC_{50} can be interpolated visually from the graph, but only when within the range of the data. Probit analyses can be used, although differences between endpoints derived analytically by probit analysis are not often practically different from those obtained by visual interpolation.

4. Microarthropod bioassay

4.1 Overall report

Simple Whole-Soil Bioassay Based on Microarthropods

Stephen C. Sheppard and William G. Evenden

Bulletin of Environmental Contamination and Toxicology
(in press)
(see Expansion of Details, Part M1)

Development of bioassays for whole soils remains a challenge. It is complicated by the heterogeneity of the solid, liquid, and gas phases of the soil matrix and the complexity of the many interactions of organisms with soils. Many bioassays for soils avoid some of these difficulties by using soil extracts. We sought to develop bioassays for whole soils, with emphasis on simplicity coupled with reliability (Sheppard and Evenden 1992; Sheppard *et al.* 1993).

Soil invertebrates are good subjects for whole-soil bioassays (Koehler 1992; Paoletti *et al.* 1991; Tomlin 1975). They are exposed to soil contaminants by contact, by direct ingestion of soil solids and soil water, and through food-chain transfers. They have short life-cycles and are sufficiently mobile that they can be extracted with simple, heat gradient devices (Crossley and Blair 1991). Their use in bioassays is somewhat limited by our lack of full understanding of their ecological relationships (van Straalen and Denneman 1989). Earthworms are perhaps the simplest to study, and earthworm bioassays typically involve survival counts and may include some physiological measurements. Relatively large numbers of earthworms are needed to obtain acceptable precision in survival counts (Sheppard and Evenden 1992). This is a statistical issue; to get meaningful counts of surviving earthworms, a large number of animals must be tested. Smaller invertebrates, such as microarthropods, occur in very large numbers in soil, and for this reason we directed our interests towards them.

Microarthropods have been used for many years in studies of the side effects of pesticides in soils (Koehler 1992; Tomlin 1975), and several studies have used them for other contaminants. Pirhonen and Huhta (1984) found microarthropods to be more sensitive to two types of oil in soil than either Enchytraeidae or Nematoda. The data of Neuhauser *et al.* (1989) seem to indicate that microarthropods were less variable indicators than earthworms for the impacts of oily waste in field plots. Bengtsson *et al.* (1985) and Posthuma *et al.* (1992) report on detailed investigations of the responses of microarthropods to metal-polluted soil. Denneman and van Straalen (1991) specifically developed a bioassay for metals using soil-dwelling mites, but their protocol did not include soil.

The objective was to develop a simple bioassay based on counts of microarthropods. Following the multispecies approach of Sugiura (1992) and the general experimental concept of Huhta *et al.* (1991), we developed a microcosm method and report here the protocol and the initial indications of sensitivity using mercury (Hg) and iodine (I).

Materials and Methods

Two pieces of apparatus, constructed from simple laboratory supplies, are required (Figure 4.1). The first, which we refer to as a mesocosm, is a plastic-lined wooden box with inside dimensions of 0.6 x 1.2 m and 20-cm deep. The bottom 2 cm is filled with gravel, nominally 2 to 10 mm diameter, to provide drainage. The next 12 cm is a mixture of fresh forest litter and soil. We have used a mixture of 46% (by volume) commercial sterilized potting soil, 46% litter horizon from a mixed boreal aspen and birch stand, and 8% of a sandy calcareous agricultural soil. The litter provides the initial arthropod population and substrate. The top 2 cm of the mesocosm is a layer of potting soil in which are planted seeds of a shade-tolerant lawn grass blend.

During assembly, 24 empty plastic sleeves are placed vertically in the soil. These sleeves are 19-cm-long, open-ended boxes of Plexiglas with cross-section inner dimensions of 3 x 15 cm. The upper end of the sleeves are positioned just above the soil surface and are covered with an opaque, removable lid. The lower ends are open to the soil. The sleeves are inclined about 30° from vertical, and the downward facing side of the sleeves are perforated with six, 3-cm-diameter holes. These holes are covered with 2-mm-mesh nylon screens.

We positioned the mesocosms on a laboratory bench to receive indirect sunlight and continuous supplemental light from fluorescent tubes. Tap water was applied to the surface when needed. Water accumulation in the gravel could be viewed through a 5-cm acrylic vertical access tube in one corner of the mesocosm, and excess water was avoided. The mesocosm was established, and the grass was cultivated for at least one month prior to the first bioassay so that the microarthropod populations could stabilize. We have done bioassays in individual mesocosms for up to a year with consistent results.

The second piece of apparatus is a plastic petri dish, 15-cm diameter and 2.5-cm deep, to hold the contaminated soil. The lid is modified by cutting out the centre to leave only a 1-cm-wide rim, and 2-mm-mesh nylon screen is glued onto the rim. With cleaning, the lids may be reused.

The soil was prepared by partial drying and grinding to pass a 4-mm mesh. Few viable microarthropods survive this treatment, but the soil can be heated to 30°C for 8 hr to eliminate indigenous meso fauna. The soil was amended with ground alfalfa at 10% by weight. We used ground, unmedicated laboratory rodent feed. The soil was then placed in the petri dish and moistened to 80% of its predetermined water-holding capacity. The petri dishes were fit, with the mesh lid downward, into the sleeves in the mesocosm.

Within 2-3 d, a considerable amount of fungal growth was visible in the petri dishes. After 11 d, the petri dishes were removed from the mesocosm and immediately placed, mesh lid downwards, in large funnels. These were supported in a plant growth chamber where temperature was controlled at 22 ± 1°C and where the lights could be lowered to 2 cm above the clear plastic bottom of the petri dishes. Under the spout of each funnel was a 50-mL beaker containing 10 mL of denatured ethanol. Parafilm was used to partially cover the beaker, but there was some ventilation so that ethanol fumes did not kill the microarthropods before they exited the soil. This apparatus operated as a Tullgren extractor and was a derivative of that described by Crossley and Blair (1991). The capture of escaping microarthropods in the ethanol was essentially complete within 24 h, and after 96 h the ethanol traps are collected and soils discarded. With care in handling the petri dishes, the traps were virtually free of detritus.

The numbers of microarthropods were counted with up to 40-fold magnification, and counts of springtails (Collembola), mites (Acari), and remaining meso fauna were recorded separately. More detailed taxonomic differentiation would be possible, especially with computer-aided keys (Moldenke *et al.* 1991), but the costs would not likely be justified by improved bioassay sensitivity. The traps generally contained many more arthropods than could be economically counted. We evaporated some of the ethanol from the traps and adjusted the volume to 10 mL, then stirred and removed an aliquot by pipette for counting. We selected the volume of the aliquot so that actual counts from control soils were 20 to 100 microarthropods. Although this procedure may have under-sampled large meso fauna, it was accurate for the 0.1 to 2 mm organisms important to this protocol. The ethanol traps may be stored, with additions of glycerol, for archival purposes.

In order to compare the sensitivity of the proposed bioassay with other methods, the soils contaminated with Hg from the experiment described by Sheppard *et al.* (1993) were used. The concentrations of Hg were initially 0, 10, 22, 46, 100, 220, 460, and 1000 mg Hg kg^{-1} soil, applied as $HgCl_2$. An acidic sand, a humus-rich garden soil and a clay were used. Aliquots of the same three soils were treated with I at concentrations of 0, 10, 22, 46, 100, 220, 460, and 1000 mg I kg^{-1} soil, applied as KI. All contaminant treatments and controls were done in triplicate within the same mesocosm. The soils were used in the bioassay shortly after they were treated, and the Hg and I concentrations were confirmed at that time by analyses. There was some loss of Hg presumably by volatilization. There was no detectable methyl mercury in the Hg-treated soils. Sheppard *et al.* (1993) indicated the relative sensitivity of other bioassays to the Hg treatments. They used earthworm survival, lettuce seed emergence and timing of bloom initiation in *Brassica rapa* as whole-soil bioassays. We present here results of these bioassays for the I-treated soils using the same methods.

Results and Discussion

The counts of microarthropods in this system are a holistic measure of the ecology of the treated soil, encompassing effects on fungal proliferation on the alfalfa substrate, invasion of fungivore and other microarthropods from the mesocosm, and then survival of those microarthropods in the contaminated soil. There may be effects at several trophic levels. Longer exposures would be needed to ensure inclusion of effects on reproductive success.

The microarthropod counts spanned several orders of magnitude (Figure 4.2), and consequently were log transformed for interpretation. Although variation was considerable, the trends were approximately monotonic, and treatments that had counts less than 50% of the controls were statistically different from the controls ($P < 0.05$ by single-degree-of-freedom contrasts, using the pooled estimate of error from analyses of variance). Whole-soil bioassays are somewhat more variable than bioassays on aqueous samples or aqueous soil extracts, and these results are considered useful. For Hg concentrations above 220 mg kg^{-1} in the sand soil, there were no microarthropods found in the samples.

The EC_{50} (the soil contaminant concentration that reduces test performance to 50% of the control) is indicated on the graphs and compared to EC_{50} for the other bioassays (Figure 4.2). The relative sensitivity of the four bioassays varied among the three soils and two contaminants, but clearly the microarthropod counts were as sensitive, and in some cases more sensitive, than the other bioassays. The costs of operation of the microarthropod bioassay is about one fifth that of the *B. rapa* bioassay, and about equal to that of the earthworm survival and lettuce seed emergence bioassays. We conclude that the simplicity and sensitivity of microarthropod bioassay is good and that this method deserves further development.

Further work is needed to examine the effectiveness of mesocosms supplied with litter and colonizing species from other habitats. The usable life time of the mesocosm is yet to be defined, as well as the importance of residual effects from one bioassay to the next in the same mesocosm. Smaller soil containers may prove to be effective, and the relationship between the size of individual soil samples and the number of replicate soil samples needs to be defined. Greater replication may markedly reduce the variation and improve precision. The method has not been tested for organic contaminants, and for this application the plastic components may have to be replaced.

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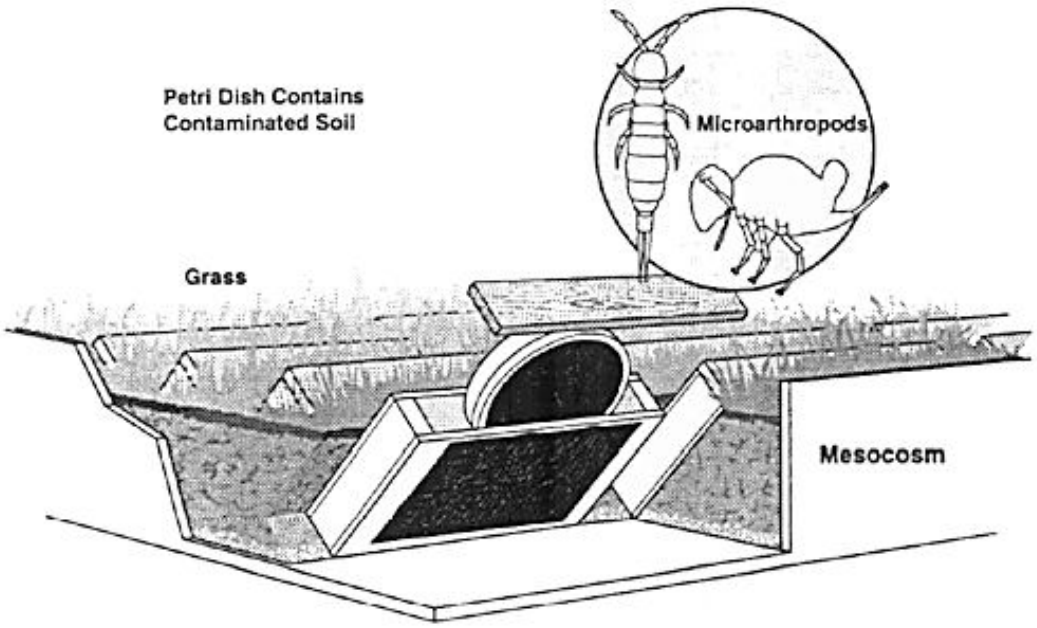

Figure 4.1: Schematic of the apparatus, showing the sleeves buried in the litter-and-soil-filled mesocosm, the modified petri dishes that contain the contaminated soil baited with ground alfalfa, and illustrations of two common springtail microarthropods that invade the contaminated soil.

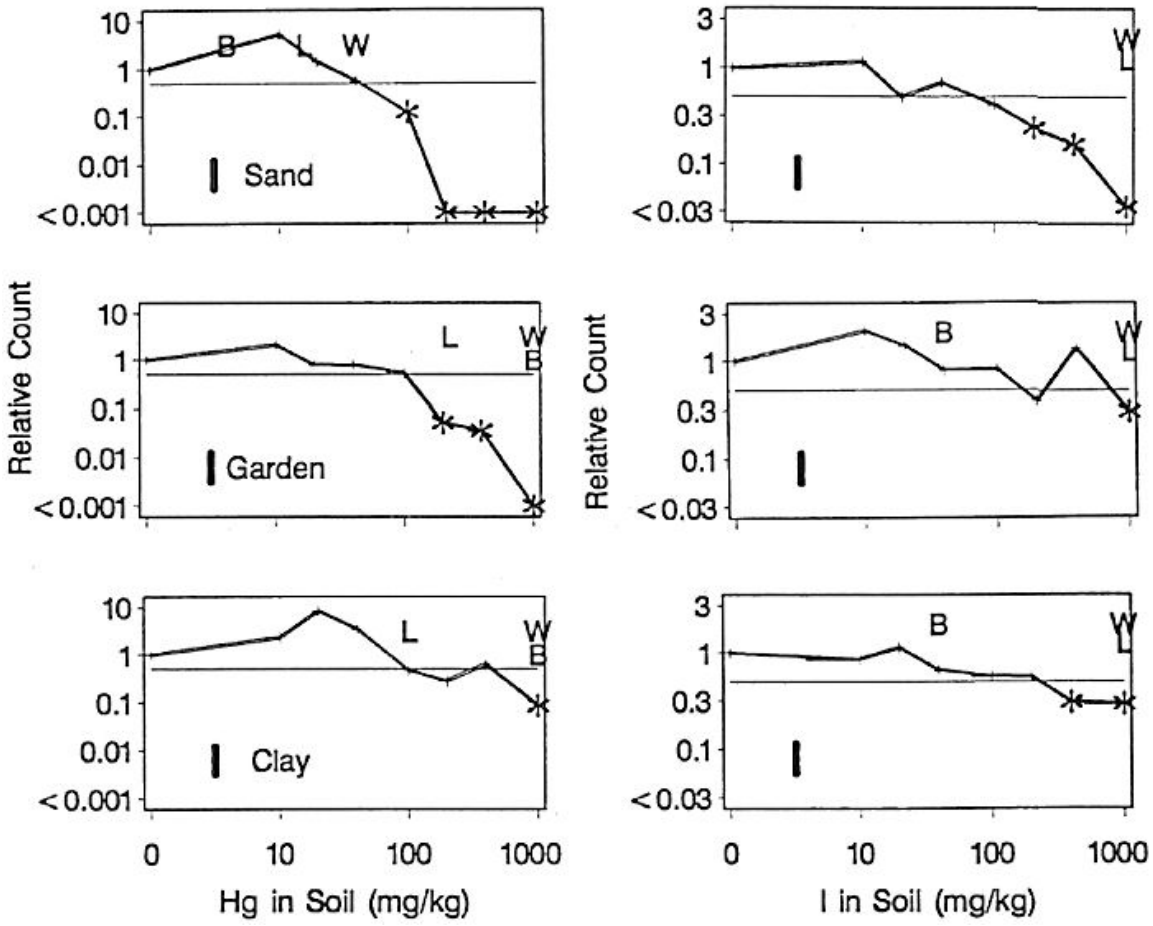

Figure 4.2: Counts of microarthropods, relative to controls, in the acidic sand (a), the garden (b) and the clay (c) soils contaminated with Hg and I. The horizontal line is 50% of the control (EC_{50} for microarthropods) and the short bold vertical line is the pooled (geometric) standard deviation. Points indicated by * were significantly lower than the control by one-tailed test ($P<0.05$). The EC_{50} for earthworm survival, lettuce emergence, and the most sensitive of either bloom or emergence counts in *B. rapa* are indicated by the position of V, L and B, respectively, along the concentration axes.

Part M1 ...Stage of development

The microarthropod bioassay as outlined is novel. There are methods in the literature that are analogous to parts of this bioassay. The idea of placing treatment samples in containers into a specific environment is analogous to litter bag decomposition studies[16] . The use of mesocosms is common [17] . Investigations of microarthropods in systems where treatment soil microcosms are placed in larger mesocosms have been reported[18] . The concept of multispecies bioassays has also been used[19]. These studies supported the concept for the method we developed.

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16. e.g., Reader R.J. and Stewart, J.M. (1972) The relationship between net primary production and accumulation for a peatland in southeastern Manitoba. Ecology 53:1024-1037.

17. e.g., Sheppard, S.C. and Evenden, W.G. 1990. Leaching of radionuclides from decaying blueberry leaves: Relative rate independent of concentration. J. Environ. Qual. 19:464-469. Taylor, B.R. and Parkinson, D. 1988. A new microcosm approach to litter decomposition studies. Can. J. Bot. 66:1933-1939.

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Frehe, C. 1984. Experimental enclosure for the testing of the effect of environmental chemicals on living communities. Verhandlungen-Gesellschaft fuer Oekologie 12:507-510.

Because there was no direct precedent for the method, the development was speculative. We designed the method based on concepts drawn from the literature and from correspondence, and proceeded to test its sensitivity relative to the other bioassays. Detailed development was not done. The results are favourable and we conclude that further development is warranted. Issues that remain include the following:

- The effectiveness of mesocosms supplied with litter and colonizing species from other habitats needs to be assessed to determine how reproducible toxicity results will be from mesocosm to mesocosm. We anticipate that some constraints may need to be placed on the sources of litter, and perhaps it will be necessary that certain soil types be tested in mesocosms containing appropriate meso fauna. For example, an acidic mesocosm may be needed to assess toxicity in an acidic soil.

- The usable life time of the mesocosm is yet to be defined, as well as the importance of residual effects from one bioassay to the next in the same mesocosm. With a growing grass cover, we anticipate that a stable meso fauna population will develop that may allow individual mesocosms to be used for several years. With care to avoid contamination of the mesocosm sleeves, we expect minimal residual or carryover effects because the meso fauna are quite mobile and opportunistic.

- Optimization of details such as the amount and type of bait (we used ground alfalfa), the exposure time in the mesocosm, the extraction time in the Tullgren system, the size of the petri dishes, and the numbers of replicates needs to be done. The bait we used is effective and sufficient, but lesser amounts may be desirable where the decomposition of the bait may affect contaminant bioavailability. (It may be useful to monitor pH to assess the effect of bait on soil chemistry.) The exposure and extraction times we used may be longer than needed, but longer-than-optimal times likely do not introduce variation. We expect that petri dishes an order of magnitude smaller in volume than ours may be useful, and this might allow more sleeves per mesocosm and greater replication.

- Criteria for an acceptable test need to developed. At present, we require mean counts in control soils greater than about 100, and a clear effect of increased contamination.

4. Microarthropod bioassay

4.3 Detailed protocol

The detailed protocol, at its present stage of development, is described in sections under the following headings.

- Source of litter
- Construction, assembly and maintenance of mesocosm
- Modification of petri dishes
- Construction of Tullgren extractor
- Basic bioassay plan
- Preparation and characterization of soil
- Preparation of treatments or dilution series
- Filling petri dishes and loading mesocosm
- Incubation conditions, midterm observations
- End of bioassay and extraction of microarthropods
- Counting of microarthropods
- Test acceptance criteria
- Disposal of materials
- Statistical summation of data

Source of litter

Litter is obtained from an uncontaminated forest site. Collected with the litter are the F and B horizons of the soil. Sites that have F and H horizons with visibly mycellial development are ideal. About 0.25 m^3 should be collected. It can be stored in a sealed plastic bag out of direct sunlight for several days before it is used.

Construction, assembly and maintenance of mesocosm

The mesocosm is shown in Figure 4.1 of the Overall Report section 4.1. The mesocosm is constructed of ¾ inch plywood lined with Plexiglas at least ⅛ inch thick. The inside dimensions of the mesocosm are 0.6 x 1.2 m and 20 cm deep. A 5-cm-diameter, 20-cm-long acrylic access tube is glued into one corner with silicon sealant. The mesocosm will be quite heavy and should either be filled where it will be used or provision made for transpiration. The bottom 2 cm of the mesocosm is filled with gravel, nominally 2 to 10 mm diameter, to provide drainage. The next 12 cm is a mixture of the fresh forest litter and soil. A mixture of 46% (by volume) commercial sterilized potting soil, 46% of the LFH soil horizon and 8% of an agricultural soil is suitable. The litter provides the initial arthropod population and substrate. Sleeves are placed in the litter mixture, as described below. The top 2 cm of the mesocosm is a layer of potting soil.

During assembly, 24 empty plastic sleeves are placed vertically in the soil. These sleeves are 19-cm long, open-ended boxes made of ¼-inch thick Plexiglas. The cross-section inner dimensions of the sleeve are 3 x 15 cm. The upper end of the sleeve is positioned just above the soil surface and is covered with an opaque, removable lid. The lower end is open to the soil. The sleeves are inclined about 30° from vertical, and the downward facing side of the sleeve is perforated with five or six, 3-cm-diameter holes. These holes are covered with 2-mm-mesh nylon screen.

After assembly, seeds of a shade-tolerant lawn grass blend are planted in the top soil and the mesocosm is covered with plastic until the seeds emerge.

The mesocosm is positioned to receive indirect sunlight and/or continuous supplemental light from florescent tubes. Tap water is applied to the surface when needed. Water accumulation in the gravel can be viewed through the acrylic access tube in one corner of the mesocosm, and excess water is avoided.

The mesocosm is established and the grass cultivated for at least one month prior to the first bioassay so that the microarthropod populations stabilize. At intervals the grass is clipped with scissors to about 10-cm height, leaving the clippings on the surface. Bioassays have been done in individual mesocosms for up to a year with consistent results.

Modification of petri dishes

Plastic petri dishes, 15-cm-diameter and 2.5-cm-deep, are used to hold the contaminated soil. The lid is modified by cutting out the centre to leave only a 1-cm-wide rim. This can be done on a band saw, with the resulting slit in the rim held by the screen. Window screen, nominally 2-mm-mesh nylon, is glued onto the rim using silicon sealant. With cleaning, the lids may be reused.

Construction of Tullgren extractor

Many variations on Tullgren or high-gradient extractors are available. The key components are a light source that will light and heat the sample on the upper side, some temperature control that will keep the lower side of the sample cooler than the top, a funnel to collect the escaping microarthropods, and an alcohol trap to capture and preserve the microarthropods. Difficulties arise when the sample dries too quickly or when alcohol fumes from the trap pass through the sample and kill the microarthropods before they escape. Numerous designs for simple extractors are available.

Plant growth chamber facilities are appropriate. Select a funnel with the large opening big enough so the petri dish can fit fully inside. The petri dish should rest at the top of the funnel, and cross supports may be used to hold the petri dish near the top of the funnel. A rack is

required that will hold enough funnels to extract all samples of a single bioassay. Design it to fit onto the floor or bench in a plant growth chamber. The lights of the growth chamber will need to be positioned about 2 cm above the petri dishes, growth chambers with a mobile light canopy are ideal. Under the funnel is a small beaker about 50 mL. A loose fitting or punctured layer of parafilm can be used to connect the beaker to the funnel so that evaporation is reduced. The extractor should not be an airtight fit from the beaker to the petri dish.

Basic bioassay plan

Bioassays will likely be applied in either of two modes. One is the experimental investigation of contaminants, where a soil of specific properties is chosen and is artificially contaminated to meet the requirements of an experimental plan. The second mode is the application to a contaminated soil collected in the field.

The use of artificially contaminated soil is the most appropriate mode for a whole-soil bioassay because an uncontaminated control soil is extremely important in interpretation of results. In practice, an uncontaminated soil with specified properties is collected. Control and treatment aliquots are handled uniformly, and the treatment aliquots are contaminated with a range of at least five concentrations typically spanning at least an order of magnitude. The specific concentrations and ranges are determined by reports in the literature. Use a geometric scaling of the concentrations and consider placing more levels near the expected-effect concentration. At the same time, a treatment at a concentration well above the expected-effect level is highly recommended so that trends and symptoms observed at the lower concentrations can be clearly related to the effects at the extreme concentration. Great care can be taken to counterbalance companion ion and solvent additions so that the only significant difference among the aliquots is the presence of the contaminant. Use a replicated design, and extra replicates may be assigned to the control.

In the second mode, the extent and identity of the contaminant(s) may or may not be known. It is very important to seek a control soil to correspond to the contaminated soil. The field agent must estimate the extent of the contamination and try to collect a soil that appears to have properties comparable to the contaminated soil yet be outside the influence of the contamination. It would be desirable to thoroughly characterise both soils to ensure similarity in non-contaminant properties and dissimilarity in contamination. When an unambiguous control soil cannot be found in the field, it is necessary to identify one in the laboratory. For this, the laboratory should keep a supply of several soils (that can be renewed with further collections) that represent a range of key properties such as pH, texture and organic matter content. In the Expansion of Details, Part P2, efforts to create a control soil by matching a blend of laboratory reference soils to a contaminated field soil are described. This is a difficult task to accomplish.

Preparation and characterization of soil

About 5 L of soil is required, varying somewhat with the mode of the bioassay and the amount of processing needed to render a homogenous, finely-divided soil for use. Storage and initial preparation of the soil for the microarthropod bioassay can likely follow protocols established for other bioassays. Spread the soil on a large flat, plastic-covered surface where lumps can be broken and the sample homogenized, and where drying to a workable moisture content is allowed. This operation would have to be modified for volatile contaminants. A workable moisture content is one where the soil will no longer adhere in clumps when handled. It is undesirable to fully dry the soil, as this affects both contaminant behaviour and soil microbial condition. The soil is then passed through a 4- to 10-cm mesh. Material retained on the mesh is normally discarded. The operator must decide what amount of force to use to grind the soil through the mesh, bearing in mind that the goal is a chemically and physically homogenous sample. The processed soil is then stored in sealed pails. Each time a sample is removed for analysis, an additional subsample is taken to measure the moisture content. Soil moisture content is defined as the weight loss on drying at 105°C for 24 h. All soil concentrations must be expressed on a soil dry weight basis.

It is likely that the bioassay of a soil will be accompanied by chemical and physical characterization of the soil to meet other needs. However, to facilitate interpretation of the microarthropod bioassay results, quantitative soil pH and at least qualitative texture and organic matter content are required. In addition, the microarthropod bioassay requires a measure of the soil moisture holding capacity. This is not interpreted as a basic property of the soil - it is an application-specific measurement. Measure by weight the amount of water retained after 24 h draining of a thoroughly wetted aliquot of the soil held in the same physical container as used in the application. In this case, the container is the petri dish. This measurement of moisture holding capacity will give higher values than field moisture retention or third-bar tension measurements. It may also be above the optimal moisture content for microarthropods in a given soil because of the effect of water on oxygen diffusion. Wet the soils in the bioassay to 80% of the measured moisture holding capacity.

Preparation of treatments or dilution series

The principles applied here are outlined in the Basic Bioassay Plan section. For artificial contamination, at least 3 replicates are recommended. If more than about seven concentrations are used, then the numbers of replicates could be reduced. Each petri dish will hold about 300 mL of soil, so that about 1 L of soil for each treatment should be prepared. Care should be taken to counterbalance companion ion and solvent additions across the treatments. This requires judgement and compromise, approaches are described in the Overall Report sections here, in the Detail section 2.2, and elsewhere[20] .

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20. Sheppard, S.C., Evenden, W.G. and Anderson, A.J. 1992. Multiple assays of uranium toxicity in soil. Environ. Toxicol. Water Qual. 7:275-294. Footnote Cont. Next Page

After addition of the contaminant, it is desirable to moisten the soil to near the moisture holding capacity and incubate it for some time. The length of time is dependent on the volatility of the contaminant and the expected reaction kinetics in the soil. Elevated temperature to 30°C may be appropriate to speed reactions for non-volatile contaminants. The incubation should last at least 7 to 14 d to pass the burst in soil respiration that follows rewetting of a dry soil.

For field contaminated soil, the objective is to provide a dilution series of the contaminated soil diluted with a control soil. As described, the choice of the control soil is very important. In the absence of a field control soil, then a laboratory reference soil or blend can be used. A broad dilution series is ideal, but unlike water samples, dilution of whole soils is limited by the physical scale of the soil particles. It is inappropriate to dilute the contaminated soil to the extent that the probability of a microarthropod encountering contaminated particles is affected. Dilution ratios, control:contaminated, of 1:0, 16:1, 8:1, 4:1, 2:1, 1:1, 1:2 and 0:1 are usually appropriate. Mixing should be very thorough, and it is appropriate to moisten the mixtures and allow them to incubate as described above.

Filling petri dishes and loading mesocosm

The soil is measured by weight into uniform aliquots of about 300 g. To these are added 10% w/w ground alfalfa-based rodent feed pellets. The soil and alfalfa are thoroughly mixed and the mixture placed in the bottom of one of the modified petri dishes. The edge of the petri dish bottom is labelled, with the label repeated in two different positions around the edge. Water is added to bring the moisture content of the soil to 80% of its moisture holding capacity, and the lids are put in place. Two small pieces of tape are useful to secure the lids to the bottoms. When all petri dishes for a given bioassay are filled, they are placed screen side downward in the sleeves of the mesocosm. The ordering of treatments in the sleeves is carefully randomized and then recorded. The lids of the sleeves are put in place.

Incubation conditions, midterm observations

The mesocosm is tended as usual during the bioassay. Within a few days, the petri dishes can be examined and fungal growth should be evident. The petri dishes should not be otherwise disturbed.

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Footnote 20 Cont.

Sheppard, S.C., Evenden, W.G. and Pollock, R.W. 1989. Uptake of natural radionuclides by field and garden crops. Can. J. Soil Sci. 69:751-767.

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End of bioassay and extraction of microarthropods

After 11 d in the mesocosm, the petri dishes are removed from the mesocosm sleeves and transferred immediately to the Tullgren extraction funnels. They are fit screen downward in the funnels, handling with care to prevent soil and detritus from falling into the ethanol trap. Ten mL of ethanol is placed in the beakers under the funnels. The lights are lowered to 2 cm from the bottom of the petri dishes. The room temperature is 22°C with strong air circulation. Within 24 h, there should be microarthropods visible by eye in the control soil beakers. The ethanol traps gain moisture because of distillation from the soil into the funnel. After 96 h, the beakers are collected and the soil disposed.

Counting of microarthropods

There are often many more microarthropods than can be economically counted. Evaporate some of the ethanol/water from the trap and adjust the volume to exactly 10 mL. A hear lamp or ambient temperatures are suitable. Stir and remove an aliquot by pipette for counting. By estimation, select the volume of the aliquot so that actual counts from control soils are 20 to 100 total meso fauna. Although this procedure may under-sample large meso fauna, it is accurate for the 0.1 to 2 mm organisms important to this protocol. The ethanol traps may be stored for archival purposes with additions of glycerol.

Use a dissecting microscope with adjustable intensity lighting from above and below the stage. Place the aliquot of ethanol in a small watch glass. It may be possible to use an aliquot small enough that the entire aliquot appears In one field of view. Magnification up to 40x is needed to differentiate smaller microarthropods from detritus, but counts can normally be done at lower power.

Count springtails (Collembola), mites (Acari), and remaining meso fauna separately. More detailed taxonomic differentiation would be possible, especially with computer aided keys[21] , but the costs would not likely be justified by Improved bioassay sensitivity. In general, the counts are dominated by springtails so that the few larger meso fauna contribute nothing meaningful to the count and can be disregarded.

Test acceptance criteria

Criteria for an acceptable test include mean counts in control soils greater than about 100 and a clear effect of increased contamination.

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21. e.g., Moldenke, A., Shaw, C. and Boyle, J.R. 1991. Computer-driven image-based soil fauna taxonomy. Agric. Ecosys. Environ. 34:177-185.

Disposal of materials

The petri dish bottom containing the soil should be disposed of in the same manner used for the contaminated soil. It is possible to clean the lids for further use. The ethanol traps will contain negligible contamination and can be archived or disposed of as an organic solvent.

Statistical summation of data

Statistical summation can follow several approaches. A replicated design allows either regression or analyses of variance (ANOVA), or a combination. The counts need to be corrected for the aliquot subsampling used to obtain counts. This is typically a tenfold multiplier. The corrected counts may span several orders of magnitude, and in many if not all cases log transformation is appropriate or required. For analysis, the SAS programs[22] are ideal, they are broadly accepted and very flexible.

The first step is a plot of the log transformed counts, with error bars, versus the contaminant concentration or dilution ratio. Log-scaled treatments should be plotted on log scale. The second step is an overall ANOVA, where the F ratio for the effect of the contaminant is calculated. If the F ratio is not significant at $P<0.05$, then further interpretation must be considered with caution. There is some controversy about the next step. Individual statistical tests, equivalent in results to t tests, on each treatment versus the control can be used. These result from the PDIFF option of the LSMEANS statement in the GLM procedure of SAS. If you can assume no possibility of enhanced counts as a result of the contaminant, a one-tailed test is appropriate. In the sense of a bioassay to detect negative environmental impact, a one-tailed test is also conservative. The multiple comparisons to the control are not an orthogonal decomposition of the overall treatment sum of squares. They are *a priori* and are conservative in the sense of a bioassay.

The statistical indications require interpretation supported by the plot. The no-observable-effect-level (NOEL) is associated with the highest-concentration position on the curve where there is no continuous trend toward poorer counts than the control. This point will never be significantly different from the control. The lowest-observable-effect-level (LOEL) is associated with the lowest concentration that had counts significantly different from the control. The LC_{10} and LC_{50} can be interpolated visually from the graph, but only when within the range of the data.

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Appendix A

Summary of some established and potential bioassays

Table A1. Selected soil bioassays (adapted from Reddy and Greene 1992) that have been sponsored by international standards organisations. The organisation acronyms are ASTM (American Society for Testing and Materials), ISO (International Standards Organization), OECD (Organization for Economic Cooperation and Development), US EPA (U.S. Environmental Protection Agency) and US FDA (U.S. Federal Department of Agriculture).

	Bioassays using vascular plants			
Sponsor agency	ASTM	ASTM	ASTM	OECD
Application	soil elutriate	whole soil	whole soil or elutriate	whole soil
Species	lettuce, radish, red clover, wheat cucumber	lettuce, radish, red clover, wheat cucumber	*Tradescantia* hybrid	16 candidate species
Endpoints	root length, EC_{50}	seedling emergence, EC_{50}	flower stalks and blooming flowers	seedling emergence, plant weight, EC_{50}
Organism selection	seed sizing	seed sizing	one clone commonly used	seed sizing
No. organisms + replicates	5 seeds, 3 reps, geometric series of >3 concentrations	40 seeds, 3 reps, >5 concentrations	--	5 seeds, 4 reps, 3 concentrations
Observation frequency	120 hr ± 0.5 hr	120 hr	daily (one flower produced/d)	14 d after 50% control germination
Volume of test vessel	100 x 15 mm petri dishes	150 x 15 mm petri dishes	--	---
Volume of test substance	20 mL elutriate/rep	100 g soil/rep	—	---

continued...

Bioassays using vascular plants, continued

Sponsor agency	US EPA	US EPA	US EPA	US EPA
Application	pesticide toxicity, whole soil, sand or filter paper	pesticide toxicity, whole soil, sand or filter paper	whole soil, hazardous wastes	aqueous wastes, elutriates from solid wastes
Species	soybean, corn, root crop and 7 others	soybean, corn, root crop and 7 others	lettuce, cultivar specified	lettuce, cultivar specified
Endpoints	seed germination, seedling emergence, EC_{25}, EC_{50}	growth, morphology development, EC_{25}, EC_{50}	seedling emergence, EC_{50}	root length, EC_{50}
Organism selection	----	plants 1-4 wk post emergence	seed sizing, 1 seed lot, untreated	seed sizing, 1 seed lot, untreated
No. organisms + replicates	10 seeds, 3 reps, 5 concentrations	5 plants, 3 reps, 5 concentrations	40 seeds, 3 rep, 5, preferably 7 concentrations	5 seeds, 3 reps, a 5, preferably 7 concentrations
Observation frequency	at 5 d for germination, weekly for emergency	weekly for at least 2 wk	120 h	120 h
Volume of test vessel	----	----	150x15 mm plastic petri dish bottom half in 30x 30 cm plastic bags	100 x 15 mm glass petri dish
Volume of test substance	----	----	100 g soil/rep	4 mL/rep

continued...

	Vascular plants, continued		Bioassay using alga
Sponsor agency	US EPA	US EPA	Environment Canada
Application	artificial soil, sand or glass beads	solution culture	soil elutriate
Species	10 crops specified	10 crops specified	*Selenastrum capricomutum*
Endpoints	seed germination, root elongation, EC_{10}, EC_{50}	weight and length of roots and shoots, EC_{10}, EC_{50}	cell concentration EC_{50}
Organism selection	seed sizing	uniform seedlings	specified strain
No. organisms + replicates	10 seeds, 3 reps, 6 concentrations	10 seedlings, 3 reps 5 concentrations	1×10^4 cells/mL 2 reps, 3 concentrations
Observation frequency	when controls 65% germinated	14 d after 50% germination	0 and 96 hr
Volume of test vessel	200 mm petri dishes	----	125 mL
Volume of test substance	----	----	125 g soil for all reps, 50 mL elutriate/rep

continued...

	Bioassays using earthworms		
Sponsor agency	ISO	ISO	OECD
Application	artificial soil	artificial soil	chemicals in artificial soil, or on filter paper
Species	*Eisenia foetida/ E. andrei*	*Eisenia foetida, E. andrei*	*Eisenia foetida*
Endpoints	survival, LC_{50}	survival, cocoon production, hatchability, juveniles/cocoon, LC_{50}, EC_{50}, NOEC	survival, LC_{50}
Organism selection	> 60 d old, with clitellum, 300-600 mg	> 60 d old, with clitellum, 250-600 mg; batches of 10 worms differ by < 1g	> 60 d old, with clitellum, 300-600 mg
No. organisms + replicates	10 worms, 4 reps geometric series of 5 concentrations	10 worms, 4 reps, geometric series of 5 concentrations	1 worm, 10 reps, geometric series of 5 concentrations
Observation frequency	7, 14 d	21 d for mortality, cocoon production, 5 wk hatchability, juveniles	14 d for soil; 48 hr, 72 hr optional for contact filter paper test
Volume of test vessel	1-2 L glass container not tightly closed	1 L glass container <15 cm diam. loosely covered with lids	1 L glass container with lid for soil; glass vial 8 cm long 3 cm diameter for contact test
Volume of test substance	500 g dry soil/rep	500 g soil/rep	750 g moist soil/rep or 1 mL solution for contact test

continued...

	Bioassays with earthworms, continued		Bioassay with arthropods
Sponsor agency	US EPA	US FDA	OECD
Application	whole soil, hazardous wastes	artificial soil	artificial soil
Species	Eisenia andrei	*Lumbricus terrestris / rubellus*	*Folsomia candida*
Endpoints	survival, EC_{50}	survival, body weight, LC_{50} EC_{50}	adult survival, offspring number, NOEC, LOEC
Organism selection	>60 d old, with clitellum, 300-500 mg, same culture	mature with clitellum	10-14-d-old juveniles
No. organisms + replicates	10 worms, 3 reps, ≥ 5, preferably 7 concentrations	10 worms, 4 reps, geometric series of 5 concentrations	10 animals, 4 reps, ≥ 5 concentrations
Observation frequency	7, 14 d	7, 14, 21, 28 d for survival, weight at start and end	4 wk
Volume of *test* vessel	1 pint glass canning jars	2.5 L *glass* container, diameter 1:2 height	100 mL glass containers
Volume of test substance	200 g soil/rep	2 kg soil/rep	30 g moist soil

continued...

Selected bioassays described in the literature for use in soils, and selected aquatic bioassays that have traits useful for soil bioassays. Very many methods have been used to investigate the effects of contaminants in soils, and all of these are candidate bioassays. Most papers we list here had the expressed intent of developing bioassays, although a few others are included that we think merit further attention.

Earthworms

Menzie, C.A. *et al.* 1992. Assessment of methods for estimating ecological risk in the terrestrial component: A case study at the Baird and McGuire Superfund site in Holbrook, Massachusetts. Environ. Toxicol. Chem. 11:245-260. In-field exposure of bait worms for 1-7 d in chambers, with scoring of results for visual sublethal and lethal effects.

van Gestel, C.A.M. *et al.* 1988. Comparison of two methods for determining the viability of cocoons produced in earthworm toxicity experiments. Pedobiologia 32:367-371. Cocoon production of *Eisenia fetida* more sensitive than adult mortality, and can be expanded to include cocoon viability. Specific methods needed to ensure reliable results.

van Gestel, C.A.M. *et al.* 1989. Development of a standardized reproduction toxicity test with the earthworm species *Eisenia foetida andrei* using copper, pentachlorophenol, and 2,4-dichloroaniline. Ecotoxicol. Environ. Safety 18:305-312. See above paper by same authors.

Other invertebrates

Bengtsson, G. *et al.* 1985. Influence of fungi on growth and survival of *Onychiurus armatus* (Collembola) in a metal polluted soil. Oecologia 68:63-68. Survival and growth measurements of specific soil springtails introduced to small amounts of contaminated soil in vials.

Berger, B. and Dallinger, R. 1993. Terrestrial snails as quantitative indicators of environmental metal pollution. Environ. Monit. Assess. 25:65-84. Snails as biomonitors, and there is an opportunity for the use of snail and slug eggs as bioassays.

Denneman, C.A.J. and van Straalen, *N.M.* 1991. The toxicity of lead and copper in reproduction tests using the oribatid mite *Platynothrus peltifer.* Pedobiologia 35:305-311. Survival, growth, reproduction and absorbed contaminant concentrations were measured in soil mites exposed to contaminated feed in a purified sand culture.

Koehler, H.H. 1992. The use of soil mesofauna for the judgement of chemical impact on

ecosystems. Agric. Ecosystem. Environ. 40:193-205. Ecological study of the response of Collembola, Acari and Enchytraeidae to contaminated soil, with comments that they are ideal for bioassay if used in an ecological setting.

Köhler, H.-R. *et al.* 1992. The 70 kD heat shock protein (hsp 70) in soil invertebrates: A possible tool for monitoring environmental toxicants. Arch. Environ. Contam. Toxicol. 22:334-338. Measurements of heat shock protein in a forest litter isopod showed good preliminary sensitivity to Pb.

Lew, K.K. *et al.* 1983. *In vivo* assay to screen for mutagens/carcinogens in the nematode C. *elegans.* Pages 139-150 *in* Proc. *In vitro* toxicity testing of environmental agents: current and future possibilities. 22-28 Sept. 1979. Monte Carlo, Monaco. Plenum Press, New York NY. Mutagenic reversion of isolates of small-sized soil nematodes to large wild types measured by filtration, after nematodes exposed to liquids (potentially soil elutriates) in petri dish cultures.

Nola, L. *et al.* 1987. Effects of atrazine on two species of Collembola (Onychiuridae) in laboratory tests. Pedobiol. 30:145-149. Survival and reproduction of defined Collembola species introduced to contaminated artificial soil but fed uncontaminated feed.

Samoiloff, M. 1990. The nematode toxicity assay using *Panagrellus redivivus.* Tox. Assess. 5:309-318. Development and to a lesser extent survival of nematodes *in* extracts of soils.

Schmidt, G.H. 1986. Use of grasshoppers as test animals for the ecotoxicological evaluation of chemicals in the soil. Agric. Ecosyst. Environ. 16:175-188. *Egg* production and maturation success were measured for grasshoppers in trays of sand (potentially soil) in rearing cages.

Weeks, J.M. 1992. The use of the terrestrial amphipod *Arcitalitrus dorriene* (Crustacea; Amphipoda; Talitridae) as a potential biomonitor of ambient zinc and copper anailabilities in leaf-litter. Chemosphere 24:1505-1522. Terrestrial amphipods (landhoppers) used as biomonitors, and there could be application as bioassay.

Plants

Aitken, R.L. *et al.* 1990. A simple bioassay for the diagnosis of aluminium toxicity in soils. Commun. Soil Sci. Plant Anal. 21:511-529. Root elongation in paired samples of soil extracts, with the activity of the toxicant eliminated in one of the pairs. For Al they use pH to remove Al toxicity, but a comparable concept may apply to other selected toxicants.

Cheung, Y.H. *et al.* 1989. Root and shoot elongation as an assessment of heavy metal toxicity

and ‘Zn equivalent value' of edible crops. Hydrobiologia 188/189:377-383. After comparison of root and shoot growth in 13 plant species, the best bioassay was measurement of root elongation of *Brassica parachinensis* in sand saturated with contaminated liquid (potentially soil elutriate), and expressed relative to results for Zn toxicity.

Dreesen, D.R. and Cokal, E.J. 1984. Plant uptake assay to determine bioavailability of inorganic contaminants. Pot culture assay where mesh-bottom units with growing plants are transplanted onto contaminated soil. Intended for relative measurements of bioavailability, but could include root growth measurements. See also Sheppard and Racz (1984, 'Effects of soil temperature on phosphorus extractability I. Extractions and plant uptake of soil and fertilizer phosphorus. Can. J. Soil Sci. 64:241-254).

Fiskesjo, G. 1985. The Allium test as a standard in environmental monitoring. Hereditas 192:99-112. Method using Allium bulbs and measuring root growth and cell division. It was developed for water and is applicable to soil extracts. The author has used it for whole soils (reported in Swedish only), where there is the difficulty of washing the roots from the soil.

Fletcher, J.S., 1990. Use of Algae versus Vascular Plants to Test for Chemical Toxicity, Plants for Toxicity Assessment, ASTM STP 1091, W. Wang, J.W. Gorsuch, and W.R. Lower, Eds., American Society for Testing and Materials, Philadelphia, pp. 33-39. Compared algae versus vascular plants and found the algae will fail to indicate effects in 20% of contaminants that do effect vascular plants.

Garten, C.T., Jr., 1990. Multispecies Methods of Testing for Toxicity: Use of the Rhizobium-Legume Symbiosis in Nitrogen Fixation and Correlations Between Responses by Algae and Terrestrial Plants, Plants for Toxicity Assessment, ASTM STP 1091, W. Wang, J.W. Gorsuch, and W.R. Lower, Eds., American Society for Testing and Materials, Philadelphia, pp. 69-84. Plant growth, modulation success, nitrogen fixation and plant nitrogen content measured. Also compared to algal tests and found poor ability of the algal test to predict effects on higher plants.

Gorsuch, J.W., Kringle, R.O., and Robillard, K.A. 1990. Chemical Effects on the Germination and Early Growth of Terrestrial Plants, Plants for Toxicity Assessment, ASTM STP 1091, W. Wang, J.W. Gorsuch, and W.R. Lower, Eds., American Society for Testing and Materials, Philadelphia, pp. 49-58. Used a suspended blotter paper technique to measure root growth in response to contaminated solutions. This could also include measurement of nastic movements. Adaptation to contaminated soils is possible.

Judy, B.M., Lower, W.R., Miles, C.D., Thomas, M.W., and Krause, G.F. 1990. Chlorophyll Fluorescence of a Higher Plant as an Assay for Toxicity Assessment of Soil and Water,

Plants for Toxicity Assessment, ASTM STP 1091, W. Wang, J.W. Gorsuch, and W.R. Lower, Eds., American Society for Testing and Materials, Philadelphia, pp. 308-318. Chlorophyll fluorescence as a sublethal indicator of plant stress.

Linder, G., Greene, J.C., Ratsch, H., Nwosu, J., Smith, S., and Wilborn D. 1990. Seed Germination and Root Elongation Toxicity Tests in Hazardous Waste Site Evaluation: Methods Development and Applications, Plants for Toxicity Assessment, ASTM STP 1091, W. Wang, J.W. Gorsuch, and W.R. Lower, Eds., American Society for Testing and Materials, Philadelphia, pp. 177-187. Description of standardized seed germination and root elongation tests.

Miles, D. 1990. The Role of Chlorophyll Fluorescence as a Bioassay for Assessment of Toxicity in Plants, Plants for Toxicity Assessment, ASTM STP 1091, W. Wang, J.W. Gorsuch, and W.R. Lower, Eds., American Society for Testing and Materials, Philadelphia, pp. 297-307. Chlorophyll fluorescence as a sublethal indicator of plant stress.

Miller, W.E. *et al.* 1985. Comparative toxicology of laboratory organisms for assessing hazardous waste sites. J. Environ. Qual. 14:569-574. A comparison of the results of many of the EPA bioassays (see Keddy and Greene 1992, Greene is one of the co-authors here). Most were plant bioassays.

Nwosu, J.U., Ratsch, H., and Kapustka, L.A. 1991. A Method for On-Site Evaluation of Phytotoxicity at Hazardous Waste Sites, Plants for Toxicity Assessment: Second Volume, ASTM STP 1115, J.W. Gorsuch, W.R. Lower, W. Wang and M.A. Lewis, Eds., American Society for Testing and Materials, Philadelphia, pp. 333-340. Essentially a five day germination and emergence test with soils from contaminated sites, the unique feature being the use of an on-site protective cover in which to do the tests.

Pestemer, V.W. and Auspurg, B. 1986. Suitability of a variety of test-plants for assessing the risks of the effects of chemicals to higher plants within the scope of the chemical law. Nachrichtenbl Deut Pflanzenschutzd 38:120-125. A comparison of several of the plant bioassays recommended by the OECD, which showed *Brassica rapa* and *Avena sativa* as superior because of sensitivity and precision.

Ratsch, H.C. and Johndro, D. 1987. The influence of two culturing techniques on toxicity of four chemicals in *Arabidopsis.* Environ. Monit. Assess. 9:155-168. Seed and total shoot yield of *Arabidopsis* grown in solution or vermiculite (potentially soil) culture with contaminated liquids (potentially soil elutriates).

Shimabuku, R.A., Ratsch, H.C., Wise, C.M., Nwosu, J.U., and Kapustka, L.A. 1991. A New Plant Life-Cycle Bioassay for Assessment of the Effects of Toxic Chemicals Using Rapid Cycling Brassica, Plants for Toxicity Assessment: Second Volume, ASTM STP 1115, J.W. Gorsuch,

W.R. Lower, W. Wang and M.A. Lewis, Eds., American Society for Testing and Materials, Philadelphia, pp. 365-375. The use of reproductive indicators (counts of seed pods) as well as height, weight and stem thickness as indicators.

Thomas, M.W., Judy, B.M., Lower, W.R., Krause, G.G., and Sutton, W. 1990. Time-Dependent Toxicity Assessment of Herbicide Contaminated Soil Using the Green Alga Selenastrum capricornutum, Plants for Toxicity Assessment, ASTM STP 1091, W. Wang, J.W. Gorsuch, and W.R. Lower, Eds., American Society for Testing and Materials, Philadelphia, pp. 235-254. Algal growth in soil elutriates.

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Microbial

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Dougherty J.M. and Lanza G.R. 1989. Anaerobic subsurface soil microcosms: Methods to monitor effects of organic pollutants on indigenous microbial activity. Tox. Assess. 4:85-104. Amylase, dehydrogenase, esterase, phosphatase and protease enzyme systems and soil protein concentrations were measured on soil slurries. the mesocosm system is also relevant for bioassay of subsurface materials.

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Chem. 30:183-192. Substrate-induced respiration and substrate-induced oxygen consumption were best of several for bioassays of soil. The other included fumigation-extraction of microbial carbon, ninhydrin-reactive nitrogen, dehydrogenase activity and ATP content.

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Jones, K.C. and Peace, E.A. 1989. The Ames mutagenicity assay applied to a range of soils. Chemosphere 18:1657-1664. Soil extracts were screened using two strains of the bacterium in the standard Ames assay.

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Rossel D. and Tarradellas J. 1991. Dehydrogenase activity of soil microflora: significance in ecotoxicology tests. Environ. Toxicol. Water Qual. 6:1733 (see also Toxicol. Environ. Chem. 30:219-220). Dehydrogenase activity was determined periodically over 64 d using buffered slurries of soil incubated 24 h with substrate and then extracted with acetone to measure absorbance. The ATP content was determined periodically over 64 d using acid extracts of soil, purified by a resin with analysis by bioluminescence from the luciferin/luciferase system. The CO_2 evolution was determined periodically over 64 d using base absorption in a closed system containing 100 g soil, measuring electrical conductivity in the trap. Esterase activity was determined periodically over 64 d using buffered suspensions of soil incubated 70 min with substrate, after which the reaction was terminated with acetone and the suspension filtered and absorbance in the filtrate measured.

Schaeffer, D.J. *et al.* 1991. Evaluation of the "reference toxicant" addition procedure for testing the toxicity of environmental samples. Bull. Environ. Contam. Toxicol. 47:540-546. Applied to liquids, but the concept may be of use for soils. The concept is to add a reference toxicant to samples of contaminated water that have been to diluted to a no-effect concentration. The response to the reference contaminant is compared to it addition in uncontaminated water, and differences in response indicate latent, synergistic or antagonistic effects of the original contaminant.

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(medical spatulas) implants in contaminated soil in the field was measured gravimetrically at intervals over one year, accounting for adhering soil.

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