SOIL TOXICITY AND BIOASSESSMENT TEST METHODS FOR ECOLOGICAL RISK ASSESSMENT

Toxicity Test Methods for Soil Microorganisms, Terrestrial Plants, Terrestrial Invertebrates and Terrestrial Vertebrates

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Integrated Risk Assessment Branch
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Now With
California Department of Fish and Game

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INTRODUCTION

Purpose

Chemical contaminants in the environment can adversely affect living organisms at levels ranging from microbes in the soil to upper trophic level vertebrates, including humans. Concern for the effects of contaminants in the environment has led to the development of risk assessment methodologies that require exposure and effect data inputs in order to characterize risk. The Office of Environmental Health Hazard Assessment (OEHHA) within the California Environmental Protection Agency (Cal/EPA) is compiling technical resources and guidance documents for use by scientists in their development of ecological risk assessments. Of particular interest are standardized methods for toxicity determination and *in situ* bioassessment that can be applied to species, populations and communities inhabiting terrestrial ecosystems.

It is noteworthy that there are currently many national and international efforts underway to develop, improve, and standardize methods of assessing soil quality, particularly for use in classifying the potential hazards of both soils and contaminant materials they may contain (van Straalen and Løkke 1997). The focus of this document is the review of those test methods and guidelines that have been standardized, either nationally or internationally, for the purpose of assessing potential effects of chemical substances released or anticipated to be released into the environment. A variety of standardized testing methods or guidelines are currently available and are reviewed in depth. There are also many more methods proposed or in development. When available, information and relevant references for non-standardized protocols and procedures are provided. It can be anticipated, however, that new standardized approaches for assessing soil toxicity will continue to be developed in the future.

Standardized toxicity tests for soil microbes, plants, invertebrates, birds, and mammals were reviewed for their ability to assess the toxicity of chemicals in soils, and for their inherent limitations and usefulness in assessing contaminant hazard to the environment. The document serves as a technical resource that reviews available standardized assessment methodologies, based on their utility in ecological risk assessments and other types of environmental assessment. This information is intended to assist environmental scientists and toxicologists in selecting test methods and interpreting test results for evaluating the toxicity of chemicals.

General Principles of Soil Ecotoxicology

The science of soil ecotoxicology is an interdisciplinary field of science that looks at the toxicological effects of chemicals or conditions (e.g., acidification) on soil ecology. Humans activities pose considerable risk to soil ecosystems.

Because of the enormous importance of understanding the influences of anthropogenic activities on the environment, the discipline of *Ecological Risk Assessment* has emerged "in response to the need to create ecologically- and toxicologically-defensible schemes to evaluate the impact of contaminants on the environment" (Calabrese and Baldwin 1993).

Many approaches to performing ecological risk assessments have been proposed and used to support environmental management; Urban and Cook 1986, Calabrese and Baldwin 1993, Bartell et al. 1992, Suter 1993, EPA 1992ab, Kendall et al. 1990, Kendall and Ackerman 1992, and Dickerson et al. 1994, to name but a few. Ecological risk assessment is further discussed below.

Soil ecology has been defined as the study of natural fluctuations in soil processes and populations of soil organisms. Soil ecosystems are incredibly complex with great heterogeneity in physical, chemical and biological characteristics and are considerably influenced by factors such as geology, topography, climate and anthropogenic activities (see Figure I-1, modified from Torstensson 1997, 1998).

TOPOGRAPHY   **CLIMATE**

- *Cultivation system*
- *Organic matter*
- *Water*
- *Soil gases*

- *Structure*   **Physical**   **Chemical**   - *Nutrients*
- *Texture*   **Characteristics** ⟷ **Characteristics**   - *Chemicals and heavy metals of natural and anthropogenic origin*
- *Temperature*
                    Biological   - *Acid/base conditions and buffering capacity*
                  Characteristics

- *Soil microorganisms*
- *Soil animals and plants*

GEOLOGY   **ANTHROPOGENIC ACTIVITIES**

Figure I-1. The complex structure of soil and its influences. Modified from Torstensson 1997, 1998

Soil is formed over time by the parental material (the chemical and physical properties of the originating rock, alluvium/colluvium or organic material), the climate (past and present, rainfall and temperature for example), the fauna and flora that have lived in it, the relief (the geomorphology and its influences, for example, on drainage), and time (Clark 1986). The soil profile is a description of the vertical cross sections of the soil that naturally occur in layers or horizons (Clark 1986). These layers or horizons result from soil formation and uniquely characterize the physico-chemical nature of individual soils. Clark (1986) describes three basic soil layers: topsoil, the topmost layer containing maximum biotic activity; subsoil, found below

the topsoil containing reduced amounts of biotic activity; and the substratum, the bottom layer containing primarily unconsolidated material merged with hard rock.

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Physical and Chemical Influences on Contaminant Bioavailability and Toxicity in Soil

The aforementioned physical and chemical parameters can markedly affect the bioavailability of contaminants to soil dwelling or other exposed organisms. Bioavailability is defined as the physicochemical access that a toxicant has to the biological processes of an organism (Allen 2002). The less the bioavailability of a toxicant, the less its toxic effect on an organism. For example, the partitioning of metals to soil may reduce their availability for mobilization and uptake by microbes, plants, and animals. Metals may become toxic to soil dwelling organisms when significant levels of free metal ions (e.g., Pb^{2+}, Zn^{2+}, Cd^{2+}) dissolved in soil porewater are available for uptake by microbes or plants. Numerous physical and chemical factors, including soil pH, organic matter, and chemical form of the element in the environment (e.g., carbonate, oxide, sulfate) affect the potential for metal ionization and availability. In addition, animals also can be exposed to soil-bound contaminants by ingestion (Allen 2002). Factors including particle size, pH of the digestive tract, and chemical form can markedly affect the degree to which a contaminant is bioavailable (DTSC 2000). Toxicity tests are a useful means to assess the bioavailability of chemicals in soil.

Ecological Risk Assessement

Predicting the adverse effects of chemicals in soil requires a conceptual framework upon which to organize and quantify the potential for risk. Ecological risk assessment estimates likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors (EPA 1992a). Applicable guidance for conducting ecological risk assessment includes (DTSC 1996a,b, and EPA 1997, 1998). Web-based resources include the following:

DTSC: http://www.dtsc.ca.gov/AssessingRisk/eco.cfm

EPA: http://www.epa.gov/oswer/riskassessment/index.htm
               http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=12460

The general principles of the ecological risk assessment process can perhaps be best illustrated by reviewing the EPA's Framework for Ecological Risk Assessment (EPA 1992a).   The

framework consists of three major phases: 1) problem formulation, 2) analysis and 3) risk characterization, which can be seen in Figure I-2, taken from the Framework (EPA 1992a).

Details of the three phases can be found in Table I-1. In summary the first phase of the ecological risk assessment, *Problem Formulation*, is a planning phase. The environmental problem is characterized in terms of exposure and effects and the ecological risk assessment is systematically planned based on the data available and the information needed to complete the assessment. Existing data is acquired and compiled and an assessment of additional data needs is made. Policy and regulatory issues are explored. The feasibility, objectives and scope of the ecological risk assessment are determined particularly in relation to any site-specific factors.

The second phase, *Analysis*, is composed of two subactivities, the characterization of exposure and the characterization of ecological effects. The exposure characterization step predicts or measures the spatial and temporal distribution of the stressor and identifies its co-occurrence or contact with the ecological components of concern. The ecological effects characterization step identifies and quantifies the adverse effects resulting from the stressor, and where possible, establishes a cause-and-effect relationship. The end results of the Analysis phase are an exposure profile and an ecological effects profile.

The third and last phase, risk characterization, is an integration phase where the exposure and effects profiles developed in the second phase are integrated to estimate the potential risk or likelihood of adverse ecological effects associated with exposure to the stressor. The risk may be described qualitatively, quantitatively, or both depending upon the data, but should describe the risk in terms of the assessment endpoint identified in the problem formulation phase. It includes a summary of the uncertainties and assumptions made during the assessment, the strengths and weaknesses of the assessment, and the ecological significance of the identified risks in terms of type, magnitude, spatial and temporal patterns, cause and effect relationships and likelihood of recovery. Ancillary data sorted on a weight-of-evidence basis may also be included. The third phase should provide a complete picture of both the analysis, the uncertainty and the results, and should pave the way for science-based risk management decisions.

In addition to the three main phases of the ecological risk assessment, interactions between risk managers and risk assessors are dispersed frequently throughout the assessment process. These discussions were integrated in the framework in order to ensure that the risk assessment will result in information relevant to the risk management requirements and that the risk assessment is ecologically relevant. In addition, early and repeated involvement in the risk assessment process will assist in ensuring that the risk manager has full and complete understanding of the assessment's conclusions, assumptions and limitations.

Also of importance to the ecological risk assessment framework is the recognition that the steps may be iterative. Data acquisition and analysis may proceed in a step-wise fashion, in tiers or with iterations of the complete process to validate exposure or toxicity assumptions and reduce uncertainty in the analysis.

**ECOLOGICAL RISK
ASSESSMENT**

Discussion Between the Risk Assessor and Risk Manager (Planning)

PROBLEM FORMULATION

A

Characterization of Exposure | Characterization of Ecological Effects

RISK CHARACTERIZATION

Data Acquisition; Verification and Monitoring

Discussion Between the Risk Assessor and Risk Manager (Results)

Risk Management
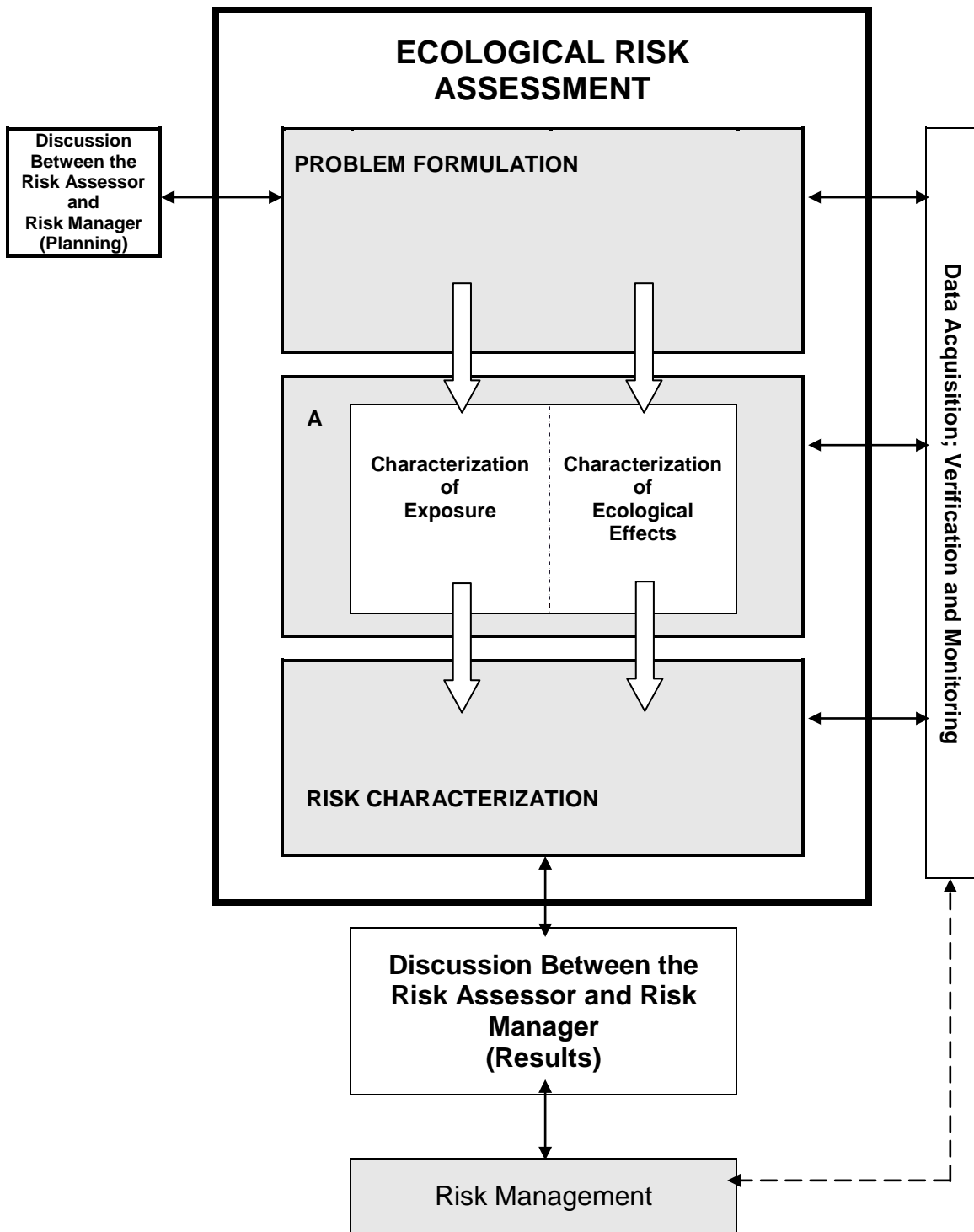
Figure I-2. Framework for Ecological Risk Assessment (Taken from EPA 1992a)

Table I-1. Critical Phases of the Ecological Risk Assessment Process

Phase I	Problem Formulation	• Determine stressor characteristics (e.g. type, intensity, duration, frequency, timing, scale) • Determine the ecosystem potentially at risk • Evaluate existing data of ecological effects • Select appropriate endpoints, considering ecological relevance, policy goals and societal values, susceptibility to the stressor • Develop a conceptual model, working hypothesis regarding how the stressor might affect the ecological components of the ecosystem
Phase II	Analysis	Characterization of exposure: • Characterize the stressor, in terms of distribution or pattern of change • Characterize the ecosystem • Analyze the potential exposure • Develop an exposure profile Characterization of ecological effects: • Evaluate the relevant effects data • Analyze the ecological response in terms of stressor –response determinations or extrapolations and causal evidence evaluation • Develop a stressor-response profile
Phase III	Risk Characterization	• Estimate the risk • Integrate the stressor-response and exposure profiles • Identify uncertainty in the analyses • Describe the risk • Summarize the risk assessment • Interpret the ecological significance

For example, the first or screening phase of the ecological risk assessment may include the comparison of plant or animal soil screening levels (e.g., EPA Ecological Soil Screening Levels – EcoSSLs 2005) to contaminant concentrations in soil. These soil screening levels are often conservative or protective levels in soil that represent either no or low potential for an adverse effect and assume high bioavailability of the chemical in soil. In some cases (e.g., Pb), these screening levels may approach background or ambient levels of metals in soil. In order to make an informed and scientifically defensible estimate of the potential for ecological risk, the risk assessment may rely on soil toxicity testing as a means to assess bioavailability, define the spatial (i.e., horizontal and vertical) extent of contamination, and develop ecologically protective remediation goals.

PART 1: TOXICITY TEST METHODS FOR SOIL ORGANISMS

INTRODUCTION TO TOXICITY TESTING

Basis for Toxicity Testing

Toxicity testing is a well-established science for comparing the hazard of one chemical to another, or for assessing the hazard of chemicals, singularly or in mixtures, to a particular test organism(s) (Ecobichon 1992). Data resulting from regulatory-mandated toxicity tests have been pivotal in developing and regulating agrochemicals, industrial chemicals, drugs, food additives, cosmetics and home products. The utility of the toxicity tests depends on both the experimental design and the conduct of the study, as well as the application of test results to risk assessment and the resulting chemical or site management.

All contaminants possess measurable physical and chemical properties that remain constant and do not vary under the same test conditions. Soils; however, are composed of unique complex mixtures of living and non-living components. Therefore, the effect of contaminants on soil and soil organisms reflects the physical and chemical properties of the contaminants *and* the interaction of the contaminants with the unique components and properties of each soil tested. The inherent capacity and varying ability of each soil to adsorb, transform, metabolize, store, sequester, and accumulate contaminants, can affect all aspects of contaminant bioavailability and toxicity. It is for this reason that standardized procedures of assessing contaminant hazards in soil have been developed. Standardized testing protocols bring a measure of control that allows comparison with other contaminants, hazardous sites or with clean reference or control soils that are required for meaningful hazard assessment.

Toxicity tests are based on the understanding that under a set of given test conditions, there is a measurable and progressive relationship between dose and effect. Toxicity tests measure an endpoint or groups of endpoints (e.g. mortality, reproductive capacity, growth rate) over a range of known concentrations of a chemical. The results are then analyzed to determine the nature of the dose-response relationship. The contaminants, concentrations, soil, test species and test conditions chosen should reflect the purpose of the test and the use of the results. Depending upon the study needs and design, contaminant concentrations used in toxicity tests may be conducted over the environmentally relevant range of anticipated soil concentrations; at a high concentration level to guarantee a response, at a low concentration level to develop a no effect level, or over a range of concentrations that will elicit all and none of the measured effect(s).

However, when performing Comprehensive Environmental Restoration, Compensation, and Liability Act (CERCLA or Superfund) ecological risk assessments, most often the risk assessor will collect soils from the field that is contaminated with a wide variety of hazardous substances. Toxicity tests are then used to assess the biovailability and toxicity of the chemicals in the environment. For example, dilutions of the field collected soils may be performed to estimate no effect or low effect levels of the hazardous substances found in the soils and as a means to establish cleanup criteria that are protective of soil dwelling organisms.

Toxicity Testing Endpoints

The endpoint measured is a reflection of the study design and purpose of the intended hazard or risk assessment. Perhaps the most common endpoint used in toxicity tests is lethality. This is a quantitative approach where the number of test organisms found dead or alive (survival) at each concentration is measured and compared to appropriate controls, such as positive, negative, solvent/carrier, or reference controls. Other common endpoints include sublethal or behavioral endpoints (e.g., movement, regeneration potential, lesions, etc.), reproductive success, functional endpoints (e.g., organic carbon utilization or transformation, nitrogen transformation, etc.) and tissue measures (e.g., bioaccumulation).

Soil Source and Characteristics

In all soil toxicity tests, the source of the soils used is critical to the study design and interpretation. The source of the soil may be a very precisely-made artificial soil to which a known amount of test compound or a potentially contaminated soil is added. Artificial soils are particularly useful when the comparison of two or more chemicals or sites is desired or when a chemical has been, or is anticipated to be, released into the environment at known concentrations, for example, at pesticide label registration rates (Stenersen 1979, Bouwman and Reinecke 1987, Inglesfield 1984).

When contamination of ambient soils is suspected, soil samples can be collected in the field and brought into the laboratory for testing. The subsequent soil toxicity test functions to assess the potential site hazard. Toxicity evaluations of field collected soils can be performed on: 1) soils collected from a potentially contaminated site, 2) soils collected from a reference site, 3) site soils spiked with compounds of concern, 4) reference soils spiked with compounds, 5) site soils diluted with artificial soil, and 6) site soils diluted with reference soils. It is important to note, however, that maintaining the integrity of soils during collection, transport and laboratory testing is extremely difficult. Soils are composed of microenvironments, each with their own redox gradient, and interacting physico-chemical and biological process. These processes are known to influence soil toxicity through their effect on microbial degradation, chemical sorption, solubility and availability, and partitioning coefficients.

Addition of Test Chemicals to Soil

Regardless of the source, the test chemical or contaminated, reference or control soils should be clearly identified in the test protocol. To the extent possible, physical and chemical properties of the test material should be ascertained and taken into consideration during study design. Critical information includes physical state, solubility in aqueous or suitable carriers (e.g., vegetable oil, organic solvents such at acetone, ethanol, or methanol) at the intended concentrations, stability of the compound in solution and in the test medium, vapor pressure, and other physico-chemical properties. For the most part, soil toxicity tests, by their very nature, do not adequately assess test chemicals with high volatility or those that are rapidly transformed biologically or chemically. Chemical equilibrium, especially in light of test material degradation or dissipation and study duration, should be considered in all study designs.

It is therefore critical that the chemical(s) of concern be characterized prior to study design. Proprieties of the test material that should be measured and/or considered include: source, composition, purity, nature and quantity of impurities, physico-chemical properties such as water solubility, vapor pressure at $25^{o}C$, structure, functional groups, nature and position of substituting groups, degree of substitution, octanol/water partition coefficient (K_{ow}), disassociation constant, degree of polarity, pH of pure and serial dilutions, soil sorption coefficient (K_s) or sorption constant (K_{oc}), organic matter constant, hydrolysis, and photolysis rate constants.

Water solubility, soil sorption, octanol-water partitioning and vapor pressure largely control physical transport and bioavailablity. This is particularly important in those test procedures where viable microbial or plant components are present or necessary to test procedures. Water-soluble compounds are transported with soil water through the test medium and become associated with the water films surrounding soil particles and other organic components such as root surfaces. Microbial degradation can occur in the micro-environments associated with the soil particles and other soil components. Plant roots can absorb contaminants from this film as well. Roots may also influence contaminant solubility through release of organic acids in the rhizosphere (e.g., via activity of mycorrhizal fungi).

As discussed above, soil sorption of organic molecules is controlled by the contaminant's properties, such as molecular weight, ionic speciation, acid-base properties, polarity and nature of functional groups, and by soil properties, such as organic matter content, clay content, clay mineralogy and nature, pH, water content, bulk density, cation exchange capacity, and percent base saturation. When contaminants, even water-soluble contaminants, are strongly bound to organic materials, they may become effectively immobilized and therefore relatively resistant to biodegradation. Highly sorbed materials may, however, be susceptible to extracellular enzymatic degradation. In some cases, highly sorbed chemicals may also displace micronutrients such as inorganic nutrient ions, from exchange sites in the soil, potentially affecting the health of biological components.

Collection of Contaminated Soils from the Field

As previously mentioned, maintaining the integrity of soils during collection, transport and laboratory testing is extremely difficult. Changes in rates or amounts of microbial degradation, chemical sorption, solubility and availability, and partitioning in soils has the potential to modify the degree of toxicity. Soils should be collected from the field following strict quality control/quality assurance (QA/QC) guidelines and standardized collection procedures. In addition to measuring the mass of chemicals of concern in each sample, other soil measurements and characteristics should be quantified. Depending on the purpose of the test and to select appropriate reference or control soils, the following parameters should be measured in each sample:

- pH
- Moisture content/soil porosity
- Bulk density
- Total organic matter/total organic carbon

- Soil type and texture (sand, silt, clay)
- Grain size/mineralogy
- Cation exchange capacity
- Exchangeable cation concentrations (potassium, calcium, sodium, magnesium)
- Salinity (as assessed by electrical conductivity)
- Macronutrient levels (nitrogen, phosphorous).

Preparation or Collection of Appropriate Control or Reference Soils

A common feature of soil toxicity tests is the use of negative, reference, and positive controls. Control groups are generally prepared and subjected to the exact experimental conditions as the treatment groups. They are used for a variety of purposes to ensure the integrity of the test system, including: 1) to measure the acceptability of the test, 2) to ensure the health and quality of the test organisms in general, and to ensure that no shifts in test organism sensitivity has occurred, 3) to ensure that test conditions (e.g., organism handling, husbandry and environmental parameters) are suitable for the test, and 4) to provide a basis or comparison for interpreting the results. The use of negative, reference, or positive treatment groups is required in some tests and only recommended in others. The use of positive control groups is often required only periodically, depending upon the frequency that the test is conducted in a particular laboratory in order to verify the sensitivity of the test organism. Regardless, the use of positive or negative treatment groups should also be addressed in the test protocol.

When collecting soils from the field and performing toxicity tests, it is critical that an appropriate reference site(s) is selected. Soil parameters (e.g., pH, grain size, organic matter, nutrient levels and others discussed above) should be as closely matched as possible. Otherwise, these parameters may influence the outcome of the test to a greater extent than the chemical contamination itself. It is also important to select an appropriate reference site to obtain "clean" soils which can be used to proportionally dilute site soils for establishing site-specific soil toxicity effects levels. We recommend range-finding or pre-sampling/analysis of site and proposed reference soils to determine whether an adequate reference site exists nearby the contaminated site of interest.

Quality Assurance

The Good Laboratory Practice Standards 40 CFR Part 160 and 40 CFR Part 792, promulgated under the Federal Insecticide Fungicide and Rodenticide Act (FIFRA) and the Toxic Substance Control Act (TSCA), respectively, as well as the equivalent standards provided by the Organization for Economic and Community Development (OECD) apply to most of the guidelines provided in this overview. This is particularly true when the US EPA or other relevant government organizations will use the data from these tests to assess the hazards of a test substance to the environment for purpose of seeking registration approval of a product. Alternatively, when testing soil from a Superfund designated or suspected contaminated sites for contamination effects, the quality principles to be followed should be identified in the Data Quality Objectives and the Quality Assurance Plan under CERCLA or other appropriate regulations.

Test Selection

Soil toxicity tests are generally designed to evaluate or detect the lethal or sublethal effects of chemicals on organisms in soil ecosystems. Test selection should be based on the relevant questions being asked. Some considerations for test selection can be found in Table 1-1.

Test Utility, Strengths and Limitations

Each study design and test protocol has uses and limitations unique to their design that are generally described in detail in the test guidelines. Overall, soil toxicity tests have significant advantages over other forms of hazard assessment and are often used in conjunction with other site assessment techniques (i.e., chemical analysis) to strengthen the overall understanding of contaminated terrestrial sites. Soil toxicity testing has the advantage of being relatively quick, simple and inexpensive to perform. Moreover, it can provide a unique insight into the complex biological functions of bioavailablity, trophic energy transfer and nutrient cycling. Soil toxicity tests are therefore important tests for studying the biological availability, movement and effects of contaminants in an ecosystem.

In addition, soil toxicity tests can also be easily used to compare the relative sensitivities of soil organisms to particular chemicals or chemical mixtures. They are particularly useful in comparing chemicals of concern or in identifying and isolating spatial and temporal distributions of soil toxicity. They can also greatly assist in understanding the effects of soil characteristics (e.g., pH, clay or organic content, salinity, etc.) on soil toxicity or bioaccumulation. This is a lot to ask of any series of tests and is an indication of their great potential and usefulness.

When soils collected on potentially contaminated sites are the test materials, the collection and handling of each test soil becomes focal to the test procedures. The resulting laboratory analysis is only as useful as the source and quality of the test material. Detailed collection, handling and documentation procedures must be included as part of the testing protocol. Any alteration of the field sample through transport, storage, sieving, mixing or sub-sampling could affect the test results.

Laboratory toxicity tests are often limited, for practical reasons, to species which are amenable to laboratory use and conditions, such as ease of husbandry, reproduction and maintenance, ease of handling and measuring meaningful endpoints, commercial availability, and knowledge of life history and biology. Species amenable to laboratory use may not have ecological significance, in terms of their functional importance in soil processes, in many or all soils, nor may they be key indicator species (Moore and Ruiter 1997, Eijsackers 1997). Further study of species comparative sensitivities and predictive capacity will allow greater guidance for species selection in toxicity tests.

Natural soils are composed of living and nonliving components in complex heterogeneous mixtures. Thus soils collected in the field are composed of multiple micro-environments with associated redox gradients and interacting physico-chemical and biological processes. Any disruption in these processes could affect the toxicity assessment of the soil by altering the

availability of the test compounds to the test organisms, microbial degradation, chemical sorption, etc. Changes in temperature, for example, can alter contaminant solubility, partitioning coefficients, chemical interactions and other physical and chemical characteristics. Maintenance of test soil integrity during collection, transport and handling, consistent soil treatment, and careful documentation of procedures all lead to improved study interpretation and hazard assessments.

Physico-chemical differences between assessment site soils and selected references soils can lead to equivocal toxicity test findings. As summarized by Giller et al. (1998), in field studies (i.e., similar to those used to support Superfund ecological risk assessment and remedial decision-making), it is nearly impossible to find or sample a "control" or "reference" site soil which differs from a contaminated soil only in terms of some level of metal or other type of contamination, but still possess the same physico-chemical and biological characteristics.

Toxicity test results from field-collected soils can also be complicated by the presence of indigenous microbial and invertebrate organisms. Microorganisms such as bacteria, molds, fungi, or algae, are necessary for some assays (i.e., carbon transformation, OECD 2000), but may be confounding factors with other assays. Invertebrates may be of the same or closely related species to one being tested or may compete with, or prey upon, the test species. The required addition of food to containers in some assays (e.g., chronic annelid toxicity tests) may provide a source of contamination to the container, as well as alter the original microorganism levels and composition. Such interference can be accounted for through careful planning for control samples and soil analyses.

Application of artificial soil studies to a contaminated site is sometimes complicated by the disparity between the artificial soil and soils in the field. The complexity of soils is well known (Eijsackers 1997). Diversity in soil type, variations in soil profiles between and within soil layers, and general lack of homogeneity within soils leads to irregular distribution of such soil aggregates as minerals, sand and clay, as well as any contaminants. The resulting spatially irregular distribution of soil components, and contaminants and their resulting interactions, can result in invalid toxicity test results if soil ecology is not considered when selecting the toxicity test and collecting soils form the field. The use of laboratory-spiked soils may not be fully representative of the soil contamination or of the physical and biological components and interactions found in the field. However, laboratory-spiked soil testing may provide the level of control necessary to isolate individual contaminants or mixtures of concern that can result in both cost and time savings by refining and focusing further field-based sample collections.

The quality of the soil toxicity test may also be affected by the nature of the contaminant of concern. Laboratory testing of highly volatile or gaseous materials is not appropriate for many soil toxicity test protocols due to the difficulty in maintaining the test material in contact with the test organisms. Some test materials may be rapidly transformed due to biological or chemical factors. Changes in chemical exposure and equilibrium during the test period due to test material breakdown or volatilization, should be controlled and/or assessed in the test's protocol and interpretation. Non-water soluble test materials may need to be dissolved in highly volatile organic solvents (e.g., acetone, methanol or ethanol) in order to incorporate the test material into the soil matrix. Soils are then well vented prior to use. Fine sand may also be used as a carrier.

Adequate solvent or carrier control samples can assist in determining whether the organic solvent used might affect the toxicity endpoint, total organic carbon levels, or other geochemical properties of the soil.

Testing protocols are often designed with procedures and materials that minimize the effects of confounding factors. As testing apparatus can affect the survival, growth and reproduction of the test organism, most test guidelines identify appropriate apparatus and equipment to be used. Care should be taken to follow the test guidelines during study conduct and describe assay procedures and equipment in detail. Deviations from the test guidelines should be identified in all study reports and their potential impacts assessed.

Conclusions

Soil toxicity and bioaccumulation tests can be important tools for decision makers, whether in approving registration requests or in assessing contaminated sites and remedial action plans. Soil toxicity test can also be used for long term monitoring programs or to assess the success of clean-up projects.

Table 1-1. Test Selection Considerations

Criteria	Considerations
Test Species	Sensitivity, exposure routes, life stage, life strategy, life form, species interaction, habitat
Chemical Factors	Volatility, concentration, distribution patterns, temporal or permanent exposure, persistence, known hazard, physico-chemical properties, water solubility, specific gravity, vapor pressure, adsorption capacity, hydrolysis in water, melting and boiling point, octanol/water partition coefficient (k_{ow})
Soil Characteristics	Soil type, soil source, soil content, soil purpose (e.g., agriculture), pH, moisture, macro- and micro-nutrient levels, water-holding capacity, existing microflora and fauna micro-climatic conditions
Test Conditions	Duration, cost-effectiveness, bias, precision, robustness, ruggedness, reproducibility, standardization, environmental applicability (temperature, pH, salinity, etc.), biological validity, statistical validity
Endpoints	Sensitivity, applicability to the chemical or anticipated exposure, chemical responsiveness, ecological realism

TOXICITY TEST METHODS FOR SOIL MICROORGANISMS, TERRESTRIAL PLANTS, TERRESTRIAL INVERTEBRATES AND TERRESTRIAL VERTEBRATES

Test Organisms: Soil Microorganisms

<u>**Overview**</u>

Soil toxicity tests using microbial organisms are generally measure the functionality of the microbial community. This is in contrast to studies in which measured endpoints include individual organism lethality/survival or reproductive success, or the bioaccumulation of test material. Chemical toxicity to microbial communities is often measured in terms of a change in the community's ability to decompose organic matter and release plant nutrients. Microbial soil toxicity studies are commonly conducted by adding the test material to a soil core containing the naturally occurring microbial community to assess the effects of the chemicals(s) on the ability of the community to maintain its functionality. When previously contaminated soils are the target of interest, microbial community function is compared against that of control soils. Inhibition of microbial community activity is thus a measure of adverse effect.

A listing of standardized and proposed microbial test protocols or guidelines can be found in Table 1-2. Individual reviews of these protocols are found later in this chapter.

<u>**Microbial Toxicity Tests**</u>

Although many microbial toxicity tests have been described (Carter 1993, Bitton and Koopman 1986, Bulich 1986, Jenkinson et al 1979, Tate and Jenkinson 1982, Holme-Hansen 1973, Xu and Dutka 1987, van Beelen and Doelman 1996, Dumontet and Mathur 1989), few are currently standardized for the purpose of assessing and regulating the effects of substances on the microbial community. In addition, most have not been subjected to strenuous intra-laboratory comparisons (round robin or ring testing) and have not been compared against more traditional soil toxicity tests (such as, earthworm toxicity tests). The validity and standardization of many of these tests have not yet been established such that they allow chemical comparisons or site comparisons that would be adequate for regulatory decision-making.

The U.S.EPA ecological risk assessment guidance (EPA 2005) does not recommend using soil microbes as assessment endpoints in ecological risk assessments. The rationale includes the lack of field validated methodology and the great spatial and temporal variation in microbial responses, which makes it difficult to evaluate the ecological consequences of any measured change in activity (Kapustka 1999, USEPA 2004). Hence, Superfund- or CERCLA-related ecological risk assessments rarely, if ever, evaluate endpoints related to soil microbial activity.

While the U.S. EPA does not yet recommend consideration of microbial endpoints as standard assessment endpoints in ecological risk assessment, there are advantages of considering microbial endpoints as lines-of-evidence in the assessment of soil toxicity. Recently, Broos et al. (2005) compared the toxicological sensitivity of a variety of microbial and plant growth bioassays. Some of the microbial bioassays used in the study also are discussed herein. The authors concluded that symbiotic nitrogen fixation by soil microbes was a useful endpoint (i.e.,

both robust and toxicologically sensitive) for assessing the toxicity of metals to soil-dwelling organisms. Most microbial toxicity tests have the advantage of being of short duration and cost-effective. They often require minimal resources in terms of personnel training, reagents and equipment. In addition, they can assess a wide range of environmental conditions and chemical effects through varying study design, laboratory conditions and endpoint assessment. The large number of effect endpoints is of particular interest for their assistance in elucidating not only the adverse effects on microbial health but also on the potential mechanism of effect. In general, endpoints are designed to assess microbial community populations and activities through measuring biomass, populations, activities and diversity. A brief survey of microbial toxicity endpoints and their assessment goals can be found in Table 1-3.

One of the oldest and most common endpoints as an assessment of microbial community health is the measure of soil microbial biomass. Over the years, a number of methods for measuring microbial biomass have been proposed (see Anderson and Domsch 1978 for a brief summary). The measurement of adenosine triphosphate (ATP) is currently one of the most widely accepted and used methods for measuring microbial biomass (Xu and Dutka 1987).

Microbial plate count methods have also been used to measure microbial communities. Microbial number or species counts alone, however, can bias results as they generally fail to provide data on meaningful effects and results are often limited to those species which can be easily cultured in the laboratory. Other more recent measures of microbial community diversity (e.g., Zak et al. 1994, Dubranic and Zak 1999, Dinel et al. 1990, Vestal and White 1989) should be explored more fully, as they offer more realistic approaches to using community diversity as a means of assessing contaminant impacts on soil microbial community function.

Microbial community health has also been assessed by its ability to cycle key nutrients including carbon, nitrogen, sulfur and phosphorous. The ability of select microbes to mineralize these nutrients is critical to ecosystem well being and, in the case of some processes such as nitrification and sulfur oxidation, are exclusively limited to microbial activity. Carbon-transformation and nitrogen-transformation procedures have been standardized and published (OECD 2000ab) as well as a bioluminescent assay (ASTM 1998). Detailed reviews of these methods can be found below.

Microbial community health has also been assessed through a number of microbial substrate biodegradation assays (Nannipieri et al 1990, Dobbins et al. 1992). In these assays, easily measured or radiolabelled substrates are incubated in test soils and degradation by microbial communities is determined as a measure of microbial health and activity. In addition, there are a large number of miscellaneous microbial activity assays, such as enzyme inhibition, respiration, ATP content, adenylate energy charge (AEC), incorporation of radiolabelled nucleic acids, and calorimetry that, with time, may become more standardized and usable to environmental effect assessments and regulatory decision making.

Table 1-2. Standardized soil testing protocols and guidelines for microorganisms

Species / Duration	Test Guideline Title	Test Organism	Life Stage	Test Duration	Endpoint	Reference
Microorganism						
Acute						
	Soil Microorganisms: Carbon Transformation Test	Soil microbes	N/A	28-100 days	Rate of respiration [mean carbon dioxide released (mg carbon dioxide/kg dry weight soil/h) or mean oxygen consumed (mg oxygen/dry weight soil/h)].	**[OECD] Organization for Economic Cooperation and Development. 2000. OECD Guideline For The Testing Of Chemicals: Soil Microorganisms: Carbon Transformation Test. No. 217. Paris, France. 21 January 2000.**
	Soil Microorganisms: Nitrogen Transformation Test	Soil microbes	N/A	28-100 days	Nitrate production (mg nitrate/kg dry weight soil/day).	[OECD] Organization for Economic Cooperation and Development. 2000b. OECD Guideline For The Testing Of Chemicals: Soil Microorganisms: Carbon Transformation Test. No. 218. Paris, France. 21 January 2000
	Luminescence Bioassay	*Photobacterium phosphoreum* Strain NRRL B-11177	N/A	Approx. 1 hour	Quantitative reduction in light output of luminescent marine bacteria (i.e., IC20 or the calculated concentration of sample that would produce a 20% reduction in the light output of exposed bacteria over a specified time)	[ASTM] American Society for Testing and Materials. 1996. Standard Guide for Assessing the Microbial Detoxification of Chemically Contaminated Water and Soil Using a Toxicity Test with a Luminescent Marine Bacterium. Annual Book of Standards D 5660-96. West Conshohocken, PA. May 1996.
	Soil Microbial Community Toxicity Test	Soil microbes	N/A	28 days	Ammonification and nitrification (measured as NH_3 and NO_3 concentration per gram of soil, respectively) and respiration (CO_2) eflux	[EPA] US Environmental Protection Agency. 1987. Soil Microbial Community Toxicity Test. EPA 40 CFR Part 797.3700. Toxic Substance Control Act Test Guidelines; Proposed rule. 28 September 1987.

(Continued)

Table 1-2. Continued.

Species / Duration	Test Guideline Title	Test Organism	Life Stage	Test Duration	Endpoint	Reference
Microorganism						
Chronic						
	Soil-Core Microcosm Test	Soil microbes	N/A	12 weeks or longer	Effect of chemicals on 1) growth and reproduction of either naturally occurring vegetation or crop(s) of interest, 2) nutrient uptake and cycling within the soil/plant system, 3) potential bioaccumulation (enrichment) of test material into plant tissue and 4) the potential for and rate of transport of the chemical through soil to ground water	[EPA] US Environmental Protection Agency. 1996. Ecological Effects Test Guideline OPPTS 850.2450 Terrestrial (Soil-Core) microcosm Test. EPA 712-C-96-143, April 1996 [ASTM] American Society for Testing and Materials. 1987. Standard Guide for Conducting a Terrestrial Soil-Core Microcosm Test. Annual Book of Standards E 1197-87. West Conshohocken, PA. November 1987.

Table 1-3. Microbial toxicity and activity tests, endpoints and assessment.

Tests	Endpoint	Assessment Goal	Reference
Microbial Biomass Tests			
	Microbial Biomass - Adenosine triphosphate (ATP) synthesis	Microbial biomass as measured by ATP	Jenkinson et al. 1979, Tate and Jenkinson 1982, Holme and Hansen 1973, Xu and Dutka 1987
	Microbial Biomass – Chloroform fumigation technique	Microbial biomass as measured by recolonizing microbial population decomposition of microbial cells	Jenkinson and Powlson 1976, Brookes and McGrath 1984
	Miscellaneous Biomass Assays	Microbial biomass as measured by physiological or separation methods	See Anderson and Domsch 1978 for a brief overview, Dumontet and Mathur 1989
Microbial Population Tests			
	Microbial Populations – Plate count method	Growth and biosynthesis	Olsen and Thornton 1982, Duxbury and Bicknell 1983
	Microbial growth assays	Population growth and cell motility	
Microbial Activity Tests			
	Carbon transformation	Growth and biosynthesis, nutrient cycling, respiration	OECD 2000a, Anderson and Domsch 1978.
	Nitrogen transformation	Growth and biosynthesis, nutrient cycling, nitrification	OECD 2000b
	Sulfur transformation	Growth and biosynthesis, nutrient cycling, sulfur mineralization	Strickland and Fitzgerald 1983
	Nitrogen Cycling	Growth and biosynthesis, nutrient cycling, nitrification	Wainwright 1978, Powell and Prosser 1986
	Luminescence bioassay	Cellular metabolism	ASTM 1996, Microbics Corporation 1992, Bulich 1986

(Continued)

Table 1-3. Continued.

Tests	Endpoint	Assessment Goal	Reference
	Miscellaneous microbial substrate biodegradation assays	Measure substrate degradation by microbial communities as an indicator of microbial health and activity	Nannipieri et al. 1990, Dobbins et al. 1992
	Miscellaneous Microbial Activity assays	Activity and enzyme inhibition, including dehydrogenase assay, respiration, ATP content, adenylate energy charge (AEC), incorporation of radiolabelled nucleic acids, calorimetry (heat production), enzyme activity	Christensen et al. 1982, Dutka and Bitton 1986, Dumontet and Mathur 1989, Dutton et al. 1988, Eiland 1985, Ladd 1985, Lenhard 1968, Nannipieri et al. 1980, Orgenics Ltd. 1985ab, Sparling 1981, Tyler 1974
Microbial Diversity Tests			
	Biological activity	Microbial functional diversity	Zak et al. 1994
	Fungilog activity	Fungal functional diversity	Dubranic and Zak 1999
	Lipid signatures	Microbial diversity	Dinel et al. 1990, Vestal and White 1989

Microbial Soil Core Test

In this assay, intact soil core microcosms are used to test the environmental fate and transport, and ecological effects of chemicals that may enter the terrestrial ecosystem at site-specific or regional levels. Specifically this approach is intended to assess the potential ecological impacts and environmental transport and fate of chemicals applied to agricultural soils or accidental chemical spills in natural soils through the measurement of plant growth and reproduction. Microbiological assessment is obtained as it pertains to nutrient uptake and cycling within the soil and plant.

Microbial Community Test

This approach is used to assess the toxicity of chemical substances and mixtures to microbial populations indigenous to the soil. Surface soil is incubated with the test substance and analyzed for NH_3 and NO_3 concentrations and CO_2 efflux rate to determine microbial health as a function of ammoniafication, nitrification and respiration processes, respectively.

Standardized Soil Toxicity Tests: Microbial

The following tests are reviewed in this section :

- Soil Microorganisms: Carbon Transformation Test
- Soil Microorganisms: Nitrogen Transformation Test
- Microbial Detoxification of Chemically Contaminated Water and Soil
- Using a Toxicity Test with a Luminescent Marine Bacterium
- Soil Microbial Community Toxicity Test
- Terrestrial Soil-Core Microcosm Test

Toxicity Test: Soil Microorganisms: Carbon Transformation Test

Summary Description:	This test is based on the understanding that soil microorganisms play a critical role in soil health by breaking down and transforming organic matter. Interference in these biochemical processes could adversely affect nutrient cycling and soil fertility. This assay measures the effect of chemicals of interest on carbon transformation in aerobic surface soils under laboratory conditions favorable to microbial metabolism. Soils are treated, homogenized and incubated in the dark at room temperature for up to 100 days. Soils are periodically sampled and glucose-induced respiration rates measured as carbon dioxide released or oxygen consumed. Results are compared against control samples or a dose-response is prepared. Changes in respiration reflect changes in size and activity of microbial communities through both chemical stress and carbon starvation.
Source:	
Standardized:	Yes, adopted 21 January 2000
Reference:	[OECD] Organization for Economic Cooperation and Development. 2000. OECD Guideline For The Testing Of Chemicals: Soil Microorganisms: Carbon Transformation Test. No. 217. Paris, France. 21 January 2000.
Targeted Assessment:	Designed to investigate the long-term potential effects of a single exposure of a chemical (principally pesticides and other crop-protection products but possibly other contaminants) on carbon transformation activity of soil microorganisms. Detailed study designs for agrochemicals and non-agrochemicals can be found in the test method.

Summary At-A-Glance

Test location:	Laboratory
Test System:	
Test Species:	Soil microbial community
Lifestage:	NA
Strain:	NA
Soil Type Used:	Sandy soil (between 50-75% sand) low in organic matter (organic content 0.5-1.5%) should be used. Soil pH should be between 5.5-7.5 with microbial biomass, measured as carbon content, at least 1% of total soil organic carbon. Soil is designed to minimize chemical adsorption and maximize chemical availability to

	microorganisms and generally represent a worst-case scenario. Other soils types (e.g., highly acidic) may be recommended based on test purposes.
Test Conditions/ Procedures:	The sieved (particle size $\leq$ 2mm) soil samples are treated with the test chemical and homogenously mixed or left untreated (controls). Common carriers, when needed, include water and fine quartz sand. Soils are then incubated as either a bulk sample or in a series of individual, equally sized subsamples under conditions that allow rapid microbial metabolism [in the dark at room temperature (20±2 ^{o}C)]. Soils should be adjusted and maintained for moisture content (40-60% of the maximum water holding capacity) throughout the test with distilled, dionionized water. A minimum of three replicate per treatment are recommended. Soils are generally then sampled at 0, 7, 14, and 28 days and tested for glucose-induced respirations rates. Results are measured and reported as mean carbon dioxide released (mg carbon dioxide/kg dry weight soil/h) or mean oxygen consumed (mg oxygen/dry weight soil/h) and as percent deviation from the control or % inhibition. Results are evaluated using generally acceptable statistical methods (F-test, 5% significance level) or a dose-response curve is prepared, as appropriate to the study design.
Test Duration:	28 days minimum to a maximum of 100 days or until the difference in carbon dioxide released or oxygen consumption consumed between treated and control samples is less than 25%, whichever is shorter.
Test Endpoint:	Glucose-induced respirations rates [mean carbon dioxide released (mg carbon dioxide/kg dry weight soil/h) or mean oxygen consumed (mg oxygen/dry weight soil/h)].
Test Validity:	Since test results are based on relatively small differences (i.e., average value ± 25%) between treatment and control groups, large variations in controls can lead to false results. Variation between replicate control samples should therefore be less than ± 15%. When a geometric series of concentrations are used, the concentrations tested should cover the range needed to determine the Effective Concentration (EC)$_X$ values.
Records Required:	Detailed soil collection, history and use pattern records, soil depth of sampling, soil storage (including any pre-incubation) records, sand/silt/clay content (% dry wt), soil pH in water, organic carbon content (% dry wt), nitrogen content (% dry wt, cation exchange capacity (mmol/kg), pre- and post-incubation microbial biomass (% of total carbon), test material identification and application, and test conditions, test results.
Training Requirements:	Nominal

Equipment Requirements:	Nominal for standard soil laboratory
Test History and Use:	This test was based on the recommendations of the European and Mediterranean Plant Protection Organization (EPPO 1994), but took into consideration other guidelines (BBA 1990, EPA 1987, SETAC-Europe 1995). Details of the test were finalized in an OECD workshop on Soil/Sediment Selection, held at Belgirate, Italy, in 1995 (OECD 1995).
Test Benefits and Limitations:	This test is standardized and quick, easy and inexpensive to conduct. This test was originally developed for test materials for which the type and amount reaching the soil could be anticipated (e.g., agrochemicals with known application rates), however by using a series of concentrations for other test materials a dose-response curve can be prepared and corresponding EC_X calculations can be calculated.

Toxicity Test: Soil Microorganisms: Nitrogen Transformation Test

Summary Description:	This test is based on the understanding that soil microorganisms play a critical role in soil health by breaking down and transforming organic matter. Interference in these biochemical processes could adversely affect nutrient cycling and soil fertility. This assay measures the effect of chemicals of interest on nitrogen transformation in aerobic surface soils under laboratory conditions favorable to microbial metabolism. Soils are sieved, amended with powdered plant meal (i.e., to ensure a nitrogen source) and treated with the test substance or left untreated (control). Samples are incubated under conditions to ensure adequate gas exchange and prevent water loss in the dark at room temperature for at least 28 days. Soils are periodically sampled (0. 7, 14, 28 days) and extracted with an appropriate solvent and nitrate measured. Results are compared against control samples and/or a dose-response is prepared. Nitrate forms following the degradation of the carbon-nitrogen bonds. Therefore, if nitrate production is equal in treated and control samples, major carbon degradation pathways are presumed to be intact and functional. Changes in nitrogen transformation, however, may reflect changes in size and activity of microbial communities through chemical stress.

Source:	
Standardized:	Yes, adopted 21 January 2000
Reference:	[OECD] Organization for Economic Cooperation and Development. 2000b. OECD Guideline For The Testing Of Chemicals: Soil Microorganisms: Carbon Transformation Test. No. 218. Paris, France. 21 January 2000.
Targeted Assessment:	Designed to investigate the long-term potential effects of a single exposure of a chemical (principally pesticides and other crop-protection products but possibly other contaminants) on nitrogen transformation activity of soil microorganisms. Because nitrate formulation takes place subsequent to carbon transformation, results also allow some estimation of the function of the major carbon transformation pathways. Detailed study designs for agrochemicals and non-agrochemicals can be found in the test method.

Summary At-A-Glance

Test location:	Laboratory
Test System:	

Test Species:	Soil microbial community
Lifestage:	NA
Strain:	NA
Soil Type Used:	Sandy soil (between 50-75% sand) low in organic matter (organic content 0.5-1.5%) should be used. Soil pH should be between 5.5-7.5 with microbial biomass, measured as carbon content, at least 1% of total soil organic carbon. Soil is designed to minimize chemical adsorption and maximize chemical availability to microorganisms and generally represent a worst-case scenario. Other soils types (e.g., highly acidic) may be recommended based on test purposes.
Test Conditions/ Procedures:	The sieved (particle size ≤ 2mm) soil samples are amended with a suitable organic substrate [e.g., powdered lucerne-grass-green meal (main component: *Medicago sativa*)] with a C/N ration between 12/1 and 16/1 at a recommended ratio of 5 g of lucerne per kilogram of soil (dry weight). Soils are then treated with the test chemical and homogenously mixed or left untreated (controls). Common carriers, when needed, include water and fine quartz sand. Soils are then incubated as either a bulk sample or in a series of individual, equally sized subsamples under conditions that allow rapid microbial metabolism (in the dark at room temperature ($20\pm2\ ^{\circ}$C)). Soils should be adjusted and maintained for moisture content (40-60% of the maximum water holding capacity) throughout the test with distilled, dionionized water. A minimum of three replicate per treatment are recommended. Soils are generally then sampled at 0, 7, 14, and 28 days and extracted by shaking samples for 60 minutes with a suitable solvent (e.g., 0.1 M potassium chloride solution). The mixture is then centrifuged or filtered and the liquid phase analyzed for nitrate. Results are measured and reported as mean mg nitrate/kg dry weight soil/day and as percent deviation from the control. Results are evaluated using generally acceptable statistical methods (F-test, 5% significance level) or a dose- response curve is prepared, as appropriate to the study design.
Test Duration:	28 days minimum to a maximum of 100 days or until the difference in nitrate formation between treated and control samples is less than 25%, whichever is shorter.
Test Endpoint:	Nitrate production (mg nitrate/kg dry weight soil/day).
Test Validity:	Since test results are based on relatively small differences (i.e., average value $\pm$ 25%) between treatment and control groups, large variations in controls can lead to false results. Variation between replicate control samples should therefore be less than $\pm$ 15%. High nitrogen quantities in some test substances may contribute to

	the nitrate quantities form during the test, particularly at high concentrations. Appropriate controls must be developed and accounted for with these chemicals. When a geometric series of concentrations are used, the concentrations tested should cover the range needed to determine the EC_X values.
Records Required:	Detailed soil collection, history and use pattern records, soil depth of sampling, soil storage (including any pre-incubation) records, sand/silt/clay content (% dry wt), soil pH in water, organic carbon content (% dry wt), nitrogen content (% dry wt, cation exchange capacity (mmol/kg), pre- and post-incubation microbial biomass (% of total carbon), test material identification and application, and test conditions, test results.
Training requirements:	Nominal
Equipment Requirements:	Nominal for standard soil laboratory
Test History and Use:	This test was based on the recommendations of the European and Mediterranean Plant Protection Organization (EPPO 1994), but took into consideration other guidelines (BBA 1990, EPA 1987, SETAC-Europe 1995). Details of the test were finalized in an OECD workshop on Soil/Sediment Selection, held at Belgirate, Italy, in 1995 (OECD 1995).
Test Benefits and Limitations:	This test is standardized, as well as quick, easy and inexpensive to conduct. This test was originally developed for test materials for which the type and amount reaching the soil could be anticipated (e.g., agrochemicals with known application rates), however by using a series of concentrations for other test materials a dose-response curve can be prepared and corresponding EC_X calculations can be calculated.

Toxicity Test: Microbial Detoxification of Chemically Contaminated Water and Soil Using a Toxicity Test with a Luminescent Marine Bacterium

Summary Description:	This test method is design as a rapid evaluation of the toxicity of wastewaters or aqueous extracts from contaminated soils and sediments to a luminescent marine bacterium. In this study the bioluminescent marine bacteria *Photobacteria phosoreum* is exposed to the test wastewater or aqueous soil/sediment extract and the inhibition of light output measured over a specified time and compared to controls. The reduction in light output is an indication of the toxicity of the test material to this specific microbe and has implications on the biodegradation of the test material and is often used to assess samples resulting from biotreatability studies and procedures. Also known as the Microtox® Assay System.
Source:	
Standardized:	Yes, approved 10 March 1996
Reference:	[ASTM] American Society for Testing and Materials. 1996. Standard Guide for Assessing the Microbial Detoxification of Chemically Contaminated Water and Soil Using a Toxicity Test with a Luminescent Marine Bacterium. Annual Book of Standards D 5660-96. West Conshohocken, PA. May 1996.
Targeted Assessment:	A rapid assessment of acute toxicity of a soil extract, and its associated contaminant(s), to the bioluminescent marine bacteria *Photobacteria phosoreum* as a measure of toxicity through toxicity inhibition analysis. Results are reported as the IC20, or 20% inhibitory concentration in produced light output readings.

Summary At-A-Glance

Test location:	Laboratory
Test System:	
Test Species:	*Photobacterium phosphoreum*
Lifestage:	NA
Strain:	Strain NRRL B-11177 (source limited to Microbics Corporation, Carlsbad, CA, USA)
Soil Type Used:	Soil extract
Test Conditions/ Procedures:	Test soil samples are collected or prepared according to normal procedures to reduce loss of volatile components or according to study design. All sample containers should be of borosilicate

glass, acid rinsed according to the method and capped with TFE-fluorocarbon-lined caps. Aqueous soil extracts are prepared by removing pore water, drying the soils and then reconstituting in an appropriate diluent by shaking for 16 hours. The sample is centrifuged and then decanted to provide the test extract. Soil samples and resulting extracts should be examined for suspended solids and color. Filtration may be appropriate but might impact the test results. The extract is measured for pH, dissolved oxygen (DO), conductivity and salinity. The sample salinity is adjusted to 2% NaCl to allow growth of the bacterial strain. Any adjustments to pH and DO must be noted in order to interpret results correctly. A series of test cuvettes are then prepared in a temperature-controlled area ($15 \pm 0.5 \, ^{o}C$) with the osmotically adjusted test extract in serial dilutions using non-toxic water. Allow 5-10 minutes for the samples to reach thermal equilibrium. The bacterial reagent is then reconstituted (temperature $5.5 \pm 1 \, ^{o}C$) and dispensed into a series of non-treated test cuvettes within 5 minutes of reconstitution. After mixing, the bacteria is allowed to reach a stable light output by incubating the bacteria undisturbed in the cuvettes for 15 minutes at $15 \, ^{o}C$. The cuvettes are then read with the photometer (see equipment below) at 0, 5, 15 and 30 minute exposure periods (accurately timed). The diluted test extracts are then added to the cuvettes and the cuvettes read again at 5, 15 and 30 minute exposure periods (accurately timed) or at time periods of greatest sensitivity. Note: some organics react very quickly 5-10 minutes while some metal contaminated soils can continue reacting > 30 minutes. Data is analyzed and reported using these procedures as the IC20, or the 20% inhibitory concentrations in light output readings.

Test Duration:	Approximately 1 hour
Test Endpoint:	Quantitative reduction in light output of luminescent marine bacteria (i.e., IC20 or the calculated concentration of sample that would produce a 20% reduction in the light output of exposed bacteria over a specified time)
Test Validity:	This assay was evaluated by an 18 laboratory, 4 round robin, intralaboratory comparison study using 6 blind samples (coefficient of variation = 17.8%) (Casseri et al. 1983), but has not been validated against more standard soil and microbial assays. In addition to normal negative controls, the use of reference toxicant controls, such as phenol or zinc sulphate, is recommended for validation of data produced with different lots of reagent (i.e., bacteria, reconstitution solution, and diluent) and over time. Any adjustments in procedures (e.g., timing, filtration, pH, etc.), must be noted and accounted for through the use of control samples in order for accurate data interpretation and particularly for

	comparative analysis with other test results. Duplicate sample series can also assist in establishing test validity.
Records Required:	Description of test sample (e.g., source, characterization, collection, handling) and subsequent extraction, source and characterization of dilution water, bacterial reagent information (e.g., lot, batch, date received, storage, reconstitution), description of the assay procedures (including temperature, pH, DO, salinity, read times), photometric readings and statistical analysis.
Training requirements:	Careful training and experience in the intricacies of the test method and demonstration of competence in procedures and timing may be necessary as operator error is an important source of error.
Equipment Requirements:	Microbics Corporation (Carlsbad, CA, USA) is currently the only known supplier of the reagents (test organism *Photobacterium phosphoreum* strain NRRL B-11177) required for this assay. The corresponding analyzer is also limited to two companies at this time, Microbics Corporation and Pharmacia LKB (Gaithersburg, MD, USA)
Test History and Use:	A large body of work has developed using this assay since approximately 1980 (ASTM 1996). Historically the assay has been used in wide number of applications beyond soil toxicity assessments including assessing the toxicity of wastewaters and extracts from soils and sediments prior to and after bioremediation. Combined with respirometry, total organic carbon, biochemical oxygen demand or spectrophotometry, this assay can be beneficial in determining the biogradability of a contaminant in water, soil or sediment.
Test Benefits and Limitations:	Standardized and rapid toxicity assessment that is amendable to monitoring soil treatment over time. Soils which are highly colored (especially red or brown) or contain high suspended solid concentrations may not be suitable for this assay due to interference of the suspended material with the test procedures, although some corrective factors can be applied. Soil extracts must be adjusted for salinity of the test organism and may also be adjusted for pH which may alter contaminant availability and reaction with the bacteria.

Toxicity Test: Soil Microbial Community Toxicity Test

Summary Description:	This guideline is used to assess the toxicity of chemical substances and mixtures using natural soils to microbial populations indigenous to the soil. Surface soil is sieved and supplemented with ground, dry alfalfa. The test substance, if soluble, is added as a solution to moisten the soil, or is added in a manner that best simulates its anticipated mode of entry in nature. All soil samples are then incubated in darkness at approximately $22^{\circ}C$. Soils are then sampled on days 5 and 28 and analyzed for NH_3 and NO_3 concentrations and CO_2 efflux rate to determine microbial health through measuring ammonification, nitrification and respiration, respectively.
Source:	
Standardized:	Yes, 1987
Reference:	[EPA] US Environmental Protection Agency. 1996. Ecological Effects Test Guideline OPPTS 850.5100 Soil Microbial Community Toxicity Test. EPA 712-C-96-161, April 1996.
Targeted Assessment:	This guideline is used to assess the toxicity of chemical substances and mixtures using natural soils to microbial populations indigenous to the soil.

Summary At-A-Glance

Test location:	Laboratory
Test System:	
Test Species:	Soil microbial community
Lifestage:	NA
Strain:	NA
Soil Type Used:	Soils should possess a pH of 4 to 8, an organic matter content between 1 and 8 percent, a cation exchange capacity greater than 7 meq/100g and consist of less than 70% sand. Soil collection should be limited to the surface layer (top 15 cm excluding the litter layer) and should not have received any fertilizer or pesticide applications within the past 24 months.
Test Conditions/ Procedures:	Surface soil, excluding the litter layer, is collected and allowed to air dry until sievable (approximately 12% water content). Large objects are manually removed and soil sieved through a 2-mm mesh screen into an inert container (such as a glass wide-mouth jar). A control sample should be immediately analyzed for the endpoints, NH_3, and NO_3 concentrations. Soils are then

supplemented with dried, ground alfalfa that has been passed through a 0.6-mm mesh screen and mixed thoroughly. The test substance is then added as a single application solution in distilled water (with appropriate solvent as required) to moisten the soil, or is added in a manner that best simulates its anticipated mode of entry in nature. Solution concentrations should be adjusted to achieve final water content of 10 kPa. Soils can then be covered with a polyethylene cover that permits gas exchange but reduces water moisture loss or left uncovered and watered to the original weight every 7 days. Controls should receive a similar amount of water without the test substance. Appropriate solvent or carrier controls should be added as appropriate. Soil samples are then incubated in darkness (to prevent photosynthesis by algal or moss growth) at approximately 22°C or a temperature that the soil microbes are accustomed in their soil environment. One soil sample is analyzed on day 5 for NH_3 and NO_3 concentrations and then discarded. A second sample is analyzed on day 5 for CO_2 evolution and the reincubated under the same conditions until day 28 when it is analyzed for NH_3 and NO_3 concentrations and CO_2 efflux to determine microbial health through measuring ammonification, nitrification and respiration, respectively, as measures of the ability of the microbial community to decompose organic matter and release plant nutrients. Concentration response curves and EC_{50} concentrations for all three variables are developed and assessed to determine whether the test material is toxic to the community residing in that particular soil. Multiple soil sources can be tested.

Test Duration:	28 days
Test Endpoint:	NH_3, and NO_3 concentrations (concentrations per gram of soil) and CO_2 efflux rate to determine microbial health through measuring ammonification, nitrification and respiration, respectively, as a measure of the soil microbial community to decompose organic matter and release plant nutrients.
Test Validity:	When a geometric series of concentrations are used, the concentrations tested should cover the range needed to determine the EC_X values. Results should be compared to appropriate controls. For a particular test substance, a test is defined as the exposure of the selected soil to a duplicate series of five concentrations of the test substance in a minimum of five replicate containers per concentration with appropriate controls.
Records Required:	Test methods and conditions, soil pH, endpoint results and statistical analysis.
Training requirements:	Nominal
Equipment	Nominal for standard soil laboratory

Requirements:	
Test History and Use:	This guideline is one of a series of test guidelines developed by the Office of Prevention, Pesticides and Toxic Substances, US EPA for use in testing of pesticides and toxic substances and the development of test data that must be submitted to the Agency for review under Federal regulations and has been harmonized with publications of the National Technical Information Service (NTIS) and Organization for Economic Cooperation and Development (OECD).
Test Benefits and Limitations:	This test is standardized and quick, easy and inexpensive to conduct and can be used for test materials for which the type and amount reaching the soil could be anticipated (e.g., agrochemicals with known application rates), or for toxic substances whose exposure is not anticipated.

Toxicity Test: Terrestrial Soil-Core Microcosm Test

Summary Description:	In this test an intact soil-core containing the natural assemblages of biota surrounded by the boundary material is collected from the site or region of interest. Soils may be agricultural soils or contaminated site soils. Agricultural soils are planted with the crops and associated grasses and broad leaves of interest. Contaminated soils are selected to include vegetation representative of the site or region. Soil cores are collected, the test chemical and seeds applied (as appropriate) and maintained for 12 or more weeks under laboratory or green house conditions of light, temperature and moisture simulating those of the site or region of interest. Microcosms are then monitored for ecological effects and test chemical fate, including transformation products and analyzed according to standard statistical means according to study design. Ecological effect measures include plant growth (e.g., primary productivity, crop yield, and total biomass) and health (e.g., plant stress or lesions), and nutrient loss or uptake (e.g., leachate measures of primary nutrients). Environmental fate measures include chemical analysis of leachate, soil or plants for the parent compound and appropriate transformed by-products.
Source:	
Standardized:	Yes, EPA approved April 1996, ASTM approved 25 September 1987, published November 1987, re-approved 1998
Reference:	[EPA] US Environmental Protection Agency. 1996. Ecological Effects Test Guideline OPPTS 850.2450 Terrestrial (Soil-Core) Microcosm Test. EPA 712-C-96-143, April 1996 [ASTM] American Society for Testing and Materials. 1987. Standard Guide for Conducting a Terrestrial Soil-Core Microcosm Test. Annual Book of Standards E 1197-87. West Conshohocken, PA. November 1987.
Targeted Assessment:	This guide is intended to define the requirements and procedures for using microcosms to test the environmental fate, ecological effects and environmental transport of chemicals that may enter the terrestrial ecosystem at either a site-specific or possibly regional level. Specifically this guide is intended to assess the potential ecological impacts and environmental transport and fate of chemicals applied to agricultural soils or accidental chemical spills in natural soils through the measurement of plant growth and reproduction, nutrient uptake and cycling within the soil/plant ecosystem, bioaccumulation of chemicals into plants, and potential transport of chemicals through soils.

Summary At-A-Glance

Test location:	Laboratory, greenhouse or environmental chamber, or field
Test System:	
Test Species:	The interacting community of autotrophs, omnivores, herbivores, carnivores, and decomposers found within an intact soil profile. The size of the test system is determined by the size of the soil core and the space needed for vegetative growth.
Lifestage:	NA
Strain:	NA
Soil Type Used:	Intact, undisturbed soil-cores containing the natural assemblages of biota surrounded by the boundary material typical of the region or site of interest. Cores should be of a depth to allow a full growing season for the natural vegetation or crop selected, without causing the roots to become root-bound.
Test Conditions/ Procedures:	In this test an intact soil-core containing the natural assemblages of biota surrounded by the boundary material is carefully collected from the site or region of interest. Care is taken to collect cores representative of the ecosystem. Cores should be of a depth to allow a full growing season for the natural vegetation or crop selected, without causing the roots to become root-bound, usually 45-60cm deep by 15-17cm in diameter. Disturbances to the soil core during extraction, transport, storage and test material application (if required) should be minimized. Disturbances to the soil coil architecture and ecosystem are minimized by collecting and maintaining the core in test-defined polyethylene tubes packed in insulated beads in a movable cart. Soil cores are treated with the chemical(s) of concern, if a contaminated site is not being used, and maintained in a greenhouse or growth chamber where environmental conditions such as light, temperature, moisture, etc, can be controlled. Test substances should be radiolabeled whenever possible. Test substances should be applied in the manner consistent with the expected release into the environment. Temperature regimes, photoperiods, and light intensities are usually designed to simulate those of the typical growing season in the region of interest. When agricultural chemicals or soils are of interest the top 20 cm of the core are added to topsoil and the core is seeded with the appropriate crop seeds along with grasses or broad leaves typically grown together as an agricultural crop in the region of interest. Seeds should be sown in a manner similar to actual farming practices, e.g., depth, cover, density, etc. For natural soils, e.g., contaminated sites, top soil is not amended and the cores are selected to incorporate a representative collection of

naturally occurring vegetation.   Soil cores are maintained for approximately 12 weeks to simulate the growing period, but may be longer for slowly maturing crops. Microcosms are watered as dictated by study needs using either distilled water or filtered rainwater. If analytical analysis of resulting leachate is part of the study design, a pre-application sample should be taken prior to chemical application. Radiolabeled test chemical may be required to distinguish between parent material, transformed material and materials naturally occurring in the test system.

Physiochemical properties of the soil and contaminant of concern are used of tailor the study design, including any range finding. Soil core microcosms test may be conducted to compare against field plots. In these studies, efforts should be exercised to maintain the soil cores under the same environmental conditions of temperature, light and moisture as possible. Any sources of variation should be identified and accounted for through appropriate statistical analysis.

Test Duration:	12 weeks or longer depending upon study design, such as crop maturation rate or growing period.
Test Endpoint:	Effect of chemicals on 1) growth and reproduction of either naturally occurring vegetation or crop(s) of interest, 2) nutrient uptake and cycling within the soil/plant system, 3) potential bioaccumulation (enrichment) of test material into plant tissue and 4) the potential for and rate of transport of the chemical through soil to ground water
Test Validity:	This test has been validated by comparing test results with data derived form a series of multi-year field plot tests for a limited number of compounds and conditions (Jackson et al. 1978, Tolle et al. 1982, 1983, Van Voris et al.1982, 1984, 1985ab). In the laboratory test validity can be maintained by randomizing treatments, treating 10 replicates per concentration treated as five replicate pairs and by maintaining appropriate controls.
Records Required:	Soil collection and history records, soil characterization and classification according to methods referenced in the guidelines, test substance characterization data, light intensity, temperature and other test conditions and treatments throughout the test period, plant productivity and health, nutrient losses and chemical fate analysis of leachate, plant material and soil as appropriate to study design, and results of statistical analysis
Training requirements:	Minimal to moderate, including a good understanding of soil ecosystem functioning
Equipment Requirements:	Specialized microcosm collection and incubation materials and equipment may be required

Test History and Use:	This guideline was based on the results of a multi-year soil-core microcosm test correlated with data derived from a series of multi-year plot tests for a limited number of materials (Van Voris et al. 1984, 1985ab, and Tolle et al. 1983). In addition, they were developed to assist US federal agencies assessing the hazard of a substance to the environment.
Test Benefits and Limitations:	Standardized and relatively inexpensive to conduct. Microcosm tests are most helpful in assessing the impact of a chemical release or spillage after preliminary knowledge of chemical properties and biological activities have been obtained. This test has the benefit over test tube and single species toxicity tests, by presence of a natural assemblage of organisms, which provide a higher order of ecological complexity capable of providing information on system component interactions and ecological processes. Like other laboratory studies, it also has the benefit of permitting investigator control over environmental conditions such as light, moisture, temperature, etc. that allows for comparative assessments between treatment groups and chemicals of interest. This test system has limitations related to scale and sampling, which in turn constrain both a) the type of ecosystems and species assemblages on which information can be gained and b) the longevity of the test system. This test will generally not provide absolute measures of toxicity, but provides an important approach to comparing responses among and between treatments. Although not designed for forested ecosystems, this assay could be modified for forested and other unique ecosystems. This test is also not designed for use with volatile or gaseous materials, or with non-water soluble contaminants. Carriers other than water are not recommended, however when needed, acetone or ethanol can be considered when choosing appropriate carrier controls. In addition, soil characteristics play an important role in how the microcosm responds to the test substance. Soil heterogeneity can contribute to a loss of sensitivity of the test. By using sufficient treatment groups, responses among and between groups can be evaluated rather than relying on the absolute value measured, thus increasing the value of this test system.

Test Organisms: Plants

<u>Overview</u>

Soil toxicity tests using plants generally measure adverse effects on seeds or plants. Effects on individual plants are used to extrapolate possible population or community level effects. For example, some species may be tolerant of a given toxicant, while others are highly sensitive. In natural ecosystems, changes in species diversity or in abundance may also influence the distribution and abundance of dependent wildlife species. Phytotoxicity (i.e., lethal or sublethal response of plants to a toxicant) tests have been developed for many types of plant species, with the majority being crop or domesticated species. Nevertheless, standard phytotoxicity tests can be adapted for use with native or undomesticated species, if necessary. Phytotoxicity testing may be used when performing ecological risk assessments, evaluating the efficacy of a selected remedial action, developing soil quality criteria, or establishing soil cleanup criteria. The most toxicologically sensitive life-stage in plants is thought to be represented by the early life-stages, from seed germination and emergence to early root and shoot development.

Standardized phytotoxicity test protocols or guidelines can be found in Table 1-4. Individual reviews of these protocols can be found later in this chapter.

Phytotoxicity tests may be performed by spiking a given toxicant to various substrates, including solutions, filter paper, artificial soils, sand, quartz beads, potting soils, or natural (i.e., field collected) soils. Field collected soils may be collected for the purpose of toxicant addition or to assess the toxicity of the soil itself. As previously mentioned, field collected soils must be obtained and stored with care, as storage and handling may change the physicochemical properties of the soil or alter chemical bioavailability. Field soils are often characterized as highly heterogeneous, even within discrete sampling locations. When assessing the potential phytotoxicity of field collected soil samples, care should be taken to collect representative samples with similar soil properties or attributes.

Factors to consider when selecting appropriate test species include relative sensitivity, life history requirements, and relevance to site conditions. Standard protocols recommend consideration of two plant groups: dicotyledons (plants with two embryonic seed leaves) and monocotyledons (plants with one embryonic seed leaf). Seeds should be sorted and selected to reduce within treatment variability. For example, a good commercial seed supplier should be found, identical seed lots/batches should be used, and miscolored or obviously misshapen, poor quality seeds should be removed.

Prior to the test, soil samples are treated with the test chemical and homogenously mixed or left untreated (controls). A large industrial mixer or cement mixer can be used to thoroughly mix the soil when modifying it by the addition of sand, clay, nutrients, or the test substance. Common carriers, when needed, include water and organic solvents (e.g., acetone). Seeds used in the study should be from the same batch/lot. Depending on the test protocol, replicate samples are created to measure within treatment and among treatment variation. Typically, a minimum of five replicates per treatment is recommended for definitive tests that provide statistically validated effects concentration 50% (EC50), EC25, or no observable effect concentration

(NOEC) values. Water is usually applied to control and treated plants throughout the duration of the test. The source of light can be natural or artificial, within specified parameters. Recommended environmental conditions include 14 hours/light per day, 20 – 30 C air temperature, and humidity greater than 30%. The duration of the test is tied to the selected endpoint. For example, seedling emergence tests are normally conducted for twice the length of time required for seed germination.

Phytoxicity endpoints are typically segregated into quantitive measurements and semi-quantitative observations. Examples of quantitative measurements include: number of seedlings that germinate and emerge from soil, time to seedling emergence, percent survival, plant height, radicle (root) length, and dry weights of above-ground vegetation and roots. Semi-quantitative or qualitative endpoints (as % observed or rated) include: visual observations of abnormal changes in growth, development, and morphology compared to reference or control. Results are evaluated using generally acceptable statistical methods (i.e., ANOVA). A subjective scoring system, based on the semi-quantitative or qualitative visual observations of plant health, may also be developed. Regression analysis, including linear and non-linear parameterizations, may be used, depending on the robustness of the collected data. Typically, a definitive test (i.e., a test carried out following a range-finding test) includes at least 10 exposure levels, 3-6 replicates per treatment, and 5-10 seeds or individual plants per replicate.

Confounding factors to consider when interpreting the data must be considered. Phytotoxicity tests are extremely sensitive to the physicochemical characteristics of the test soils (e.g., pH, nutrient levels, grain size, % organic matter, cation exchange capacity). Care should be taken to ensure that site and reference soils are as closely matched in physicochemical characteristics as possible. Soil properties such as soil texture, soil water-holding capacity, soil structure, degree of soil aeration, or soil-borne pathogens can limit seedling emergence or plant growth.

Standard Phytotoxicity Tests

A number of standardized protocols have been developed for assessing phytotoxicity. Protocols are published by various national and international entities including the ASTM International (ASTM), the U.S. Environmental Protection Agency (USEPA), the Organisation for Economic Co-operation and Development (OECD), Environment Canada, and the International Standards Organization (ISO). These standards or guidelines generally address seedling emergence tests, early seedling growth tests, root elongation tests, the *Brassica* Life-Cycle Test, or the Woody Species Growth and Development Test (Table 1-4). Each test is summarized as follows:

Seedling Emergence Tests

Several protocols or guidelines for evaluating seedling germination and emergence are available (ASTM 1994, USEPA 1996, ASTM 2003, OECD 2003). Groups of seeds or seedlings are exposed for up to 28 days (depending on the length of time until germination). Endpoints include number of seedlings that emerge, time to seedling emergence, and percent survival. Visual observations including color (e.g., chlorosis, mottling), tissue death (i.e., necrosis), vigor, and wilting may be collected.

Early Seedling Growth Tests

Several protocols or guidelines for evaluating early seedling growth are available (ASTM 1994, USEPA 1996, ASTM 2003, OECD 2003). Groups of seeds or seedlings are exposed for up to 28 days (depending on the length of time until germination). Endpoints include measures of plant height, root length, and dry or wet weights of above-ground vegetation and roots. Visual observations including color (i.e., chlorosis, mottling), tissue death (necrosis), vigor, and wilting may be collected.

Root Elongation Tests

The USEPA (1996) has published a standard guide for assessing root elongation (growth) of early seedlings. The test is conducted until 65% of the control seeds have germinated. At the termination of the test, seed germination and root length lengths are recorded. Toxicants are known to adversely affect the growth and development of the root systems of plants.

Brassica Life-Cycle Test

ASTM International (2003) has published a standard guide for assessing the entire life-cycle of *Brassica rapa* plants under laboratory conditions. As a further check of the toxicity of a particular chemical or soil amendment, seeds derived from the 1st generation of a Brassica Life-Cycle Test, may themselves be tested for potential generational effects following chronic exposure of the parental plants. *Brassica rapa* can complete its entire life-cycle within 42 days. As such, a variety of endpoints can be assessed, including mortality, growth, reproduction, and developmental effects. Endpoints include plant mass, plant height, stem diameter, number and length of stems, number of siliques (i.e., seed pods), and number and size of seeds.

Woody Species Growth and Development Test

ASTM International (2003) has published a standard guide for assessing the growth and development of woody species under laboratory conditions. Phytotoxic effects that occur as a consequence of impairment of photosynthesis or shoot/root growth can be quantified. Test plants, collected such that similar sized and staged plants are used, are planted in the test medium. Quantitative endpoints include measures of plant mass, plant height, root length, and root mass. Semi-quantitative or qualitative endpoints include parameters such as plant color, root condition, and shoot condition also can be assessed. The test duration is variable and depends upon the species investigated.

Table 1-4.　Standardized soil testing protocols and guidelines for plants

Species / Duration	Test Guideline Title	Test Organism	Life Stage	Test Duration	Endpoint	Reference
Plants						
Acute or Subchronic						
		Seedling	Early	14 to 28 days	Assessment of germination, seedling emergence, biomass (fresh or dry shoot weight, or shoot height) and visual detrimental effects (chlorosis, necrosis, wilting, mortality, leaf and stem developmental abnormalities)	[OECD] Organization for Economic Cooperation and Development. 2003. OECD Guideline For The Testing Of Chemicals: Proposal for Updating Guideline 208, Terrestrial Plant Test 208: Seedling Emergence and Seedling Growth Test. No. 208. Paris, France. 03 September 2003. [ASTM] American Society for Testing and Materials. 1994. Standard Practice for Conducting Early Seedling Growth Tests. Annual Book of Standards E 1598-94. West Conshohocken, PA. April, 1994. [EPA] US Environmental Protection Agency. 1996. Ecological Effects Test Guideline OPPTS 850.4100. Terrestrial Plant Toxicity, Tier I (Seedling Emergence) EPA 712-C-96-153, April 1996. Draft [EPA] US Environmental Protection Agency. 1996. Ecological Effects Test Guideline OPPTS 850.4225. Seedling Emergence, Tier II EPA 712-C-96-363, April 1996. Draft
		Seedling	Early	until 65% of control seeds have germinated	Seed germination rate, root length	[EPA] US Environmental Protection Agency. 1996. Ecological Effects Test Guideline OPPTS 850.4225. Seedling Emergence, Tier II EPA 712-C-96-363, April 1996. Draft
		Germinated seeds	Early	14 days	Mass and length of roots, shoots, and entire plants	[EPA] US Environmental Protection Agency. 1996. Ecological Effects Test Guideline OPPTS 850.4225. Seedling Emergence, Tier II EPA 712-C-96-363, April 1996. Draft
		Woody species (cutting or immature plant)	Early to maturing	Variable	Total plant weight, number of shoots or leaves, etc. Visual detrimental effects (same as above)	[ASTM] American Society for Testing and Materials. 2003. Standard Guide for Conducting Terrestrial Plant Toxicity Tests. Annual Book of Standards E 1963-02. West Conshohocken, PA. April, 2003.

Table 1-4. Continued.

Species / Duration	Test Guideline Title	Test Organism	Life Stage	Test Duration	Endpoint	Reference
Chronic						
	Brassica Life-Cycle	Plant	Whole life-cycle	42 days	Wet and dry foliar and root weights, maximum foliar height, stem diameter, number and length of axillary stems, number of siliques, and number and size of seeds.	[ASTM] American Society for Testing and Materials. 2003. Standard Guide for Conducting Terrestrial Plant Toxicity Tests. Annual Book of Standards E 1963-02. West Conshohocken, PA. April, 2003

Standardized Soil Toxicity Tests: Plants

The following tests are reviewed in this section :

- Early Seedling Growth Test
- Terrestrial Plant Toxicity Tests
       Seedling Emergence
       Root Elongation
       Brassica Life Cycle
       Woody Plant Species Growth and Development

Phytotoxicity Test: Early Seedling Growth Test

Summary Description:	The test provides a standard procedure for measuring the toxicities of various chemical substances, industrial and domestic effluents, industrial and domestic sludges, as well as site soils. The test provides data as to whether the test substance or site soil either inhibits or enhances the growth of terrestrial plants. The first days of seedling growth are often the most sensitive stages of plant development. Separate groups of seeds/seedlings are exposed to different concentrations/percents of test substances for at least 21 days post-emergence. The study is terminated no later than 28 days post-planting, and seedling heights, above ground dry weights, and/or root lengths are determined. Visual assessment of plant condition also may be appropriate for determining phytotoxicity effects. A no effect soil concentration, based on the reference and experimental samples, is determined.
Source:	
Standardized:	Yes, published 1994
Reference:	[ASTM] American Society for Testing and Materials. 1994. Standard Practice for Conducting Early Seedling Growth Tests. Annual Book of Standards E 1598-94. West Conshohocken, PA. April, 1994.
Targeted Assessment:	Designed to investigate early life stage developmental toxicity. The first days of seedling growth are often the most sensitive stages of plant development.

Summary At-A-Glance

Test location:	Laboratory greenhouse
Test System:	
Test Species:	Plants; procedure recommended for assessing a variety of dicotyle and monocytle species (e.g., lettuce, rape, vetch, ryegrass, wheat, sorghum, and a variety of vegetable species).
Lifestage:	Seedling
Strain:	NA
Soil Type Used:	If artificial or natural soil is used, it should be sieved, to remove large objects (i.e., > 2mm, including stones, sticks)
Test Conditions/ Procedures:	The sieved (particle size $\leq$ 2mm) soil samples are treated with the test chemical and homogenously mixed or left untreated (controls). A large industrial mixer or cement mixer can be used

	to thoroughly mix the soil when modifying it by the addition of sand, clay, nutrients, or the test substance. Common carriers, when needed, include water and organic solvents (e.g., acetone). Seeds used in the study should be from the same batch/lot. When purchasing seeds it is recommended that the seeds are from stock which have not been treated with fungicide, insecticide, or chlorine. A minimum of 15 seeds per experimental treatment is recommended. A minimum of five replicates per treatment is recommended for definitive tests that provide statistically sound EC50, EC25, or NOEC values. Water is applied to control and treated seedlings throughout the duration of the test. The source of light can be the sun or artificial (i.e., lights capable of delivering 300 to 400 umole/m2/s of visible light). Recommended environmental conditions include 14 hours/light per day, 20 – 30 C air temperature, and humidity greater than 30%. Results are evaluated using generally acceptable statistical methods (i.e., ANOVA). A subjective scoring system, based on visual observations of plant health, may also be developed.
Test Duration:	28 days maximum or a minimum 21 days post seedling emergence.
Test Endpoint:	Quantitative: number of seedlings that emerge, time to seedling emergence, percent survival, plant height, radicle (root) length,and dry weights of above-ground vegetation and roots. Qualitative (as % observed or rated): abnormal changes in growth, development, and morphology compared to reference or control.
Test Validity:	Percentage change of treated seedlings from the control seedlings that are less than 10% typically are not staisticaly significant. Additional, definitive tests may be required to appropriately bracket the EC50 value or NOEC. The test is considered acceptable if the mean control or reference seedling growth does not exhibit phytotoxicity or developmental effects, and survival throughout the duration of the exposure period is at least 90%
Records Required:	Detailed soil collection, history, soil depth of sampling, soil storage, soil source chemical and physical properties, laboratory environmental conditions, test species description, test conditions, test results, and photographic documentation.
Training Requirements:	Nominal
Equipment Requirements:	Nominal for standard soil laboratory
Test History and Use:	The standard practice is under the jurisdiction of ASTM Committee E-47 on Biological Effects and Envronmental Fate and is the direct responsibility of Subcommittee E47.11 on Plant

	Toxicity. Current standard approved February 15, 1994; published April 1994.
Test Benefits and Limitations:	This test is standardized and fairly inexpensive to conduct. The test can be adapted to perform a comparison of site soils to reference soils in order to assess potential phytotoxicity. The test is extremely sensitive to the physicochemical characteristics of the test soils (e.g., pH, nutrient levels, grain size, percent organic matter, cation exchange capacity), therefore it is difficult to collect site and reference soils that are closely matched in all these parameters, while differing significantly in the level of a particular chemical of concern.

Phytotoxicity Test: Terrestrial Plant Toxicity Tests

Summary Description:	The test provides a standard procedure for establishing phytotoxicity of organic and inorganic substances, determining the phytotoxicity of environmental samples, determining the phytotoxicity of sludges and hazardous wastes, assessing the impact of discharge of toxicants to land, and assessing the effectiveness of remediation efforts. The test soils can either be collected from the field or amended with the selected toxicant. Plants are exposed from 96 hours to several months, depending on the selected species, endpoints, and protocol. For tests lasting more than 2 weeks, nutrient additives may be warranted, depending on the test objectives, in order to maximize the potential for plant growth and development. The test includes protocols for assessing seedling emergence, root elongation, the *Brassica* life-cycle, and woody plant species growth and development.
Source:	
Standardized:	Yes, published 2003
Reference:	[ASTM] American Society for Testing and Materials. 2003. Standard Guide for Conducting Terrestrial Plant Toxicity Tests. Annual Book of Standards E 1963-02. West Conshohocken, PA. April, 2003.
Targeted Assessment:	Designed to investigate both early-life stage developmental toxicity and later life stage toxic effects. The tests include short-term physiological endpoints (i.e., biomarkers), short-term tests conducted during early life stages to assess survival, growth and development; and life-cycle toxicity tests that emphasize reproductive success.

Summary At-A-Glance

Test location:	Laboratory greenhouse
Test System:	
Test Species:	Plants; procedure recommended for assessing a variety of dicotyle and monocytle species (e.g., lettuce, rape, vetch, ryegrass, wheat, sorghum, and a variety of vegetable species). The majority of species routinely used in phytotoxicity tests has been limited to agronomic plants. The standard also provides guidance for assessing the *Brassica rapa* life-cycle, as well as woody plant species growth and development.
Lifestage:	Seedlings or cuttings

Strain:	NA
Soil Type Used:	If artificial or natural soil is used, it should be sieved, to remove large objects (i.e., > 2mm, including stones, sticks). Natural soil (free of chemical contamination), commercial potting soil, synthetic soil mixes, or washed quartz may be used depending on need. The standard provides the caveats that (1) natural soils are not easily demonstrated to be free of toxic substances, (2) commercial potting soils may not be appropriate for the growth and development of some species, and (3) synthetic mixes, i.e., quartz sand or glass beads, are not representative of real world conditions.
Test Conditions/ Procedures:	The sieved (particle size ≤ 2mm) soil samples are treated with the test chemical and homogenously mixed or left untreated (controls). A large industrial mixer or cement mixer can be used to thoroughly mix the soil when modifying it by the addition of sand, clay, nutrients, or the text substance. Common carriers, when needed, include water and organic solvents (e.g., acetone). Seeds used in the study should be from the same batch/lot. When purchasing seeds it is recommended that the seeds are from stock which have not been treated with fungicide, insecticide, or chlorine. Test conditions should include negative (i.e., carrier or deionized water) and positive controls (i.e., boron as boric acid). It is recommended that test condition monitoring include: soil water holding capacity and other physicochemical characteristics (beginning of test), and light irradiance level, air temperature, relative humidity, soil pH (duration of test). *Seedling Emergence* A minimum of 25 seeds per experimental treatment is recommended. A five replicates per treatment (i.e., soil sample, additive, or amendment; positive control, negative control) is recommended for definitive tests that provide statistically sound EC50, EC25, or NOEC values. Alternatively, range-finding or maximum challenge tests may be performed. Water is applied to control and treated seedlings daily, achieving 85% saturation throughout the duration of the test. The source of light can be the sun or artificial (i.e., lights capable of delivering 100 to 200 umole/m2/s of visible light). Recommended environmental conditions include 16 hours/light per day, 20 – 30 C air temperature, and humidity greater than 30%. Results are evaluated using generally acceptable statistical methods (e.g., ANOVA, linear regression models). *Root Elongation* Seeds are incubated in etiher growth pouches, petridishes with

filter paper, or seed trays saturated with the appropriate treatment. A total of 50 seeds is used per concentration (i.e., elutrate, spiked deionized water, negative control, and positive control).

Brassica *Life Cycle*
Seeds are germinated in a test medium which may be formulated soil, a reference siol, or contaminated soil. Soils are amended either pre-emergence or post-emergence, and at various temporal spacings or concentrations. Data are collected throughout the testing period and produced seeds can be further tested for generational effects. Each replicate container (i.e., including a minimum of five per treatment) is to include 1 to 5 seeds.

Woody Plant Species Growth and Development
Obtain cuttings or plants of uniform size and weight (pretest procedures). Each replicate container should be planted with one plant. Five replicates of each soil sample, sample dilution, additive, or amendment treatment, a positive control, and a negative control are tested. The normal time required to achieve amounts of shoot and root growth acceptable for statistical characterization of the test species should be determined.

Test Duration:	*Seedling Emergence* Normal time required to achieve 90% germination should be determined for the species tested. Test duration should be approximately twice the time to seedling emergence. *Root Elongation* 120 hours. Brassica *Life Cycle* 42 days. *Woody Plant Species Growth and Development* Twice the length of time determined for a particular species to achieve amounts of shoot and root growth acceptable for statistical characterization. Growth, depending on the type of woody plant species (e.g., poplar, conifers), varies substantially.
Test Endpoint:	*Seedling Emergence* Germination percentage, shoot height, root length, dry shoot mass, dry root mass. *Root Elongation* Root length, root mass (i.e., dry weight) Brassica *Life Cycle*

Including but not limited to: germination, emergence, foliar height, stem diameter, internode length, leaf legth and width, brancing morphology, bolt timing, intial flowering date, silique development, chlorosis, stunting, and survivial. Special care must be taken to avoid injuring the plants during measurements. Termination of the test measurements include, but are not limited to: wet and dry foliar and root weights, maximum foliar height, stem diameter, number and length of axillary stems, number of siliques, and number and size of seeds.

Woody Plant Species Growth and Development
Quantitative Endpoints: Total plant weight, number of shoots, leaves, shoot and root dry/wet weights, others, as necessary.
Qualitative Endpoints: visual observations of shoot condition, root condition, chlorosis, and others.

Test Validity:	Percentage change of treated seedlings from the control seedlings that are less than 10% typically are not statistically significant. Additional, definitive tests may be required to appropriately bracket the EC50 value or NOEC. The tests are considered acceptable if the mean control or reference endpoints do not exhibit phytotoxicity or developmental effects, and germination/growth/survival throughout the duration of the test exposure period is at least 80-90%.
Records Required:	Detailed soil collection, history, soil depth of sampling, soil storage, soil source chemical and physical properties, laboratory environmental conditions, test species description, test conditions, test results, and photographic documentation.
Training Requirements:	Nominal
Equipment Requirements:	Nominal for standard soil laboratory
Test History and Use:	The standard practice is under the jurisdiction of ASTM Committee E-47 on Biological Effects and Envronmental Fate and is the direct responsibility of Subcommittee E47.11 on Plant Toxicity. Current standard approved February 15, 1994; published April 1994.
Test Benefits and Limitations:	The tests are extremely sensitive to the physicochemical characteristics of the test soils (e.g., pH, nutrient levels, grain size, % organic matter, cation exchange capacity), therefore it is difficult to collect site and reference soils that are closely matched in all these parameters, while differing significantly in the level of a particular chemical of concern. Caution must be used in all interpretations of causality to ensure that the measured differences in response are attributable to the toxic chemicals and not due to

matrix or nutrient effects, the physicochemical properties of the soil, or soil pathogens. Testing involving volatile compounds requires specialized procedures due to rapidly changing exposure concentrations from volatilization (i.e. use of closed test chambers).

Test Organisms: Soil Invertebrates

Overview

Soil toxicity tests using invertebrate species generally characterize lethality, reproductive success, or bioaccumulation, in contrast to the functionality studies where the study endpoint is the function of the test species within the ecosystem. Invertebrate soil toxicity studies are commonly conducted by adding a known number of test organisms to the test material (either field-collected soils or spiked-artificial soils) to assess the effects of contaminants on the test organisms. Results are measured as the number of organisms surviving (or dead) or observations of sub-lethal behaviors and/or reproductive success (number of cocoons or juveniles). A listing of standardized and proposed invertebrate test protocols or guidelines can be found in Table 1-5. Individual reviews of these protocols can be found later in this chapter.

Earthworm Tests

By far the most common invertebrate test organisms used to assess soil and contaminant toxicity are members of the Family Lubricidae, i.e., earthworms. Earthworms are important members of the soil fauna and demonstrate a number of traits that make them particularly useful in assessing hazardous materials in soils. Soils are composed of living and non-living components existing in complex, heterogeneous mixtures. Earthworms maintain close physical contact with all soil components, including other soil biomass (e.g., microorganisms, other invertebrates, vegetative material, detritus, etc.). In addition to direct physical contact, earthworms ingest large quantities of soil. Earthworms can constitute up to 92% of total soil biomass and are important in nutrient cycling through breakdown and transformation of organic matter (Bouché 1988). Earthworms are easy and inexpensive to maintain in the laboratory and have minimal equipment and personnel training requirements. Equipment is generally limited to appropriate containers and environmentally controlled space. Other equipment may be specific to the endpoints of interest (e.g., chemical analysis of tissue concentration). The use of earthworms in hazard assessment, therefore, offers a particularly unique opportunity to assess a wide range of issues associated with hazardous materials in soils through the control and manipulation of laboratory (e.g., temperature, pH, moisture, salinity) and exposure conditions (e.g., soil type and source, contaminant concentration). Assessment of both direct toxicity and bioaccumulation under either acute or chronic exposures scenarios are possible in laboratory or field conditions.

Because earthworms constitute important dietary components in a variety of vertebrate and invertebrate species, such as birds, mammals, reptiles, amphibians, fish, insects and centipedes (Macdonald 1983), earthworm toxicity tests can be used to assess bioavailability and to estimate food web transfer and impacts. Major changes in the abundance of critical soil organisms, such as earthworms, could have serious adverse effects on the ecosystem. Not only would there be a reduction in their abundance for species depending upon them as a food source, particularly during reproduction and rearing of offspring, but proper transfer of trophic energy and nutrient cycling (ASTM 1997) depends on their presence near the base of the food web.

Although true earthworm field test protocols and testing are relatively rare, contaminated soils can be brought in from the field and used in several earthworm toxicity tests allowing assessment

of a wide range of soil sources. This is particularly helpful when assessing sites with known or suspected spatial (i.e., horizontal or vertical gradients or localized hotspots) or temporal distribution (e.g., pesticide application scenarios) of contaminants, or when the effect of such factors as temperature, pH, moisture or other soil characteristics (e.g., particle size, organic content, clay content, etc.) are of interest or are suspected contributors to soil toxicity. In addition, artificial soils amended with known contaminants at anticipated field concentrations (e.g., registered label pesticide rates) or in a series of concentrations can be used to assess potential impacts or to compare the relative toxicities of several chemicals by comparing calculated median lethal concentrations (LC_{50}).

Although several earthworm species are available for testing, the earthworm species *Eisenia fetida* (the common redworm) is recognized as a critical test species by both national and international authorities and utilized in a variety of approved soil toxicity guidelines and standards (e.g., ASTM 1997, EPA 1996, ISO 1993, 1996, OECD 1984, 2004) and is currently recognized by EPA as species used to screen hazardous waste sites (Greene at al. 1989). Although not a typical soil invertebrate, being more commonly found in compost-rich environments, this species is nonetheless widely considered representative of soil fauna, and earthworms in particular (Edwards 1983, Greg-Smith, et al. 1992, and SETAC 1988) and is the most common laboratory species used in toxicity testing of soils. *E. fetida* exists in two morphologically similar races, *E. fetida fetida* and *E. fetida andrei*. Both are used in testing, although *E. fetida fetida* is often preferred.

E. fetida is particularly amenable to laboratory testing due to its relatively short reproductive cycle. Cocoons hatch in 3-4 weeks and resulting worms reach maturity in 7-8 weeks at 20 ^{o}C. *E. fetida* is readily available commercially or can be bred easily in the laboratory in a wide range of rich organic waste materials. It is considered very prolific with a cocoon production of 2-5 cocoons per worm per week with each cocoon producing several worms.

In addition to being an easily maintained laboratory species, there is a wealth of established *E. fetida* test data available that assesses toxicity, reproduction and bioaccumulation in laboratory tests of a variety of organic and inorganic compounds (e.g., Marquenie et al. 1987, Neuhauser et al. 1985abc, Stafford and Edwards1985, Stenersen 1979, Beyer et al.1985, Bouwan and Reinecke 1987, Hartenstein et al. 1980, Inglesfield 1984). When four species of earthworms (including *E. fetida*) were compared in their sensitivity to ten organic compounds (representing six classes of chemicals), it was found that although the sensitivity of different species may vary, the selection of earthworm test species did not markedly affect the chemical's overall toxicity assessment (Neuhauser et al. 1985a)

Other Invertebrate Test Species and Reference Information

Other species are often necessary when the geochemical properties of the soil or other testing conditions are not within the tolerance of *E. fetida* or when a more representative species is needed for the soils in question. The species *Enchytraeus albidus* and *E. luxuriosus* are relatively new terrestrial oligochaete species (Enchytraeidae, Clitellata, Annelida) used in toxicity testing (OECD 2000d).   Species of the genus *Enchytraeus* are ecologically relevant soil-dwelling annelids species for many ecotoxicological tests. While enchytraeids are often found in soils

containing earthworms, they are also often abundant in many soils where earthworms are absent and may be a more representative species of naturally occurring soils of interest. The *Enchytraeidae Reproduction Test* (OECD 2004) has been evaluated by a number of laboratories (ring-tested) and found to be reproducible (Kula 1996). Like earthworms, Enchytraeids are amenable to laboratory conditions but have the practical advantage of a significantly shorter generation time. Test duration is only 4-6 weeks for Enchytraeids while it is 8 weeks for an earthworm reproductive test. Like other soil tests, no allowance is made in this test to account or correct for test substance degradation. Concentration stability of the test compound throughout the test period cannot be assumed.

The use of a wide variety of other soil dwelling invertebrate species in toxicity tests has been reported in the literature. However, these tests are not standardized or published by widely recognized standardization organizations or regulatory agencies. In 1992, a group of soil fauna scientists from the UK, Denmark, Germany, The Netherlands, and Sweden (and later Poland, Hungary and the Czech Republic) convened to develop test systems for the early detection and evaluation of sublethal effects of chemicals on organisms in soil ecosystems. The EU research and development project became known as SECOFASE, Development, Improvement and Standardization of Test Systems for Assessing Sublethal Effects of Chemicals On Fauna in the Soil Ecosystem (1993-1996). The result of their efforts was an important text entitled *Handbook of Soil Invertebrate Toxicity Tests* (Løkke and van Gestel 1998). Although the tests provided in this text are still gaining recognition in the regulatory community, this book provides clearly presented procedures describing animal husbandry and culturing, soil standardization, procedural controls and statistical analysis. A list of test protocols described in the Handbook can be found in Table 1-6.

Review of Standardized Toxicity Test Methods

A note about the limitations of using test method reviews: As a review, test details, particularly in study design, test material application and laboratory conditions and procedures, are by necessity not included in sufficient detail to conduct a study. Standardized testing procedures are readily available from the referenced source and should be referred to as needed for details of study conduct and interpretation.

Table 1-5. Handbook of Soil Invertebrate Toxicity Tests

Species	Test Descriptions
Enchytraeid: Oligochaeta	
Cognettia sphagnetorum	Sublethal toxicity test
Annelida: Oligochaeta	
Eisenia fetida	Sublethal toxicity test
Aporrectoda calignosa	Sublethal toxicity test
Acari: Oribatida (Orbatid mite)	
Platynosthrus peltifer	Sublethal toxicity test in soil
Platynosthrus peltifer	Sublethal toxicity test on plaster of Paris with dietary exposure
Collembola: Isotomidae (Springtails)	
Folsomia candida	Sublethal toxicity test: growth and reproduction
Folsomia fimetaria	Sublethal toxicity test
Isotoma viridis	Sublethal toxicity test: growth
Coleoptera: Staphylinidae (Beetles)	
Philonthus cognatus	Sublethal toxicity test – mature beetles
Philonthus cognatus	Sublethal toxicity test – larval stage beetles
Philonthus cognatus	Semi-field test – larval stage beetles
Myriapoda: Chilopoda (Centipedes)	
Lithobius mutabilis	Sublethal toxicity test
Diplopoda: Polydesmidae (Millipedes)	
Brachydesmus superus	Sublethal toxicity test
Isopoda: Porcellionidae (Woodlouse)	
Porcellio scaber	Sublethal toxicity test: growth
Porcellio scaber	Sublethal toxicity test: reproduction
Nematoda: Cephalobidae (Nematodes)	
Plectus acuminatus and *Heterocephalobus pauciannulatus*	Sublethal toxicity test: Competition between nematode species
Acari: Gamasida (Gamasid mite)	
Hypoaspis aculeifer	Sublethal toxicity test: Predation of a gamasid mite on the collembolan *Folsomia fimetaria*

(Løkke and van Gestel 1998)

Table 1-6. Standardized soil testing protocols and guidelines for invertebrates

Species / Duration	Test Guideline Title	Test Organism	Life Stage	Test Duration	Endpoint	Reference
Earthworms						
Acute						
	Earthworm, Acute Toxicity Tests	*Eisenia fetida*	Adult	***Filter Paper Test:* 48-72 hours** *Artificial Soil Test*: 14 days	Mortality and other noted abnormal behaviors	[OECD] Organization for Economic Cooperation and Development. 1984. OECD Guideline For The Testing Of Chemicals: Earthworm, Acute Toxicity Test. No. 207. Paris, France. April 1984.
	Toxicity in Earthworms					[EEC] European Economic Community. 1985. Directive79/8331, V, Part C: Methods for the Determination of Ecotoxicity – Level 1. DG XI/127-129/82, Rev. 1: Toxicity for Earthworms. Commission of the European Community, Brussels.

(Continued)

Table 1-6. Continued.

Species / Duration	Test Guideline Title	Test Organism	Life Stage	Test Duration	Endpoint	Reference
Earthworms						
Subchronic						
	Earthworm Subchronic Toxicity Test	*Eisenia fetida*	Adult	28 days	Mortality and other noted abnormal behaviors, pathological conditions, or weight loss	[EPA] US Environmental Protection Agency. 1996. Ecological Effects Test Guideline OPPTS 850.6200 Earthworm Subchronic Toxicity Test. EPA 712-C-96-167, April 1996
	Soil toxicity or Bioaccumulation Tests with the Lumbricid Earthworm *Eisenia fetida*	*Eisenia fetida*	Adult	14-28 days	Endpoints are dependent on purpose of the test and study design but may include animal weight, lethality, sublethal behaviors, pathological changes (segmental constriction, lesions, stiffness, etc.), reproduction, tissue accumulation, etc. Other endpoint analysis may include kinetic studies with estimate uptake, depuration rates, and time to steady state, lipid normalization and normalizing soil concentrations of non-ionic organics to total organic carbon. Reproductive endpoints might include number and growth of young worms, rate of clitellum development, number of cocoons produced, cocoon mass, number of hatchlings per cocoon, and biomass of hatchlings.	[ASTM] American Society for Testing and Materials. 1998. Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests with the Lumbricid Earthworm *Eisenia fetida.* Annual Book of Standards E 1676-97. West Conshohocken, PA. February 1998.

(Continued)

Table 1-6. Continued.

Species / Duration	Test Guideline Title	Test Organism	Life Stage	Test Duration	Endpoint	Reference
Earthworm						
Chronic						
	Earthworm Reproduction Test (*Eisenia fetida/andrei*)	*Eisenia fetida/andrei*	Adult	8 weeks observation Adult earthworms are removed after the fourth week	Adult mortality and other signs of toxicity, reproductive success as measured by number of juveniles produced.	[OECD] Organization for Economic Cooperation and Development. 2004. OECD Guideline For The Testing Of Chemicals: Test No. 222, Earthworm Reproduction Test (*Eisenia fetida/andrei*). Paris, France.

Standardized Soil Toxicity Tests: Invertebrates

The following tests are reviewed in this section:

- Earthworm Acute Toxicity Tests
- Earthworm Sub-chronic Toxicity Test
- Soil toxicity or Bioaccumulation Tests with the Lumbricid Earthworm, Eisenia fetida
- Earthworm Reproduction Test (Eisenia fetida/andrei)
- Enchytraeidae Reproduction Test

Toxicity Test: Earthworm, Acute Toxicity Tests

Summary Description:	This method describes two procedures: a simple paper contact toxicity test designed as an initial screen to identify those chemicals likely to be toxic to earthworms and therefore needing further testing and the follow-up acute toxicity study in artificial soil. The screening test exposes earthworms to concentrations of the test substance on moist filter paper over 48-72 hours as a simple, easy, reproducible test to identify potentially toxic chemicals. The artificial soil test exposes earthworms to the test substance in a precisely defined artificial soil over a range of concentrations. Results, measured as mortality, are assessed on day 7 and 14 after application. Results for both studies are analyzed by probit analysis or equivalent, and expressed as the Lethal Concentration 50 percent of the population (LC_{50}) in mg/cm2 or mg/kg (dry wt) for the paper contact and artificial soil test, respectively.
Source:	
Standardized:	Yes, 1984
Reference:	[OECD] Organization for Economic Cooperation and Development. 1984. OECD Guideline For The Testing Of Chemicals: Earthworm, Acute Toxicity Test. No. 207. Paris, France. April 1984.
Targeted Assessment:	The two tests in this guideline are designed as simple, easy tests to assess the acute toxicity (dose-response) of a variety of water soluble or insoluble chemicals to earthworms (*Eisenia fetida)*. The response is measured as mortality and reported in terms of the dose-response curve and median LC_{50}. The exposure route is through filter paper contact or artificial soil contact, respectively.

Summary At-A-Glance

Test location:	Laboratory
Test System:	
Test Species:	Earthworms; recommended test species *Eisenia fetida* although other species may be used if methodology is available.
Lifestage:	Adult; at least 2 months old with clitellum and an individual wet weight of 300 to 600 mg.
Strain:	*Eisenia fetida fetida* is recommended although the subspecies *Eisenia fetida andrei* and other species may be used if methodology is available.

Soil Type Used:	*Filter Paper Test:* No soil, moistened filter paper 80-85 g/m^2, approximately 0.2 mm thick, medium grade. *Artificial Soil Test*: a precise mixture of 10% sphagnum peat (dry, finely ground, pH 5.5-6.0), 20% kaolin clay (> 30% kaolinite preferred), 70% industrial sand (> 50% of particles should be between 50 and 200 microns), adjusted to pH 6.0 ± 0.5 by addition of calcium carbonate. Dry constituents are thoroughly mixed and moisture content brought to approximately 35% with deionized water.
Test Conditions/ Procedures:	Preliminary range-finding and definitive tests covering the range of the LC_X can be conducted for both tests. *Filter Paper Test:* Three hours prior to being placed in the test vials, worms are placed on moist filter paper so they can void their guts, and are then washed and dried before use. Meanwhile, test vials are prepared by adding the appropriate test compound dissolved in water or suitable solvent, systematically drying the vials, re-moistening with deionized water, and sealing with caps with a small ventilation hole. Control vials using the appropriate solvent without the chemical are similarly prepared. The earthworms are placed, one per vial, with at least 10 replicates into the prepared vials. Vials are then incubated at $20 \pm 2\ ^oC$ in the dark and earthworm mortality and other behaviors assessed at 48 and, optionally, 72 hours. *Artificial Soil Test*: The test mediums should be prepared before use by spraying the test chemical dissolved in appropriate water or organic solvent over the artificial soil or through using fine ground sand as a carrier and then ventilating the treated soil. Control soil should be similarly treated with the appropriate solvent/carrier only. For each replicate, 10 worms, which have been conditioned for 24 hours in an artificial soil and then quickly washed before use, are placed on 750 g of the test medium in a covered glass container to prevent drying and kept under test conditions for 14 days. Four replicates per treatment are recommended. Test conditions are $20 \pm 2\ ^oC$ in continuous light (to ensure worms remain in the test medium throughout test duration). Mortality and other behaviors are assessed at 7 and 14 days. Soil moisture should also be recorded at study completion. Results of both tests are subject to dose-response reporting and probit analysis to determine LC_X. When 2 consecutive concentrations in a geometric series (at a ratio of at most 2.0) result in 0 and 100% mortality, these two values are sufficient to indicate the range in which the LC_{50} falls.

Test Duration:	*Filter Paper Test:* 48-72 hours *Artificial Soil Test*: 14 days, mortality assessed at day 7 and 14.
Test Endpoint:	Mortality; measured when gentle mechanical stimulus to the front end produces no response; any other behavioral or pathological symptoms should also be reported.
Test Validity:	Mortality in controls should not exceed 10% at the end of either test. Periodically, laboratory test conditions should be assured with the use of a positive test reference substance, such as chloracetamide
Records Required:	Test animal source, condition and husbandry, test material identification and application, test conditions, soil moisture, test medium preparation, test results.
Training Requirements:	Nominal
Equipment Requirements:	Nominal laboratory equipment
Test History and Use:	There are many methods of toxicity testing to earthworms, including spot application, forced feeding, and immersion tests (Edwards 1983). This guideline proposes two relatively simple standardized assays to assess acute toxicity in earthworms to chemicals of concern.
Test Benefits and Limitations:	Both tests in this guideline are standardized and relatively quick and easy to conduct. The initial paper contact toxicity test is simple and highly reproducible with recommended species and functions best as an initial screening study either for comparing relative toxicities and/or selecting/ranking for further testing. The artificial soil test is more eco-relevant and representative of natural exposure of earthworms to chemicals and also allows for chemical comparisons. Like all test protocols using *E. fetida*, this recommended species is not a typical soil species, but is a surface species frequenting highly organic medium such as compost piles. However, its susceptibility to chemicals resembles that of true soil-inhabiting species and it is highly amendable to laboratory testing. *E. fetida* has a short life cycle (hatching from cocoons in 3-4 weeks), reaches maturity quickly (7-8 weeks at 20 ^{o}C), and is very prolific (producing 2-5 cocoons per week each producing several worms). It is commercially available and lives and breeds in a wide-range of organic waste material. The same strain should be used when comparing chemical hazard. (Note: *E. fetida* is commonly known as the redworm and is readily used commercially for composting and as fishing bait.)

Toxicity Test: Earthworm Subchronic Toxicity Test

Summary Description:	Test chambers are filled with appropriate amounts of treated or contaminated soils. Acclimated earthworms, species *Eisenia fetida*, are placed in the test chambers and allowed to ingest the test mixture soil *ad libitum*. Test chambers are examined every 7 days for at least 28 days for earthworm mortality and morbid worms removed. Test substance concentrations are generally over a range in concentrations resulting in the development of concentration-response curves. Final results are frequently calculated as the LD_{50}, EC_{50}, LOEC and NOEC or as a direct comparison to control soils.
Source:	
Standardized:	Yes, April 1996
Reference:	[EPA] US Environmental Protection Agency. 1996. Ecological Effects Test Guideline OPPTS 850.6200 Earthworm Subchronic Toxicity Test. EPA 712-C-96-167, April 1996
Targeted Assessment:	The tests in this guideline are designed as simple, easy tests to assess the sub-chronic toxicity (dose-response) of a variety of water soluble or insoluble chemicals to earthworms (*Eisenia fetida)*. The response is measured as mortality and reported in terms of the dose-response curve and median lethal concentration (LC_{50}) or as a comparison to control soils. Exposure routes include direct contact or ingestion of the treated or contaminated soil. Behavioral symptoms, such as general activity and location within the soil matrix are also noted.

Summary At-A-Glance

Test location:	Laboratory
Test System:	
Test Species:	Earthworms; recommended test species *Eisenia fetida andrei* although other species may be used if methodology is available.
Lifestage:	Mature adults with clitellum at anterior end, 300-600 mg in weight at study start
Strain:	*Eisenia fetida andrei*
Soil Type Used:	When using an artificial soil, it is defined as a precise mixture of 69% No. 70 mesh silica sand, 20% kaolin clay, 10% sphagnum peat moss and 2% calcium carbonate. Dry constituents are thoroughly mixed and moisture content brought to approximately 35% with deionized water.

Test Conditions/ Procedures:	Preliminary range-finding and definitive tests covering the range of the LC_X should be conducted.
	Test chambers are filled with appropriate amounts of treated or contaminated soils. Acclimated earthworms, species *Eisenia fetida*, are placed in the test chambers and allowed to ingest the test mixture soil *ad libitum* under continuous lighting, relative humidity above 85% and temperature of $22\pm2\ ^oC$. A minimum of 30 worms per concentration in three replicate groups of 10 earthworms each should be used plus a negative control group in uncontaminated soil. Distribution of individuals among test treatments should be random. Test material concentration should be assessed at the beginning of the study and at each 7 day observation period. Test chambers are examined at days 7, 14, 21 and 28 for earthworm mortality and morbid worms removed. Missing earthworms should be considered dead. Any adverse differences in weight loss, behavior (such as position and activity within the soil) and pathological condition (such as lesions, swellings or ulcerated areas) should also be noted. Test substance concentrations are generally over a range in concentrations resulting in the development of concentration-response curves. Final results are frequently calculated as the LD_{50}, EC_{50}, LOEC and NOEC or as a direct comparison to control soils.
Test Duration:	28 days
Test Endpoint:	Mortality; measured when gentle mechanical stimulus to the front end produces no response; any other adverse condition such as weight loss, behavioral changes or pathological symptoms should also be reported.
Test Validity:	Mortality in controls should not exceed 10% at the end of either test. Periodically, laboratory test conditions should be assured with the use of a positive test reference substance, such as chloracetamide
Records Required:	Test animal source, condition and husbandry, test material identification and application, test conditions (including soil pH and test material concentrations, temperature, humidity, soil moisture, test medium preparation, etc.), mortality and adverse condition observations, analytical and statistical test results.
Training requirements:	Nominal
Equipment Requirements:	Nominal laboratory equipment
Test History and Use:	There are many methods of toxicity testing to earthworms, including spot application, forced feeding, and immersion tests (Edwards 1983). This guideline proposes a relatively simple standardized assays to assess subchronic toxicity in earthworms to

chemicals of concern.

Test Benefits and Limitations:	The test is standardized and relatively quick and easy to conduct. It appears to be reproducible with recommended species and functions best as an initial screening study either for comparing relative toxicities and/or selecting/ranking for further testing. The artificial soil test is more eco-relevant and representative of natural exposure of earthworms to chemicals and also allows for chemical comparisons.
	Like all test protocols using *E. fetida*, this recommended species is not a typical soil species, but is a species frequenting highly organic medium such as compost piles. However, its susceptibility to chemicals resembles that of true soil-inhabiting species and it is highly amendable to laboratory testing. *E. fetida* has a short life cycle (i.e., hatching from cocoons in 3-4 weeks), reaches maturity quickly (7-8 weeks at 20 ^{o}C), and is very prolific, producing 2-5 cocoons per week each producing several worms. It is commercially available and lives and breeds in a wide-range of organic waste material. The same strain should be used when comparing chemical hazard. (Note: *E. fetida* is commonly known as the redworm and is readily used commercially for composting and as fishing bait.)

Toxicity Test: Soil toxicity or Bioaccumulation Tests with the Lumbricid Earthworm *Eisenia fetida*

Summary Description:	Field-collected or spiked artificial soils are uniformly prepared. Acclimated test organisms, species *Eisenia fetida* of the same source and uniform age, are placed on the prepared soil samples and allowed to burrow into the soil. Endpoints are measured at the frequency specified in the protocol and could include animal weight, lethality, sublethal behaviors, pathological changes (e.g., segmental constriction, lesions, stiffness, etc.), reproduction, tissue bioaccumulation, etc. Results are analyzed by standard statistical methods to calculate the LC_{50} or EC_{50} when a range of concentrations is used or analyzed to compare treatment to controls when single concentrations are used. The lowest observed effect concentration (LOEC) or no observed effect concentration (NOEC) may also be calculated. Bioaccumulation tests results are reported as the magnitude of contaminant concentration above Day 0 tissue analysis. Other endpoint analysis may include kinetic studies with estimate uptake, depuration rates, and time to steady state, lipid normalization and normalizing soil concentrations of non-ionic organics to total organic carbon.

Source:		
	Standardized:	Yes, published February 1998
	Reference:	[ASTM] American Society for Testing and Materials. 1998. Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests with the Lumbricid Earthworm *Eisenia fetida.* Annual Book of Standards E 1676-97. West Conshohocken, PA. February 1998.
Targeted Assessment:		The test is designed to assess lethal or sublethal toxic effects on earthworms or bioaccumulation of contaminants in earthworms in a short term test (7-28 days) in terrestrial systems.

Summary At-A-Glance

Test location:		Laboratory
Test System:		
	Test Species:	*Eisenia fetida,* other species may be used if appropriate husbandry is known and available, as appropriate to test purpose
	Lifestage:	Uniform maturity and of the same source, usually fully clitellate, sexually mature adults
	Strain:	NA

Soil Type Used:	Artificial or field-collected soils, depending upon study purpose and design. Artificial soils are recommended to consist of 10% sphagnum peat moss (that portion passing through a 2.36 mm screen), 20% kaolin clay (97% kaolinite with particle size under 40 μm) and 70% silica sand (Grade 70, 97.1% particle size of 0.053-0.3 mm) adjusted to pH of 7.0 ± 0.5 with calcium carbonate.
Test Conditions/ Procedures:	Soils should be collected or prepared and characterized for pH, percent organic matter, cation exchange capacity, total nitrogen, particle size distribution (i.e., percent sand, silt, clay), and water content. Contaminant concentration may or may not need to be analyzed prior to testing. Negative and reference soils are normally needed for proper study interpretation and are treated like treated soils in all aspects except contamination. Replication and soil source is dependent on study design. See the guideline for further details. Soils should be tested as soon as possible after collection. The day before test start, soils are screened to remove large objects and to aid in creating a homogeneous sample, adjusted to correct moisture content, spiked with the test material, if appropriate, and allowed to equilibrate to test environmental conditions. Generally three replicates per batch are prepared. On day 0, test organisms (i.e., 10 worms per 200 g dry weight of soil per container) are placed on the soil surface and allowed to burrow into the soil. Lack of burrowing should be considered a reaction to the presence of the test material. Test systems are then maintained covered with small hole(s) to minimize moisture loss but allow gas exchange, under constant temperature (between 10-26 $^{\circ}$C) and continuous light (400 lux) for the test duration. Animals are generally not fed in tests under 28 days of observation, but may be included in longer protocols. The frequency and endpoints measured are assessed as specified in the protocol, but may include animal weight, lethality, sublethal behaviors, pathological changes (e.g., segmental constriction, lesions, stiffness), reproduction, tissue bioaccumulation, etc. Results are analyzed by standard statistical methods to calculate the LC_{50} or EC_{50} when a range of concentrations is used or analyzed to compare treatment to controls when single concentrations are used. The lowest observed effect concentration (LOEC) or no observed effect concentration (NOEC) may also be calculated. Bioaccumulation tests results are reported as the magnitude of contaminant concentration above either Day 0 tissue analysis. Other endpoint analysis may include kinetic studies that estimate uptake, depuration rates, and time to steady state, lipid normalization and normalizing soil concentrations of non-ionic organics to total organic carbon. This test procedure can be adjusted to meet study needs. One

	described alternative includes the addition of Bermuda grass planted in the test cylinders to simulate natural vegetation during the test period.
Test Duration:	Generally 14-28 days, but shorter or longer times may be used
Test Endpoint:	Endpoints are dependent on purpose of the test and study design but may include animal weight, lethality, sublethal behaviors, pathological changes (e.g., segmental constriction, lesions, stiffness), reproduction, tissue accumulation, etc. Other endpoint analysis may include kinetic studies with estimate uptake, depuration rates, and time to steady state, lipid normalization and normalizing soil concentrations of non-ionic organics to total organic carbon. Reproductive endpoints might include number and growth of young worms, rate of clitellum development, number of cocoons produced, cocoon mass, number of hatchlings per cocoon, and biomass of hatchlings.
Test Validity:	Test results may be considered unacceptable for the following reasons: 1) if continuous lighting intended to maximize exposure was not used, 2) if all treatment groups were not treated identically, 3) organisms were not cultured at the same temperature, 4) soil geochemical properties were not within the tolerance for the species, 5) appropriate negative and carrier (solvent) controls were not used, 6) the solvent concentrations used adversely affected the survival, growth or reproduction of the test organism, 7) test organisms were not from the same source or of the same species, 8) treatments and test organisms were randomly assigned, 9) each container did not receive the same amount of soil (based on dry weight), 10) temperature was not in the acceptable range and 11) negative controls did not survive, grow or reproduce as required for the test species and design.
Records Required:	Chemical and physical data for soil, soil collection/preparation, transportation, storage, and mixing, chemical application, animal husbandry and culturing, worm biomass at beginning and end of study, test environmental conditions and methods used, general observations and statistical results.
Training requirements:	nominal
Equipment Requirements:	nominal
Test History and Use:	This method is the standardization of generally reported procedures used to assess the toxicity of a wide range of contaminants (e.g., Marquenie et al. 1987, Neuhauser et al. 1985abc, Stafford and Edwards1985, Stenersen 1979, Beyer et al.1985, Bouwan and Reinecke 1987, Hartenstein et al. 1980, Inglesfield 1984) and based on both OECD and EPA (Greene et al. 1989) guidelines for screening hazardous chemical and waste

	sites.
Test Benefits and Limitations:	Standardized, quick, easy and inexpensive to conduct. Like other *E. fetida* tests, environmental test conditions are limited to the tolerance range of the species. And no allowance is made in this test to account for or correct for test substance degradation. Concentration stability of the test compound throughout the test period cannot be assumed.

Toxicity Test: Earthworm Reproduction Test (*Eisenia fetida/andrei*)

Summary Description:	In this test, adult earthworms of the species *Eisenia fetida fetida* or *E. fetida andrei* are exposed to a range of concentrations of the test substance applied as a single application to the surface or mixed into the surface depending upon purpose of the test. The range of concentrations should be selected to elicit both lethal and sub-lethal effects over an 8-week exposure period. During the first 4 weeks, adult growth, mortality and other toxic effect endpoints are recorded. During the second 4 weeks, the adults are removed from the samples and the number of offspring counted in the soil. The reproductive outputs of worms exposed to the test substance are compared to reproductive output of controls in order to determine the no observed effect concentration (NOEC). Where possible, data are also analyzed by regression model to determine the concentration that would cause X% reduction in reproductive output (EC_X).

Source:	
Standardized:	Yes, 2004
Reference:	[OECD] Organization for Economic Cooperation and Development. 2004. OECD Guideline For The Testing Of Chemicals: Test No. 222: Earthworm Reproduction Test (*Eisenia fetida/andrei*). Document No. 222. Paris, France.
Targeted Assessment:	This test guideline is design to assess the effect of chemicals on reproductive output (and other sub-lethal end points) of the earthworm species *Eisenia fetida fetida* and *Eisenia fetida andrei*. This test is not suitable for volatile substances.

Summary At-A-Glance

Test location:	Laboratory
Test System:	
Test Species:	Earthworms; recommended test species *Eisenia fetida fetida* or *E. fetida andrei* .
Lifestage:	Adult; between 2 months and one year old with clitellum and an individual wet weight of 300 to 600 mg at test start. Selected worms should be from a synchronized culture with a relatively homogenous age structure (age should not differ by more than 4 weeks).
Strain:	*Eisenia fetida fetida* is recommended although the subspecies *Eisenia fetida andrei* and other species may be used if methodology is available.

Soil Type Used:	An artificial soil is used prepared according to OECD Guideline 207 (OECD 1984) and composed of a precise mixture of 10% sphagnum peat (dry, finely ground, pH 5.5-6.0), 20% kaolin clay (> 30% kaolinite), 70% industrial sand (> 50% of particles should be between 50 and 200 microns), adjusted to pH 6.0 ± 0.5 by addition of calcium carbonate. Dry constituents are thoroughly mixed and moisture content brought to between 40-60% of the maximum water holding capacity with deionized water.
Test Conditions/ Procedures:	Worms should be acclimated to the artificial soil substrate and standard feed for 1-7 days prior to study start. When the test material is to be mixed into the soil, the test material should be incorporated immediately before the worms are added. When the test material is to be applied to the soil surface, the worms are added prior to the test material and allowed to bury into the soil prior to application to prevent any direct exposure to the test substance. The test material can be dissolved in an appropriate water or organic solvent or incorporated through using fine ground sand as a carrier, as necessary. Solvents should be appropriately vented off as needed prior to study initiation. Control soil should be similarly treated with the appropriate solvent/carrier only. For each replicate (see the test procedure for details regarding study design and replicate number), 10 worms, should be weighed individually and placed into 500-600 g of the prepared test medium in a covered glass container to prevent drying and kept under test conditions for the specified time periods. Test conditions are 20 ± 2 ^{o}C under controlled light-dark cycles (preferably 16 hours light:8 hours dark) with 400-800 lux illumination in test container area. Moisture content should be monitored throughout the test period and adjusted as needed. Soil moisture should be with in 10% of starting moisture content at study completion. Test containers are not aerated. Once per week feeding (see test procedures for further feeding details) provide sufficient gas exchange. On day 28 living adult worms are removed from the test medium, counted, and weighed. Any changes in behavior or morphology are noted. The soil samples, excluding adult worms but including any cocoons, are incubated for another 4 weeks under the same test conditions. At the end of the second four weeks, the numbers of juvenile worms produced are counted and any harmful signs noted. Results of both tests are subject to study design (see test guidelines for details) but commonly result in dose-response reporting and probit analysis to determine EC_X.
Test Duration:	8 weeks observation, the adult earthworms are removed after the fourth week

Test Endpoint:	Adult mortality and other signs of toxicity, reproductive success as measured by number of juveniles produced.
Test Validity:	This test has been tested by a number of laboratories (ring-tested) and found to be reproducible (Kula 1996ab). Test validity can be assessed through the use of controls as follows: each control replicate should produce ≥ 30 juveniles by the end of the test, the coefficient of variation of control reproduction should be ≤ 30% and adult control mortality over the initial 4 weeks should be ≤ 10%. The periodic use of a reference substance (e.g., carbendazim) is advised to be run parallel to the toxicity test of a test substance at least twice a year, or more frequently when testing is being carried out at a lower frequency, to ensure the response of the test organism has not changed significantly over time. When a geometric series of concentrations are used, the concentrations tested should cover the range needed to determine the EC_X values. See the test guidelines for further detail.
Records Required:	Test animal source, condition and husbandry, test material identification and application, test conditions, soil pH and moisture, test medium preparation, test results including worm weights, number (i.e., adult and juvenile surviving), any morphological observations, and statistical analysis.
Training requirements:	Nominal
Equipment Requirements:	Nominal for standard soil laboratory
Test History and Use:	This test was based on a number of other international and national guidelines that contain provisions useful to the performance of this test (OECD 1984, ISO 1993, 1996, SETAC 1998, EPA 1996).
Test Benefits and Limitations:	This test has been evaluated by a number of laboratories (i.e., ring-tested) and found to be reproducible (Kula 1996). It is quick, easy and inexpensive to conduct. Like other soil tests, no allowance is made in this test to account for or correct for test substance degradation. Concentration stability of the test compound throughout the test period cannot be assumed.

Toxicity Test: *Enchytraeidae* Reproduction Test

| Summary Description: | In this test, adult Enchytraeidae worms of the species *Enchytraeus albidus* are exposed to a range of concentrations of the test substance applied as a single application to the surface or mixed into the surface depending upon purpose of the test. The range of concentrations should be selected to elicit both lethal and sub-lethal effects over an 8-week exposure period. During the first 4 weeks, adult growth, mortality and other toxic effect endpoints are recorded. During the second 4 weeks, the adults are removed from the samples and the number of offspring found in soil are counted. The reproductive outputs of worms exposed to the test substance are compared to reproductive output of controls in order to determine the no observed effect concentration (NOEC). Where possible, data are also analyzed by regression model to determine the concentration that would cause X% reduction in reproductive output (EC_X). |

Source:

Standardized:	Yes, 2004
Reference:	[OECD] Organization for Economic Cooperation and Development. 2004. OECD Guidelines for the Testing of Chemicals: Test No. 220: Enchytraeid Reproduction Test. Document No. 220. Paris, France.
Targeted Assessment:	This test guideline is design to assess the effect of chemicals on reproductive output (and other sub-lethal end points) of Enchytraeidae worms of the species *Enchytraeus albidus.* This test is not suitable for volatile substances.

Summary At-A-Glance

Test location:	Laboratory
Test System:	
	Enchytraeidae worms; recommended species *Enchytraeus albidus*.
	Adult; between 2 months and one year old with clitellum and an individual wet weight of 300 to 600 mg at test start. Selected worms should be from a synchronized culture with a relatively homogenous age structure (age should not differ by more than 4 weeks).
	Enchytraeidae worms; recommended species *Enchytraeus albidus*, other species (e.g., *E. luxurious*) may be used if methodology is available.

Soil Type Used:	An artificial soil is used prepared according to OECD Guideline 207 (OECD 1984) and composed of a precise mixture of 10% sphagnum peat (dry, finely ground, pH 5.5-6.0), 20% kaolin clay (> 30% kaolinite), 70% industrial sand (> 50% of particles should be between 50 and 200 microns), adjusted to pH 6.0 ± 0.5 by addition of calcium carbonate. Dry constituents are thoroughly mixed and moisture content brought to between 40-60% of the maximum water holding capacity with deionized water.
Test Conditions/ Procedures:	Worms should be acclimated to the artificial soil substrate and standard feed for 1-7 days prior to study start. When the test material is to be mixed into the soil, the test material should be incorporated immediately before the worms are added. When the test material is to be applied to the soil surface, the worms are added prior to the test material and allowed to bury into the soil prior to application to prevent any direct exposure to the test substance. The test material can be dissolved in an appropriate water or organic solvent or incorporated through using fine ground sand as a carrier, as necessary. Solvents should be appropriately vented off as needed prior to study initiation. Control soil should be similarly treated with the appropriate solvent/carrier only. For each replicate (see the test procedure for details regarding study design and replicate number), 10 worms, should be weighed individually and placed into 500-600 g of the prepared test medium in a covered glass container to prevent drying and kept under test conditions for the specified time periods. Test conditions are $20 \pm 2\ ^{o}C$ under controlled light-dark cycles (preferably 16 hours light:8 hours dark) with 400-800 lux illumination in test container area. Moisture content should be monitored throughout the test period and adjusted as needed. Soil moisture should be with in 10% of starting moisture content at study completion. Test containers are not aerated. Once per week feeding (see test procedures for further feeding details) provide sufficient gas exchange. On day 28 living adult worms are removed from the test medium, counted, and weighed. Any changes in behavior or morphology are noted. The soil samples, excluding adult worms but including any cocoons, are incubated for another 4 weeks under the same test conditions. At the end of the second four weeks, the numbers of juvenile worms produced are counted and any harmful signs noted. Results of both tests are subject to study design (see test guidelines for details) but commonly result in dose-response reporting and probit analysis to determine EC_X.
Test Duration:	8 weeks observation, the adult worms are removed after the fourth week

Test Endpoint:	Adult mortality and other signs of toxicity, number of juveniles produced.
Test Validity:	This test has been tested by a number of laboratories (ring-tested) and found to be reproducible (Kula 1996ab). Test validity can be assessed through the use of controls as follows: each control replicate should produce ≥ 30 juveniles by the end of the test, the coefficient of variation of control reproduction should be ≤ 30% and adult control mortality over the initial 4 weeks should be ≤ 10%. The periodic use of a reference substance (e.g., carbendazim) is advised to be run parallel to the toxicity test of a test substance at least twice a year, or more frequently when testing is being carried out at a lower frequency, to ensure the response of the test organism has not changed significantly over time. When a geometric series of concentrations are used, the concentrations tested should cover the range needed to determine the EC_X values. See the test guidelines for further detail.
Records Required:	Test animal source, condition and husbandry, test material identification and application, test conditions, soil pH and moisture, test medium preparation, test results including worm weights, number (adult and juvenile surviving), any observations, and statistical analysis.
Training requirements:	Nominal
Equipment Requirements:	Nominal for standard soil laboratory
Test History and Use:	This test was based on a number of other international and national guidelines that contain provisions useful to the performance of this test (OECD 1984, ISO 1993, 1996, SETAC 1998, EPA 1996).
Test Benefits and Limitations:	Although earthworms have been the historic invertebrate test species of choice, soil-dwelling annelids of the genus *Enchytraeus* are ecologically relevant species for ecotoxicological testing. While Enchytraeids are often found in soils containing earthworms and are often abundant in many soils where earthworms are absent and may be a more representative species of naturally-occurring soils of interest. This test has been evaluated by a number of laboratories (i.e., ring-tested) and found to be reproducible (Kula 1996). Like earthworms, Enchytraeids are very amenable to laboratory conditions but have the practical advantage of a significantly shorter generation time and test duration is only 4-6 weeks for enchytraeids while it is 8 weeks for

an earthworm reproductive test. Like other soil tests, no allowance is made in this test to account for or correct for test substance degradation. Concentration stability of the test compound throughout the test period cannot be assumed.

Test Organisms: Soil Vertebrates

<u>Overview</u>

Standardized vertebrate toxicity tests are generally single species laboratory tests. Measured endpoints include individual organism lethality/survival, reproductive success, behavioral abnormalities or the bioaccumulation of test material in target tissues or whole bodies. In general, only a few vertebrate species spend the majority of their time throughout the year in close contact with the soil ecosystem. Examples would include some small mammals, such as moles and shrews, some larger mammals, such as badgers, and several species of snakes and amphibians. Many other vertebrates, including some avian species, may spend significant amounts of time surrounded by soil during shorter but measured segments of their life history, such as during hibernation, aestivation, and reproduction. However, still more vertebrate species are exposed regularly or periodically to the soil environment during the course of preening, dust bathing, foraging and other normal maintenance and reproductive activities.

Standardized toxicity tests for vertebrate species have not been developed with the single view of assessing the effects of anticipated or known soil contaminants on likely soil inhabiting vertebrate species. Standardized tests rarely have the luxury of being conducted using the animals and all the exposure routes of concern. Instead, standardized tests have been designed to assess a variety of chemicals through one route of exposure at a time to select laboratory species, such as mice, rat, Coturnix quail, chicken, dog and rabbit species, under laboratory conditions. The economics of convenience, reduced costs, reduced variability in animals, ready animal availability, ease in handling and control that this approach brings has allowed for various exposure routes to be compared and relative toxicity determined for a wide variety of chemicals, many of them now known to contaminate soils. Recently, several tests have been expanded and standardized to include non-traditional species as wildlife species representatives to assess ecological effects. For example, mallards and Northern bobwhites have become accepted representatives of aquatic and terrestrial avian species, while *Peromyscus* sp. (e.g., deer mice, white-footed mice) have become accepted representatives of small mammals. Standardized tests have also been adapted to many other wildlife vertebrate species specific to a chemical, site or exposure route of concern and a body of toxicity literature is beginning to emerge using native vertebrate species (Tucker et al. 1971, Schafer 1972, Schafer et al. 1973, 1983, Hudson et al. 1979, Hill and Camardese 1984, Romijn 1995, Elliot et al. 1997). Such data supports the evaluation of chemical effects across species. Tests may also be conducted to distinguish differences in chemical susceptibility between sexes and among age classes. However, standardized toxicity tests seldom mimic real-life exposure scenarios. Meaningful data can be obtained that supports a broader ecological risk assessment when study results are judiciously extrapolated to other species, exposure routes, dosages, frequency of contact, etc. When extrapolating it is critical to understand the pharmaco- or toxicokinetics of the test substance, its biotransformation and its elimination from the species of concern.

Standardized vertebrate toxicity test protocols or guidelines can be found in Table 1-7.

Table 1-7. Standardized Vertebrate Toxicity Tests

TEST NAME	Source of Test Guideline		
	US EPA OPPTS Number[1]	OECD Number	ASTM Number
Ecological Effects - Terrestrial Wildlife Test Guidelines			
Avian acute oral toxicity test	850.2100		E555-92
Avian dietary toxicity test	850.2200	205	E857-87 (1997)
Avian reproduction test	850.2300	206	E1062-86
Wild mammal acute toxicity	850.2400		
Field testing for terrestrial wildlife (draft)	850-2500		
Health Effects – Acute Toxicity Test Guidelines			
Acute toxicity testing-background	870.1000	420, 423, 425	
Acute oral toxicity	870.1100	401	E1163.98
Acute dermal toxicity	870.1200	402	
Acute inhalation toxicity	870.1300	403	
Acute inhalation toxicity with histopathology	870.1350		
Health Effects – Subchronic Toxicity Test Guidelines			
90-Day oral toxicity	870.3100	408	E1372-95 (1999)
Subchronic nonrodent oral toxicity 90-day	870.3150	409	
Repeated dose dermal toxicity--21/28 days	870.3200	410	
Subchronic dermal toxicity--90 days	870.3250	411	E1103-96 (2000)
Subchronic inhalation toxicity	870.3465	413	E1373-92 (1996)
Preliminary developmental toxicity screen	870.3500		

(Continued)

Table 1-7. Continued

TEST NAME	Source of Test Guideline		
	US EPA OPPTS Number[1]	OECD Number	ASTM Number
Inhalation developmental toxicity study	870.3600		
Prenatal developmental toxicity study	870.3700	414	E1483-92 (2000)
Reproduction and fertility effects	870.3800	416	
Health Effects – Chronic Toxicity Test Guidelines			
Chronic toxicity	870.4100	452	E1619-95 (1999)
Carcinogenicity	870.4200	451	
Combined chronic toxicity carcinogenicity	870.4300	453	E1811-96
Health Effects – Genetic Toxicity Test Guidelines			
Mouse biochemical specific locus test	870.5195		
Mouse visible specific locus test	870.5200		
Detection of gene mutations in somatic cells in culture	870.5300	476	
Standard guide for performing the mouse lymphoma assay for mammalian cell mutagenicity			E1280-97
Standard guide for performance of the Chinese hamster ovary cell/hypoxanthine guanine phosphoribosyl transferase mutation assay			E1262-88 (1996)
In vitro mammalian cytogenetics	870.5375	473	
In vivo mammalian cytogenetics tests: spermatogonial chromosomal aberrations	870.5380		
In vivo mammalian cytogenetics tests: Bone marrow chromosomal analysis	870.5385	475	
In vivo mammalian cytogenetics tests: Erythrocyte micronucleus assay	870.5395	474	
Rodent dominant lethal assay	870.5450	478	

(Continued)

Table 1-7. Continued

TEST NAME	Source of Test Guideline		
	US EPA OPPTS Number[1]	OECD Number	ASTM Number
Rodent heritable translocation assays	870.5460	485	
Unscheduled DNA synthesis in mammalian cells in culture	870.5550	482	
In vitro sister chromatid exchange assay	870.5900	479	
In vivo sister chromatid exchange assay	870.5915		
Health Effects – Neurotoxicity Test Guidelines			
Delayed neurotoxicity of organophosphorus substances following acute and 28-day exposure	870.6100	418, 419	
Neurotoxicity screening battery	870.6200		
Developmental neurotoxicity study	870.6300		
Schedule-controlled operant behavior	870.6500		
Peripheral nerve function	870.6850		
Neurophysiology: Sensory evoked potentials	870.6855		
Health Effects – Special Studies Test Guidelines			
Domestic animal safety	870.7200		
Metabolism and pharmacokinetics	870.7485	417	
Dermal penetration	870.7600		
Immunotoxicity	870.7800		
Pharmacokinetic test	870.8223		
Dermal pharmacokinetics of DGBE and DGBA	870 8245		
Oral/dermal pharmacokinetics	870.8320		

(Continued)

Table 1-7. Continued

TEST NAME	Source of Test Guideline		
	US EPA OPPTS Number[1]	OECD Number	ASTM Number
Oral and inhalation pharmacokinetic	870.8340		
Toxicokinetic test	870.8500		
Developmental neurotoxicity screen	870.8600		
Subchronic oral toxicity test	870.8700		

1 US EPA Office of Prevention, Pesticide and Toxic Substances (OPPTS) has harmonized and blended testing guidelines and requirements that existed in Office of Pollution Prevention and Toxics (OPPT) and appear in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP), which appear in publications of the National Technical Information Service (NTIS) and the Guidelines published by the Organization for Economic Cooperation and Development (OECD). The new OPPTS numbers are provided. All guidelines and their previous CFR and NTIS number can be found on the EPA web site (EPA 1996f).

As can be seen, very few test guidelines have been adapted to assess ecological effects of contaminants on wildlife, and among those, none are adapted to assessing contaminated soils, but rather utilize technical grade materials usually over a range of doses/concentrations.

Avian Toxicity Test

Although few avian species are soil vertebrates in the sense that they primarily occupy soil habitats, exposure to soil contaminants can occur both directly and indirectly to a wide variety of avian species. Exposure can occur through multiple routes including inhalation, digestion, and dermal exposure. Inhalation of soil particles can occur through preening, dust bathing, and in some cases during activities associated with reproduction, for example when excavating soil nest cavities (e.g., some kingfishers and swallows) or utilizing soil in nest construction. Similar activities also lead to dermal and oral exposures to soil contaminants. In addition to direct exposure to contaminants when soil particles adhere to food items, digestive exposure also occurs indirectly through secondary toxicity when soil contaminants are present in or bioaccumulate in food items. Birds also may consume contaminated soil particles directly when digesting grit particles, a necessary activity for proper digestion in some species. Therefore non-laboratory avian species have frequently been used to assess the toxicity of potentially hazardous materials (Tucker et al. 1971, Schafer 1972, Schafer et al. 1973, 1983, Hudson et al. 1979, Hill and Camardese 1984, Romijn 1995, Elliot et al. 1997).

Avian toxicity testing has been driven primarily by the assessment of the potential ecological effects of pesticides. A number of standardized testing guidelines are available including Avian Acute Oral Toxicity Test, Avian Dietary Toxicity Test, and Avian Reproduction Test. Test guidelines generally utilize the Northern bobwhite (*Colinus virginianus*) and mallard (*Anas platyrhynchos*) as preferred representatives of terrestrial and aquatic avian species, respectively, however many other species have been utilized. Species including feral pigeon (*Columbia livia*), ring-necked pheasant (*Phasianus colchicus*), red-legged partridge (*Alectoris rufia*), European starling (*Sturnus vulgaris*), and red-winged blackbirds (*Agelaius phoeniceus*) have been more commonly utilized but site specific exposure concerns have often dictated the use of other relatively infrequently used avian species.

Recently, scientific guidelines designed to reduce the number of animals used in acute toxicity test of all species have been harmonized within the US EPA, and where possible with OECD Guidelines (EPA 1998a,b), in order to take into full account their welfare. Recommendations to reduce the number of animals while still providing data adequate to ascertain a chemical's safety include:

1. Simultaneous monitoring of several endpoints in addition to lethality, including sublethal behavioral, physiological and biochemical endpoints;
2. Use of data from structurally related substances or mixtures;
3. Use of alternative test protocols when available, for example:
   a. the Fixed Dose Method (OECD Guideline 420; OECD 1992)
   b. the Acute Toxic Class Method (OECD Guideline 423; OECD 1996), and
   c. the Up-and-Down Method (OECD Guideline 425; OECD 1998)
4. Weight of evidence approaches to dermal and ocular irritation using existing data, where all of the available information should be used in determining the need for *in vivo* testing; and
5. Use of limit testing, or single group testing when a substance is judged to be relatively non-toxic.

Sound, scientific practices to estimate the acute oral toxicity with a reduced number of animals have been recently embraced by the regulatory community, most notably the Up-and-Down Method (OECD 1998). In this method, the LD50 is estimated using a minimal number of animals by using computer programs to facilitate animal-by-animal calculations that establish the testing sequence and provide final results. The main test by this method consists of dosing animals one at a time, at a minimum of 48-hour intervals. The results (lethality) are used to establish a sequential dosing plan, up or down for each successive dose, until specified criteria are met that allow for a estimate of LD50 using a method if maximum likelihood (OECD 1998). The number of individual animals utilized in this method may be as low as four.

Mammalian Toxicity Tests

Mammalian toxicity tests have traditionally used various tightly controlled and selected inbred strains of the laboratory mouse, rat, rabbit, and guinea pig. Other domestic species such as the domestic dog and domestic swine have also been frequent mammalian test subjects. With the

exception of the deer mice (*Peromyscus maniculatus*) and their congeneric species, few other non-laboratory species have become wide-spread nontraditional alternatives for vertebrate toxicity testing despite the occurrence of several vertebrate species who occupy "soil" habitats. Perhaps this is due to difficulties in laboratory rearing and handling of these species (moles, shrews, badgers, for example). Mice of the native genus *Peromyscus*, in addition to being the most abundant and widely distributed rodents in North America (Joyner et al. 1998), are much better suited to standard laboratory husbandry methods, where they are easily bred, maintained and handled in ways similar to other small laboratory rodents. The *Peromyscus* Genetic Stock Center at the University of South Carolina maintains more than 50 laboratory-bred, well-characterized stocks of deer mice and other peromyscine species for research and educational use (Joyner et al. 1998). In native habitats, deer mice occupy a great diversity of habitats and play a significant role in natural ecosystems. Their exposure to soil contaminants would be similar to that of avian species (see above). Exposure would occur during normal maintenance and reproductive activities through ingestion, inhalation and dermal exposure routes.

Reptile and Amphibians Toxicity Tests

Despite the presence and concern for soil inhabiting reptiles and amphibians, there are no standardized tests for reptiles and amphibians that would mimic a soil contaminant exposure route with a terrestrial soil species. The amphibian Frog Embryo Teratogenesis Assay-Xenopus (FETAX Assay, ASTM 1991) is one of the few standardized amphibian assays, but measures aquatic embryo development utilizing a frog species which may not be directly applicable to soil dwelling amphibians and amphibian life-cycle stages. Regulatory agencies have also assumed that "protection" for reptiles and amphibians is provided through the risk assessment process for birds and mammals (EPA 1996e).

Review of Standardized Toxicity Test Methods

Toxicity Tests

As can be seen in Table 1-7 very few test guidelines have been adapted to assess ecological effects of contaminants on wildlife and among those none are adapted to assessing contaminated soils, but rather utilize technical grade materials usually over a range of doses/concentrations. The test methods are standard methods for the preliminary assessment of toxicity using vertebrate species. Test methods vary depending upon the test material and its intended use and anticipated exposure routes.

- Routes of test material administration
- Species
- Frequency of treatment and observation
- Duration of exposure (acute (<20 days), subchronic (21-90 days), chronic (1-2 years))
- Endpoints measured

Vertebrate toxicity tests generally fall within one or more "classical" approaches to toxicity determination:

Acute Toxicity Test

Oral Lethal Dose 50% (LD50): Treatment groups over a graduated range of doses and a control or carrier control group, of approximately 10 animals per group, receive a single oral exposure (gavage or capsule) of the test material and lethality, signs of intoxication, and weight changes are recorded. Data are reported as the LD50 (mg/kg body weight), the empirically derived dose that is expected to result in the mortality of 50% of the animals, the 95% confidence limits and the slope of the dose-response curve.

Dermal LD50: Treatment groups over a graduated range of doses and a control or carrier control group, of approximately 10 animals per group, receive a single dermal exposure (usually held in place over a 24 hour period on bare (shaved) skin) of the test material and lethality, signs of intoxication, and weight changes are recorded. Data are reported as the LD50 (mg/kg body weight), the empirically derived dose that is expected to result in the mortality of 50% of the animals, the 95% confidence limits and the slope of the dose-response curve.

Inhalation Lethal Concentration 50% (LC50): Treatment groups over a graduated range of doses and a control or carrier control group, of approximately 10 animals per group, receive a 4 hour exposure, using an inhalation chamber, of the test material. Lethality, signs of intoxication, and weight changes are recorded. Data are reported as the LC50 (ppm), the empirically derived dose that is expected to result in the mortality of 50% of the animals, the 95% confidence limits and the slope of the dose-response curve.

Dietary LC50: Treatment groups over a graduated range of concentrations evenly mixed in the basal diet and a control or carrier control group, of approximately 10 animals per group, receive a dietary exposure of the test material and lethality, signs of intoxication, and weight changes are recorded. Data are reported as the LC50 (ppm), the empirically derived dose that is expected to result in the mortality of 50% of the animals, the 95% confidence limits and the slope of the dose-response curve. In addition bioaccumulation of the test material can be assessed through tissue analysis.

These classical *acute* approaches to the assessment of chemical toxicity are particularly useful in that they are generally of short duration, inexpensive, and relatively easy to conduct. The standardization of these procedures using a few key species has allowed the *relative* hazard, as measured by mortality, of a very large number of chemicals and even chemical mixtures to be determined.

Subchronic and Chronic Tests

While acute exposures of toxic substances can occur, wildlife living on contaminated sites are more likely to receive long term, sublethal exposures to soil contaminants. Subchronic and chronic tests therefore offer the advantage of assessing not only the relative hazard of chemical and chemical mixtures, but also to look at more realistic exposure scenarios representative of expected long-term exposures of vertebrates species to measured soil contamination levels. In addition, endpoint examination is not limited to mortality, but is expanded to include a wide

range of biological, biochemical, chemical, reproductive, genetic and behavioral endpoints chosen to be meaningful to the contaminant and site of concern. That is, subchronic and chronic tests allow for the biological nature of the toxic effects to be determined at the cellular level during low doses more realistic of actual exposure scenarios. In addition, the longer duration allows differential variation in species responses to repeated exposures to be assessed. Potential body burdens of the test material, as well as metabolized products, can be measured in longer tests. Tissue damage, target tissue, and toxicokinetics can also be determined in relationship to different exposure durations and frequency, at different concentrations.

Reproductive, Fertility and Prenatal Development Tests: In these tests laboratory conditions are maintained to allow sublethal exposure of the test material to the test animals over critical periods of reproductive activity. Measured endpoints include reproductive success (e.g., number of offspring, number of eggs hatched, quality of eggs and offspring in terms of health, weight, survival, deformities, eggshell thickness), male fertility and spermatogenesis, reproductive behavior, and multigenerational effects

Carcinogenicity Tests: Both in vitro and in vivo tests have been developed to assess the carcinogenic potential of test materials. Such testing is often mandatory for pharmaceuticals, industrial chemicals and pesticides. Test parameters vary widely in terms of duration, exposure, and test animal strain. Measured endpoints include the number of tumors and tumor types in comparison to controls.

Genetic Toxicity Test: Genetic toxicity tests measure cytotoxicity. Test methods utilize both in vivo and in vitro test systems. Measured endpoints are designed to assess cellular mutations in a variety of test systems.

Neurotoxicity Test: A battery of test methods have been developed to assess neurotoxicity. Measured endpoints include delayed neurotoxicity, and effects on peripheral nerve function, sensory perception, operant behavior, and neural development.

Other Specialized Tests: Several other specialized tests are described for laboratory vertebrates that are unique in their assessment endpoint and study design and include metabolism, pharmacokinetics, and immunotoxicity test methods.

Detailed Review of Standardized Ecological Effect Test For Vertebrates

The following section provides detailed test method review for test methods designed to assess vertebrate ecological effects. A note about the limitations of using test method reviews: As a review, test details, particularly in study design, test material application and laboratory conditions and procedures, are by necessity not included in sufficient detail to conduct a study. Standardized testing procedures are readily available from the referenced source and should be referred to as needed for details of study conduct and interpretation.

Standardized Soil Toxicity Tests: Vertebrates

The following tests are reviewed in this section:

- Avian Acute Oral Toxicity Test
- Avian Dietary Toxicity Test
- Avian Reproduction Test
- Wild Mammal Acute Toxicity Test
- Field Testing for Terrestrial Wildlife (Draft)

Toxicity Test: Avian Acute Oral Toxicity Test

Summary Description:	This test is one of a series of tests originally designed to assess the toxicity of pesticides and toxic substances to birds. In the US, this test is designed to meet the data requirements of the US EPA under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and Toxic Substance Control Act (TSCA). The purpose of this test is to develop data on the acute oral toxicity to northern bobwhite and mallard of chemical substances and mixtures of interest to assess the acute hazard of these chemicals to birds. Following a 14-day acclimation period, adult birds are gavaged with a single oral dose of the test material in a range of doses. Birds are closely monitored for signs of intoxication for at least 14 days or until all signs of intoxication are not observed for 72 hours. Bird weight and food consumption are monitored at least weekly. The mortality pattern is examined and the LD50, confidence limits, and slope of the dose-response line are determined through appropriate statistical analysis. Signs of intoxication, bird weights, food consumption, necropsy results and the mortality results are reported.
Source:	
Standardized:	Yes
Reference:	[EPA] US Environmental Protection Agency. 1996. Avian Acute Oral Toxicity Test, OPPTS 850.2100, EPA 712-C-96-139. Washington, DC. April 1996. See also [EPA] US Environmental Protection Agency. 1998b. Acute Oral Toxicity, OPPTS 870.1100 Acute Oral Toxicity, EPA 712-C-98-190. Washington, DC. August 1998. [ASTM] American Society for Testing and Materials. 1995. Standard Practice for Determining Acute Oral LD50 for Testing Vertebrate Control Agents. Annual Book of Standards E 555-95. West Conshohocken, PA.
Targeted Assessment:	Designed to investigate the acute hazard of pesticides and toxic chemicals to birds by establishing the LD50, the empirically derived dose of the test substance that is expected to result in mortality of 50 percent of the birds treated with a single oral dose under the test conditions.

Summary At-A-Glance

Test location:	Laboratory
Test System:	

Test Species:	Northern bobwhite (*Colinus virginianus*) and mallard (*Anas platyrhynchos*).
Lifestage:	Young adults, not yet mated, at least 16 weeks at time of dosing, at least 180g for bobwhite and 900g for mallard.
Sex	Not specified.
Strain:	Laboratory reared, phenotypically indistinguishable from wild stock.
Test Conditions/ Procedures:	As above. A range finding test may be conducted with fewer birds. Definitive test usually consists of 10 birds per dosage level of the test substance and the control, with a minimum of five dosage levels for the test substance spaced geometrically (60% of the next higher level ideally). Dosage levels should also be spaced to provide at least 3 levels resulting in mortality between, but not including 0 and 100 percent; at least one level should kill more than 50% and at least one level should kill less than 50% of the birds in the group. Standard laboratory conditions and housing should be used, including 15-20 ^{o}C, 10-15 air changes per hour, 45-70% relative humidity, 8:16 L:D. Good Laboratory Practice Standards (e.g., 40 CFR Part 792) apply to this guideline.
Test Duration:	For at least 14 days or until all signs of intoxication are not observed for 72 hours.
Test Endpoint:	Signs of intoxication, bird weights, food consumption, necropsy results and mortality results expressed as the LD50, the empirically derived dose of the test substance that is expected to result in mortality of 50 percent of the birds treated with a single oral dose under the test conditions.
Test Validity:	Test results are not valid if greater than 10% of control animals die during the test or if more than 5% of the total test population dies during acclimation. Test animals should be of the same age, from the same adult breeding population (stock and strain), from colonies of known breeding history, and be certified disease-free. Animals should not be selected from genetic resistant strains or have been used on a previous test. Animals should be randomly assigned to treatment levels. Conducting the test using a laboratory standard (reference toxicant) periodically or when there is any significant change in laboratory conditions (food, housing, source of birds) is recommended.
Records Required:	Signs of intoxication, bird weights, age and sex of birds, food consumption, analysis of basal diets (nutrients and potential contaminants), necropsy results, mortality results and statistical analysis records.
Training Requirements:	Nominal.
Equipment	Nominal for standard animal testing laboratory.

Requirements:	
Test History and Use:	The EPA version of this test is one of a series of tests developed by the Office of Prevention, Pesticides and Toxic Substances for test data that must be submitted for review under Federal regulations and was intended to meet testing guidelines of both the FIFRA and TSCA. It was harmonized from the Avian Acute Oral Toxicity Test (40 CFR 797.2175) and the Avian Single-Dose LD50 Test (OPP 70-1) and guidelines appearing in the National Technical Information Service (NTIS) and Organization for Economic Cooperation and Development (OECD) publications.
Test Benefits and Limitations:	This test is standardized and relatively quick, easy and inexpensive to conduct. This test was originally developed for testing individual pesticides or toxic substances in the laboratory with a single oral exposure of the test substance and may not accurately reflect substance availability in soils.

Toxicity Test: Avian Dietary Toxicity Test

| Summary Description: | The purpose of this test is to develop data on the dietary toxicity to northern bobwhite and mallard of chemical substances and mixtures of interest to assess the chronic hazard of these chemicals to birds. Following a 7-day acclimation period, young birds are provided for a 5-day exposure period with the basal diet containing the test material in a range of doses. Birds are closely monitored for signs of intoxication for at least 3 days after the exposure period or until all signs of intoxication are no longer observed or up to 21 days whichever comes first. Bird behavior, weight and food consumption are monitored. The mortality pattern is examined and the LC50, 95% confidence limits and slope of the dose-response line are determined through appropriate statistical analysis. Signs of intoxication, bird weights, food consumption, necropsy results and mortality results are reported. |

Source:

Standardized:	Yes
Reference:	[EPA] US Environmental Protection Agency. 1996. Avian Dietary Toxicity Test, OPPTS 850.2200, EPA 712-C-96-140. Washington, DC. April 1991.
	[OECD] Organization for Economic Cooperation and Development. 1984. OECD Guideline For The Testing Of Chemicals: Avian Dietary Toxicity Test. No. 205. Paris, France. 4 April 1984.
	[ASTM] American Society for Testing and Materials. 1993. Standard Practice for Conducting Subacute Dietary Toxicity Studies with Avian Species. Annual Book of Standards E 857-87 (reapproved 1993). West Conshohocken, PA.

| Targeted Assessment: | Designed to investigate the hazard of pesticides and toxic chemicals to birds by establishing the LC50, the empirically derived dietary concentration of the test substance that is expected to result in mortality of 50 percent of the birds treated with a 5-day dietary exposure under the test conditions. |

Summary At-A-Glance

| Test location: | Laboratory |
| Test System: | |

Test Species:	Northern bobwhite (*Colinus virginianus*) and mallard (*Anas platyrhynchos*) preferred, although other species are acceptable including pigeon (*Columbia livia*), Japanese quail (*Coturnix xoturnix japonica*), ring-necked pheasant (*Phasianus colchicus*), and red-legged partridge (*Alectoris rufia*).
Lifestage:	Young birds, 10-14-days old for bobwhite and 5-10 days-old for mallard at the beginning of the exposure period.
Sex	Not specified.
Strain:	Laboratory reared, phenotypically indistinguishable from wild stock.
Test Conditions/ Procedures:	As above. A range finding test may be conducted with fewer birds. Definitive test usually consists of 10 birds per dietary concentration of the test substance and 20 birds for the negative/carrier control, with a minimum of five treatment levels for the test substance spaced geometrically (60% of the next higher level ideally). Dosage levels should be chosen so that there are no compound-related mortalities or signs of effects at the lowest level. At least one concentration should kill greater than 50% of birds up to and including 100% and one concentration should kill less than 50% of test birds. Standard laboratory conditions and housing should be used. Due to the young age of the animals during testing, it is recommended that animals be purchased as eggs and hatched at the test facilities but purchase of healthy young animals is also possible. Pens should allow for a range in temperatures ranging from 22-38 °C to allow for thermoregulation by young birds. The room should be maintained at 45-70 % humidity with 14:10 L:D light cycle or continuous lighting and positioned to avoid cross contamination. Good Laboratory Practice Standards (e.g., 40 CFR Part 792) apply to this guideline.
Test Duration:	For at least 8 days or until all signs of intoxication are not observed for 72 hours or 21 days which ever comes first.
Test Endpoint:	Signs of intoxication, bird weights, food consumption, necropsy results and mortality results expressed as the LC50, the empirically derived dietary concentration of the test substance that is expected to result in mortality of 50 percent of the birds exposed under the test conditions.
Test Validity:	Test results are not valid if greater than 10% of control animals die during the test or if more than 5% of total test population dies during the 72 hours immediately preceding the test period. There must be evidence that the concentration of the test substance has been satisfactorily maintained in the test diet (it should be at least 80 % of the nominal concentration) throughout the 5-days of the exposure period. The lowest level should not result in compound-

related mortality or other observable effects. A positive standard (e.g., dieldrin run) is recommended as a means of periodically detecting possible interlaboratory or temporal variation. A laboratory standard is also recommended when there has been significant change in food, housing or source of birds.   Test animals should be of the same age, from the same adult breeding population (stock and strain), from colonies of known breeding history, and be certified disease-free. Animals should not be selected from genetic resistant strains or have been used on a previous test. Animals should be randomly assigned to treatment levels.

Records Required:	Signs of intoxication, bird weights, age and sex of birds, food consumption, analysis of basal diets (nutrients and potential contaminants), stability and homogeneity of the test substance in the diet, necropsy results, mortality results and statistical analysis records.
Training Requirements:	Nominal.
Equipment Requirements:	Nominal for standard animal testing laboratory.
Test History and Use:	The EPA version of this test is one of a series of tests developed by the Office of Prevention, Pesticides and Toxic Substances for test data that must be submitted for review under Federal regulations and was intended to meet testing guidelines of both the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and Toxic Substance Control Act (TSCA). It was harmonized from the Avian Acute Dietary Toxicity Test (40 CFR 797.2050) and the Avian Dietary LC50 Test (OPP 71-2) and guidelines appearing in the National Technical Information Service (NTIS) and Organization for Economic Cooperation and Development (OECD) publications (OECD 205 Avian Dietary Toxicity Test).
Test Benefits and Limitations:	This test is standardized and relatively quick, easy and inexpensive to conduct. This test was originally developed for testing individual pesticides or toxic substances in the laboratory as a dietary exposure of the test substance and may not accurately reflect substance availability in soils.

Toxicity Test: Avian Reproduction Test

Summary Description: The purpose of this test is to develop data on the reproductive effects to northern bobwhite and mallard of chemical substances and mixtures of interest to assess the chronic hazard of these chemicals to birds. Following a 14-day acclimation period, paired adult birds (or one male and two-three females) are provided with the basal diet containing the test material in a range of doses based on the expected field residue level and the LC50 value. During the first phase animals are provided with the test material in the basal diet for approximately 6-8 weeks. In the second phase the lighting is adjusted to bring the hens into laying condition (approximately 204 weeks). The third and final phase begins with the commencement of laying and lasts for 8-10 weeks.   Adults are presented with the test substance in the basal diets continually through all phases of the study. A withdrawal period may be added to the study period if reduced incubation is observed and need not exceed 3 weeks. Birds are closely monitored for signs of intoxication and the number of eggs laid is recorded. Bird weight and food consumption are monitored. Eggs are removed daily for incubation. Eggs should be candled at day 0 and days 11 (14) and 18 (21) for bobwhite (mallards). Hatchlings should be observed for 14 days and weighed on day 14.   Signs of intoxication, bird weights, food consumption, necropsy results, number and quality of eggs laid, eggshell thickness, hatchability, fertility (viable embryos), hatchling survival and any mortality results reported. Tissue analysis may be appropriate for substances suspected of bioaccumulating or being persistent in the environment.

Source:

Standardized: Yes

Reference: [EPA] US Environmental Protection Agency. 1996. Avian Reproduction Test, OPPTS 850.2300, EPA 712-C-96-141. Washington, DC. April 1996.

[OECD] Organization for Economic Cooperation and Development. 1984. OECD Guideline For The Testing Of Chemicals: Avian Reproduction Test. No. 206. Paris, France. 4 April 1984.

[ASTM] American Society for Testing and Materials. 1991. Standard Practice for Conducting Reproductive Studies with Avian Species. Annual Book of Standards E 1062-86 (reapproved 1991). West Conshohocken, PA.

| Targeted Assessment: | Designed to investigate the chronic hazard of pesticides and toxic chemicals to birds by assessing the reproductive hazard to birds. |

Summary At-A-Glance

Test location:	Laboratory
Test System:	
Test Species:	Northern bobwhite (*Colinus virginianus*) and mallard (*Anas platyrhynchos*).
Lifestage:	Young adults approaching their first breeding season, at least 7 months old, all birds should be within 1 month of each other in age.
Sex	Not specified.
Strain:	Laboratory reared, phenotypically indistinguishable from wild stock.
Test Conditions/ Procedures:	As above. Prerequisites for the test include water solubility, vapor pressure, avian dietary LC50 of the test substance as well as structural and behavioral characteristics of the test substance. Definitive test usually consists of 12 replicate pens of birds (8 if multiple females per pen) per dietary concentration of the test substance with a minimum of three treatment levels for the test substance and 20 for the control group. Dosage levels should be chosen based on expected environmental residue levels and the LC50. Standard laboratory conditions and housing for breeding birds, incubating/hatching eggs and maintaining brood pens should be used as outlined in the guidelines. Lighting is critical to study success and should be carefully controlled. Lighting should begin with a short day (7-8 h/day) and be gradually (15 min/day) increased to 16-17 h light to induce egg laying. The dark cycle should not be interrupted in the first phase. Pens should be positioned to avoid cross contamination. Good Laboratory Practice Standards (e.g., 40 CFR Part 792) apply to this guideline.
Test Duration:	For at least 8-10 weeks of egg laying preceded by a 6-8 week pre-egg laying treatment period. Hatchlings should be observed for at least 14 days.
Test Endpoint:	Signs of intoxication, bird weights, food consumption, necropsy results, number and quality of eggs laid, eggshell thickness, hatchability, fertility (viable embryos), hatchling survival and any mortality results are reported. Tissue analysis may be appropriate for substance suspected of bioaccumulating or being persistent in the environment.
Test Validity:	Test results are not valid if bobwhite chick or mallard duckling egg productivity does not average 12 or 10, respectively, 14-day

old survivors per pen over a 10 week period, if average eggshell thickness in control group is less than 0.19 mm and 0.34 mm for bobwhite and mallards, respectively, or if greater than 10% of control adult animals die during the test. There must be evidence that the concentration of the test substance has been satisfactorily maintained in the test diet throughout the exposure period. Test animals should be pen-reared, from the same source and strain, obtained from "U.S. Pullorum-Typhoid Clean" classified stock from colonies of known breeding history, and be certified disease-free. Animals should not be selected from genetic resistant strains or have been used on a previous test. Animals should be randomly assigned to treatment levels. Animals should not be used in greater than 3% of animals become debilitated during the health observation period.

Records Required:	Bird age, any signs of intoxication, adult and 14-day old bird weights, food consumption of adults, percentage of hens laying eggs, number of eggs laid, number and percent of cracked eggs, percent viable embryos and number of eggs set, percent live 18-day embryos of viable embryos, percent and number of hatchlings that are normal, percent 14-day old survivors of normal hatchlings, number of 14-day old survivors per hen, eggshell thickness, analysis of basal diets (nutrients and potential contaminants), stability and homogeneity of the test substance in the diet, and necropsy and mortality results and statistical analysis records
Training Requirements:	Nominal.
Equipment Requirements:	Nominal for standard animal testing laboratory.
Test History and Use:	The EPA version of this test is one of a series of tests developed by the Office of Prevention, Pesticides and Toxic Substances for test data that must be submitted for review under Federal regulations and was intended to meet testing guidelines of both the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and Toxic Substance Control Act (TSCA). It was harmonized from the Bobwhite Reproduction Test (40 CFR 797.2130), Mallard Reproduction Test (40 CFR 797.2150) and the Avian Reproduction Test (OPP 71-4) and guidelines appearing in the National Technical Information Service (NTIS) and Organization for Economic Cooperation and Development (OECD) publications (OECD 206 Avian Reproduction Test).
Test Benefits and Limitations:	This test is standardized and moderately quick, easy and inexpensive to conduct. This test is standardized and relatively quick, easy and inexpensive to conduct. This test was originally developed for testing individual pesticides or toxic substances in

the laboratory as a dietary exposure of the test substance and may
not accurately reflect substance availability in soils.

Toxicity Test: Wild Mammal Acute Toxicity Test

Summary Description:	This test is one of a series of tests originally designed to assess the toxicity of pesticides and toxic substances to mammals. In the US, this test is designed to meet the data requirements of the US EPA under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and Toxic Substance Control Act (TSCA). The purpose of this test is to develop data on the acute toxicity to wild mammals of chemical substances and mixtures of interest to assess the acute hazard of these chemicals when they are suspected of having considerable variations in toxicity between mammalian species, when they are suspected of interfering with rumen fermentation in wild ruminants, or when secondary toxicity is suspected. Test parameters and reported results should be determined based on the scarcity of animals, expected chemical use patterns and the desired endpoint (acute oral LD50, dietary LC50, or dietary no-effect level) established through consultation with the Agency and registrant.

Source:

Standardized:	Yes
Reference:	[EPA] US Environmental Protection Agency. 1996. Wild Mammal Acute Toxicity Test, OPPTS 850.2400, EPA 712-C-96-142. Washington, DC. April 1996. See also [EPA] US Environmental Protection Agency. 1998b. Acute Oral Toxicity, OPPTS 870.1100 Acute Oral Toxicity, EPA 712-C-98-190. Washington, DC. August 1998.
Targeted Assessment:	Designed to investigate the acute hazard of pesticides and toxic chemicals to wild mammals by establishing the acute oral LD50, dietary LC50, or dietary no-effect level as determined by the expected use pattern and established through consultation with the Agency and registrant.

Summary At-A-Glance

Test location:	Laboratory
Test System:	
Test Species:	Mammalian species representing those found in the area affected by the proposed use pattern, in no cases should threatened or endangered animals be used in testing.
Lifestage:	Not specified.
Sex	Not specified.
Strain:	Pen-reared or wild caught, phenotypically indistinguishable from

wild stock.

Test Conditions/ Procedures:	Test conditions will vary depending upon the species, exposure route and desired endpoint.
Test Duration:	Not specified.
Test Endpoint:	Undetermined, but may include LD50 (in mg/kg) or LC50 (in ppm) with 95% confidence limits or estimated maximum tolerated dose, methods of calculation, slope of the dose response line.
Test Validity:	Not specified.
Records Required:	Age of each animal and how determined, mean body weights for each test and control group at initiation and termination of test, number of animals in each sex of animals tested, total food consumption for each test and control group, test diet, dose schedules, mortality, number and circumstances of accidental injuries or deaths, LD50 (in mg/kg) or LC50 (in ppm) with 95% confidence limits or estimated maximum tolerated dose, methods of calculation, slope of the dose response line and test protocol.
Training Requirements:	Depends on difficulty in animal handling and husbandry.
Equipment Requirements:	Depends on difficulty in animal handling and husbandry.
Test History and Use:	Several acute oral mammalian test guidelines are available. The EPA versions of this test is one of a series of tests developed by the Office of Prevention, Pesticides and Toxic Substances for test data that must be submitted for review under Federal regulations and was intended to meet testing guidelines of both the FIFRA and TSCA. It was harmonized from previous EPA guidelines appearing in the National Technical Information Service (NTIS) (e.g., Wild Mammal Toxicity Test (OPP 71-3)) and Organization for Economic Cooperation and Development (OECD) publications.
Test Benefits and Limitations:	This standardized test guideline is only a general overview of testing requirements. Its ability to assess the toxicity of soil contaminants would depend on the final study design established through consultation with the agency and registrant but is primarily designed to assess a single exposure to a single substance and may not accurately reflect substance availability in soils.

Toxicity Test: Field Testing for Terrestrial Wildlife

Summary Description:	This test is designed to assess the toxicity of pesticides to mammals and birds under normal pesticide use and field conditions. In the US, this test is designed to meet the data requirements of the US EPA under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). The purpose of this test is primarily intended to assess if wildlife species will be adversely affected by a pesticide under normal pesticide use practices. Test conditions simulate anticipated normal pesticide use. Reported results include 1) direct poisoning and death by ingestion, dermal exposure and/or inhalation, 2) sublethal toxic effects indirectly caused by reducing resistance to other environmental stresses such as disease, weather and predation, 3) altered behavior such as abandonment of nest or young, changes in parental care, or 4) reduction in food consumption, 4) reduced food resources or alteration of habit, 5) lowered productivity through fewer eggs laid, reduced litter size, or reduced fertility. Test methods focus primarily on birds and mammals but may be adopted to amphibian, reptile and other non-targeted species if indicated.
Source:	
Standardized:	Yes
Reference:	[EPA] US Environmental Protection Agency. 1996. Field Testing for Terrestrial Wildlife (draft), OPPTS 850.2500, EPA 712-C-96-144. Washington, DC. April 1996
Targeted Assessment:	Designed to investigate the acute hazard of pesticides under normal field use to wild birds and mammals by assessing lethal, sublethal and reproductive endpoints.

Summary At-A-Glance

Test location:	Appropriate field site.
Test System:	
Test Species:	Primarily avian and mammalian species, but may be expanded to include other non-target vertebrates, invertebrates and plants of concern.
Lifestage:	Not specified.
Sex	Not specified.
Strain:	Pen-reared or wild caught, phenotypically indistinguishable from wild stock.
Test Conditions/	Test conditions will vary depending upon the pesticide of concern.

Procedures:	An initial screening study to assess potential sites and survey species present or pen studies in the field is often advised to assist in study design and interpretation. The study sites are selected from the geographical areas with the greatest risk for the species and habitats of concern based on a thorough understanding of the geographical areas and the biology of the species found associated with these areas. Sites should be selected to maximize diversity and density of wildlife species, often by maximizing edge effect. If possible, study sites should be randomly selected. The number of sites needed is determined by binomial theorem and/or by choosing worst-case sites. Control sites are recommended but may not be necessary if cause and effect can be confirmed by other means.
	Once sites are selected, pesticides are applied according to standard agricultural procedures for the pesticide. Endpoints measured include:
	1. Mortality and survival (e.g., mark-recapture of small mammals, territory mapping of birds, radio telemetry of animals, and other methods such as carcass search)
	2. Reproduction and survival of dependent young (e.g., nest monitoring, behavioral observations associated with reproduction and age structure of populations)
	3. Ancillary methods (e.g., cholinesterase inhibition, carcass analysis, environmental residues, food habit assessments, roosting and denning activities).
	Study results are reported and interpreted using sound scientific process based on the statement of concern found in the protocol and investigated through the study design.
Test Duration:	Not specified.
Test Endpoint:	Determination of the level of risk for the pesticide under normal use by utilizing species-specific results, laboratory data, and data extrapolation.
Test Validity:	Not specified.
Records Required:	Records required would depend upon study design but would include all methods and materials and the resulting study results, data handling, analysis and interpretation.
Training Requirements:	Depends on study design but would require extensive scientific judgment and field research skills.
Equipment Requirements:	Depends on site conditions and study design, particularly for pesticide application and radio-tracking needs.
Test History and Use:	This test was developed by the US EPA Office of Prevention, Pesticides and Toxic Substances for test data that must be submitted for review under federal regulations and was intended

	to meet testing guidelines of the FIFRA. It was harmonized from a previous EPA guideline appearing in the National Technical Information Service (NTIS) publications (Simulated and Actual Field Testing for Mammals and Birds, Subdivision E—Hazard Evaluation; Wildlife and Aquatic Organisms (OPP 71-5)).
Test Benefits and Limitations:	This standardized test guideline is only a general overview of testing requirements and is primarily intended to assess if wildlife species, primarily birds and mammals, will not be adversely effected by a pesticide under normal pesticide use practices. Its ability to assess the toxicity of other soil contaminants and species would depend on the final study design.

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PART 2: BIOASSESSMENT OF SOIL-DWELLING ORGANISMS

INTRODUCTION

There is an underlying assumption that a relationship exists between environmental quality and measurable physical, chemical, and biological characteristics. Bioassessment techniques and methods focus on assessing the biological components that characterize environmental quality. In order to determine the best measurable biological predictors of the environmental quality of soil, a working definition of soil quality is required. Doran and Parker (1991) in their review and search for a definition of soil quality define it as, "The capacity of a soil to function within ecosystem boundaries to sustain biological productivity, maintain environmental quality, and promote plant and animal health."

Soils are of enormous economic and environmental importance (Torstensson 1998). As an economic and life sustaining resource, arable soils are a source of food for plants and animals. Soils are the ultimate reservoir of nutrients. By receiving and decomposing organic and other material, soils act as a source of nutrients and in pollution and energy cycling. Soils act as environmental filters for cleaning water and air. However, when soils are contaminated they become a source of environmental pollution and may loose some or all of their pollutant filtering and detoxification capacity. Their productivity and energy cycling capacity can also be diminished. Although traditional soil quality assessments have focused on the agricultural capacity of soil, several recent texts have been focused on methods for assessing soil quality in relation to environmental contamination (e.g., van Straalen and Løkke 1997, van Straalen and Krivolutsky 1996, and Tarradellas et al. 1997).

If environmental contaminants have the potential to reduce the functional capacity of soils through altering, disturbing or destabilizing the biological composition and interactions of soils, methods for assessing these consequences are necessary to both understanding and predicting these effects. Bioassessment methods are composed of tools that allow us to 1) predict the potential impact of contaminants in environment, 2) to assess effect of contaminants once in the environment on the biological components necessary for soil quality and 3) to monitor these impacts over time and changing conditions. Bioassessment of soils for this document is therefore defined as:

> *measurement of biological characteristics that best predict soil quality and quantitatively or, in some cases, qualitatively measuring these characteristics.*

Bioassays are usually relatively short duration tests in which the activity or adverse effect of an environmental stressor on an ecotoxicological test system is measured using a written protocol. Bioassessment methods include both laboratory and field methods. This Part examines the laboratory and field methods of assessing communities of soil-dwelling organisms, terrestrial invertebrates, and terrestrial vertebrates, including, but not limited to, avian, mammalian, and amphibian species. As will be seen, bioassessment methods are complimentary to physico-chemical methods and together they can greatly assist in the management of environmental contaminants.

Ecological Risk Assessment : The Role of Bioassessment

Bioassessment tools and methods play a critical role in the ecological risk assessment process. In phase one, problem formulation, bioassessment endpoints can be included in the conceptual model and identify the data needed. In addition, bioassessment can assist in the preliminary characterization of the stressor and in identifying the ecosystem components potentially at risk. Bioassessment data can provide unique insights into how the stressor impacts the ecosystem which will improve the focus and efficiency of the following phases two and three. Bioassessment measures provide a common language that risk assessors and risk managers can use to describe the potential problems. In the second analysis phase, bioassessment endpoints identify and quantify the potential exposure and ecological effects by providing technical information about the stressor and its effects on the ecosystem. Bioassessment methods have a novel value over traditional toxicity tests by providing stressor-response data at the community and population level that can identify and link different levels of the biological organization and by providing insight into the structural and functional properties of the ecosystem. Such data further reduces the assumptions that may be made in developing the exposure and effects profile and allows risk assessors to distinguish between different types of stressors (climatic, nutrient and chemical, for example). In the third phase, bioassessment data reduces the uncertainty in characterizing the ecological risk by supporting the risk predictions with valid and in many cases real site data. Bioassessment methods provide a bridge whereby risk assessors and risk managers can describe and focus the risk assessment process beyond the traditional chemical toxicity testing approach.

Approaches to Soil Bioassessment

Environmental scientists and risk managers appreciate that many factors influence the effects of contaminants on the environment (e.g., soil type, climate, amount and extent of exposure, type of contaminant, number of contaminants, to name but a few) and that no single endpoint analysis is likely to predict or determine the depth and breadth of the environmental risk. Multimetric approaches have long been used and accepted in water quality assessment (Southerland and Stribling 1995, Plafkin et al 1989). The US EPA's Rapid Bioassessment Protocols (RBP) (Plafkin et al. 1989) and the Index of Biotic Integrity (Karr et al. 1986) are two examples of comparative rating systems using multiple bioassessment metrics to provide an easily understood comparative method. Multiple bioassesssment methods, although not as well developed for soils as for aquatic systems, have long been recommended as excellent tools for not only assessing chemical or pollution effects, but also as early warning systems as indicators of environmental degradation (Torstensson et al. 1998). Soil bioassessment techniques are available and can provide essential soil quality data, which can, in turn, supplement more traditional chemical monitoring to better understand the effect of contaminants and contaminated soils in the environment. In particular, bioassessment methods provide *in situ* information regarding the ultimate and cumulative biological response to the environmental contaminant in the soil ecosystem.

Recently, several efforts have been undertaken to select and rank for usefulness a series of laboratory ecotoxicity tests and on-site assays for use in terrestrial ecological risk assessments

(Léon and van Gestel 1994, Tarradellas et al 1997, Fairbrother et al. 1999, van Straalen and Løkke 1997, and van Straalen and Krivolutsky 1996).

Léon and van Gestel (1994) and Torstensson et al. (1997) have proposed a test evaluation and selection system that allowed different assays to be compared by a series of criteria, including:

1. Is the assay Practical?
   - Feasible
   - Cost-effective
   - Rapid

2. Is the assay Acceptable?
   - Standardized
   - Reproducible
   - Accurate and Precise
   - Robust under a wide range of temperature, soil type, water content, pH, etc.
   - Sensitive
   - Statistically valid with a causal relationship between contaminant and measured effect
   - Meets Good Laboratory Practice
   - Amenable to quality controls
   - Broad chemical responsiveness

3. Is the assay Ecologically Significant?
   - Ecologically realistic
   - Biologically valid

4. Is the assay Representative of the Ecosystem
   - Representative of key life history strategies
   - Representative of key functional groups
   - Representative of key taxonomic groups
   - Representative of key exposure routes

5. Is the assay Representative of Different Levels of Biological Organization with the Terrestrial Ecosystem?

6. Is the assay Uniform when compared to other assays?

Multifactoral ecotoxicological bioassessment of the soil ecosystem can be difficult and expensive to both conduct and interpret but can provide critical support to the risk assessment process.

ALTERNATIVE APPROACHES FOR ASSESSING RISK ASSOCIATED WITH SOIL CONTAMINANTS

Environmental risk assessment has a rich history of developing and applying appropriate methods and mathematical models to predict the release, transport, and environmental fate of

chemicals in soil environments required for the exposure assessment phase of risk assessment (Leon and van Gestel 1994). However, there is an acknowledged lack of test methods and standardization of ecotoxicity tests for terrestrial organisms (OECD 1989, 1991, Løkke and van Gestel 1993). Although several toxicity tests are standardized (see Section I of this document), there is a paucity of applied information and tests remain limited in comparison to aquatic systems. In recent years, however, several research programs based in Europe have been initiated to develop and validate soil effect assessment tests, including the Soil Ecotoxicity Risk Assessment System (SERAS), Netherlands Integrated Soil Research Programme (NISRP), MArk Test System (MATS)-Programme, and Sublethal Effects of Chemicals On Fauna Soil Ecosystem (SECOFASE).

Soil Ecotoxicity Risk Assessment System (SERAS)

Eijsackers and Løkke (1992) outline a risk assessment methodology specific to soil, which they entitled Soil Ecotoxicity Risk Assessment System (SERAS). In this system, tests are grouped according to different levels of biological organization:

- Physical aspects (e.g., particle size, mineral composition, organic matter, etc)
- Soil processes (e.g., nutrient and carbon cycling)
- Community structure (e.g., species diversity, abundance and trophic structure)
- Community function (e.g., predation, mutualism, etc)

Netherlands Integrated Soil Research Programme (NISRP)

Eijsackers (1989) reports on a series of research initiatives instigated in the Netherlands to develop soil effect test protocols.

MArk Test System (MATS)-Programme

In another approach, the Swedish Environmental Protection Agency initiated a research program to develop standardized research protocols for soil ecotoxicology assessment (Torstensson 1993).

Sublethal Effects of Chemicals on Fauna Soil Ecosystem (SECOFASE)

Finally in the framework of the European Community, the EC Environmental Programme has developed an international research program to develop, improve and standardize ecotoxicology tests with a number of soil organisms (Løkke and van Gestel 1993)

ROLE OF PHYSICO-CHEMICAL FACTORS IN BIOASSESSMENT

Soil Profiles

Soil is formed over time by the parental material (the chemical and physical properties of the originating rock, alluvium/colluvium or organic material), the climate (past and present, rainfall and temperature for example), the fauna and flora that have lived in it, the relief (the

geomorphology and its influences, for example, on drainage), and time (Clark 1986).   The soil profile is a description of the vertical cross sections of the soil that naturally occur in layers or horizons (Clark 1986). These layers or horizons result from soil formation and uniquely characterize the physico-chemical nature of individual soils. Clark (1986) describes three soil layers that are widely recognized as basic soil layers:
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Topsoil (A Horizon): The top most layer of soil in which the maximum biotic activity, roots and humus are normally found which often darken the soil. Top soils are frequently disturbed by human and animal activity. Topsoil can lose clay, iron, and aluminum, to the subsoil and subsequently are described as the eluviation horizon.

Subsoil (B Horizon): Directly below the topsoil, the subsoil is characterized as having less biotic activity and is usually denser from the illuvial concentration of clay (or iron, aluminum or humus).

Substratum (Horizon C): Directly below the subsoil, the substratum is composed of the underlying unconsolidated material or weathered substratum which may merge with or lie over hard rock.

The soil layers vary considerably from site to site in terms of depth, nature and properties. For example, top soils with deep weathering or in deep alluvium areas may extend 20-30m (Clark 1986). Standardized formats for describing the soil profile can be found in USDA and FAO documents and were primarily designed to assess soil quality for agricultural purposes. For ecology studies, Clark (1986) suggests that the following characteristics are most important:

- Soil Source and Depth: An accurate description of the source of the sample, the sample depth and sampling methods.
- Soil Texture: an estimate or measurement of the relative percentage by weight of sand, silt, and clay in the fine earth fraction of the soil and particle size distribution
- Soil Organic Content:
- Soil pH
- Soil salinity or alkalinity: a measure of salts in the soil as determined by measuring the conductivity of a saturation extract of the soil or by direct determination of ions present.
- Soil moisture holding capacity: this measures the influence of soil organic matter, textue, structure and content of coarse material and their influence of available water held by the soil

Other endpoints include:

- Cation exchange capacity: the ability of soil to retain cations for plant use
- Organic Carbon
- Total Nitrogen
- Exchangeable cations (Ca, Mg, Na, K)
- Hydrogen ions

- Chemical speciation
- Partitioning factors
- Adsorption
- pH/Buffer capacity

Soil profiling at different points in the site assist in determining the underlying variability in soil which are essential to understanding biological results obtained from bioassays. And like bioassays, no matter how many soil samples are taken, their use in supporting contaminated site risk assessments is only valuable if the samples are representative of the site.

COMMUNITY STRUCTURAL ASSESSMENTS

Measures of Biodiversity: An Overview

In simplest terms, biodiversity is the biological variability of natural communities. Biodiversity measures generally estimate the kinds or number of species or taxa present (abundance, richness), and their distribution (evenness) throughout the community. Diversity is one of the most basic biological measurements made of community structure and there are numerous text available to assist in sampling, measuring and calculating biodiversity (Allsopp et al. 1995, Pielou 1975, Hawkesworth and Ritchie 1993, Wilson et al. 1996, Southwood 1978, Magurran 1988, Grassle et al. 1979, Allsopp et al. 1995, NPS undated). This document will provide only a brief overview. In the context of soil bioassessment, the purpose of measuring a community's diversity is 1) to evaluate if changes in a soil community's structural relationships occur when exposed to a contaminant(s) or to other environmental conditions of concern, and 2) to determine if these changes are detrimental to the community's health and well-being. Magurran (1988) found that a log normal distribution is obtained when many groups of organisms are measured from a given habitat and the resulting species abundances are graphed against the number of individuals of each species. In communities, which are stressed or disturbed, such as in polluted situations, the resulting distribution is not log normal but is flattened out toward a geometric series (Patrick 1973). The resulting communities are composed of a few tolerant dominant species while most other species become rare. When conditions become extreme, species approach extinction and evenness also approaches a geometric series.

Hawkesworth and Ritchie (1993) describe four basic levels of biodiversity:

1. Genetic diversity - a measure of gene variation arising from spontaneous mutation or reproductive selection and is the determinant of evolutionary development and diversification.

2. Species diversity – the common measure of species variation made through measuring species presence, abundance, richness and distribution.

3. Ecosystem diversity – an assessment of variations in community or habitat structure. It includes an examination of the range of available resources, such as niche width, the turnover or the degree of change between sites of samples, differentiation or beta diversity (Southwood 1978).

4. Taxonomic Diversity – the number of phyla, orders, etc. as a measure of or prerequisite for assessing phylogenetic differences (evolutionary remoteness) among the species present.

Although diversity is a common biological measure, there are practicalities that must be considered when measuring diversity is soil communities. First, because biodiversity is a community level evaluation, it is critical that the boundaries of the community be clearly defined. Community descriptors would include spatial boundaries, temporal boundaries, and the community boundaries being assessed and their relationship to each other.

Spatial boundaries: Soil diversity measures must clearly define the area or volume of soil being assessed. An adequate definition might include collection methods, collection depth, site of collection, sample handling, etc. Because of the extreme heterogeneity of soil structure, exact soil source and post-collection treatments, such as compositing samples, is critical to any use of the resulting data.

Temporal Boundaries: Soil diversity measures must clearly indicate the time the sample was taken.

Community Boundaries: Soil diversity measures must clearly indicate the taxonomic boundaries, i.e., the taxa or species constituting the communities to be measured and their relationship to each other. Seldom are all individuals or species of a community counted in an assessment of biodiversity. In fact, it is extremely difficult to directly observe many components of the soil community, such as microbial organisms. Even when observable, taxonomic classification can also be difficult, for example with soil invertebrates. Determining diversity requires an understanding of the life history of the community of interest and the ability to observe and identify that community in order to adequately measure individual components of the community.

Secondly, the method that is used to measure diversity is also critical to the biodiversity assessment. Common methods used in soil communities include:

Number of individuals: The number of individuals is frequently counted when the difference in *size* between individuals or species is not ecologically significant to the assessment. Sometimes in small areas or communities all individuals can be counted, however it is more frequent that total populations are estimated from subsamples. Vertebrates and macro-invertebrates lend themselves most easily to assessment by measuring the number of individuals.

Biomass: The biomass of different community components is often used when the difference in size between individual or species is ecologically significant to the assessment or when individuals cannot be counted, as in some microbial assessments or colonial species. Microbial, fungal, invertebrates and plants have been used to measures diversity through biomass measures.

Function: The function of different community components is measured when the function of the species/taxon within the community is the most effective measure of ecological significance when comparing two or more samples, contaminants or sites. Microorganisms lend themselves to functional measures of biodiversity (e.g., Zak et al. 1994, Dubranik and Zak 1999).

Diversity Indices

Due to the difficulty of performing accurate counts, diversity is often estimated as a calculated index that allows different communities to be compared. Diversity is commonly measured and reported in terms of species abundance or richness (the number of species, s) and evenness (J). Because a community with many unevenly distributed species, may have the same Diversity Index measure as a community with few, but evenly distributed species, species diversity indicators and distribution indicators are generally measured separately.

There are numerous texts available to assist in sampling, measuring and calculating biodiversity (Allsopp et al. 1995, Pielou 1975, Hawkesworth and Ritchie 1993, Wilson et al. 1996, Southwood 1978, Magurran 1988, Grassle et al. 1979, and Allsopp et al. 1995, NPS undated).

Challenges arise when trying to use measures of diversity in the bioassessment of soil due to the difficulties associates with the spatial, temporal, and taxa boundaries and the inherent associated random or systematic errors of the measurement process.

Spatial Issues: Due to the micro-structure of soil environments, it is extremely difficult to assume that soil samples, no matter how well collected, are of uniform species concentrations or are representative of the site or test sample. It is often difficult to find or validate appropriate control sites when using diversity indices to compare contaminated sites with uncontaminated site.

Issues Related to Taxa: Another limitation in determining soil diversity is the ability to adequately identify species. Counting measures require great skill in species identification with many soil species. Taxonomic uncertainties in identification of soil organisms can severely influence diversity measures and comparability (Pielou 1975).

Issues Related to Sampling and Observability: As previously mentioned, soil microorganisms can be difficult to observe. Recent advances in genetic probes and antibody techniques have improved the ability to quickly and efficiently identify specific organisms in soil communities and on a larger scale (Torstensson 1998). Diversity measures are also biased by sample size (NRC 2000). The larger the sample or the area sampled, the larger the number of species generally due to the increased number of habitats that the sample represents. Even with the best effort, the rarest species are those most likely to be missed.

Temporal Issues: Because diversity measures are only a snap shot in time, quickly changing communities may also be difficult to assess using diversity measures. Microbial and invertebrate populations fluctuate rapidly in response to environmental

conditions such as temperature, rainfall, and season. Again the micro-structure nature of soils often gives limited validity to diversity measures. Additional or longer assessment periods become equivalent to additional habitats in space (Rozenzweig 1998). The dynamic nature of diversity indices therefore does not indicate the sustainability of the ecosystem (NCR 2000).

An interesting observation with respect to temporal adaptability has been made by Emlen (1974). When looking at the effect of urbanization on avian species assemblages in the Upper Sonoran Desert basin, he found that abundance increased 26-fold but diversity declined slightly. Of note, however, was the shift from native species to exotic and commensal species which accounted for the bulk of the urban area avian biomass. It is hypothesized that those species which appear to be tolerant of or capable of taking advantage of suboptimal conditions may have had more time to adjust to human activities (e.g., exotic Old World species), are genetically pre-adapted to constantly changing conditions typical of human activities, or thrive in the absence of natural predators sensitive to and eliminated by human activity (NRC 2000).

One of the most widely used measures of diversity is the **Index of Biotic Integrity** (IBI), a multimetric diversity indicator that was originally developed and tested in running water systems using freshwater fish communities (Karr et al. 1986, Karr and Chu 1999). Today it is widely used with benthic macroinvertebrate. To develop an IBI, some agreement must first be reached as to what a healthy community should look like, i.e., whether the presence or absence of certain assemblages within specific phylogenetic groups represent good or poor ecological health. Based on this understanding, site measures for select taxa distribution and relative abundance are assessed and each measure assigned a numerical value, usually an integer between 1 and 6, based on the pre-determined qualitative assessment of environmental quality. The sum of the individual scores becomes the final IBI (see Barbour et al. 1995 for additional methodology). As an additive index, additional statistical analysis is limited and generally reserved for comparisons between sites with very similar expected IBIs.

DIVERSITY MEASURES IN BIOASSESSMENT

Although diversity of microbial and fungal communities were conventionally performed by direct count from cultured plates, many bacteria, spores and fungal hyphae are difficult to culture on known agars and incubation conditions. Extraction procedures also often biased the results. Recent technical advances offer molecular techniques to identify and count non-culturable organisms (Fairbrother 2000). Newer methods are not yet standardized but include measurement of the fatty-acid profile of the soil organisms, phospholipid ester-linked fatty acid, and extracted DNA.

Although it would appear to be difficult to establish valid measures of diversity in soil communities, it is often reasonable to use relative differences in abundance or changes in abundance (Conroy 1996). Total species diversity and native species diversity are two of the indicators recommended by the National Research Council (NRC 2000). Because the loss of a species is irreversible, the NRC recommends counting species richness, which is a measure not weighted by population abundances and which hopefully avoids discounting rare species. Diversity measures have also been found to be most informative and easiest to interpret when

applied to fairly limited and well defined, taxonomic groups (Magurran 1988). Whether diversity indices are sensitive enough to detect change due to environmental stressors is subject to debate. Dennis et al. (1979) found that diversity was related to but not sensitive to pollution index in aquatic invertebrates. In this case, diversity was more sensitive to physical state and season than to Palmer's organic pollution index. The limited history of the use of diversity measures as indices of environmental perturbation in soil ecosystems may limit the usefulness of this indicator until a more thorough understanding of its implications and method difficulties, as mentioned above, are available. However, it's relatively straightforward methodology and applicability to a wide array of environments make diversity indices extremely useful indicators.

Food Webs and Food Chains

Food webs and food chains has the potential to be used to assess the relationship between community stability and environmental disturbances. Food webs and food chains are a way of describing the great complexity of functional interrelationships among species and functional groups or trophic levels within and between communities.. In stable environments, trophic levels are composed of functionally similar species or *functional analogues*, that is, closely related species partitioning the same resources (such as food) or organisms of different origins, which have converged to exploit the same resources (Solbrig 1991). Ecosystems with functional analogues are said to have functional redundancy. In ecosystem with environmental stressors and many functional analogues, the loss of one or more species within a functional group may not noticeably affect the function relationships within the community structure. Because functional redundancy may appear to be more apparent than real, the utilization and value of food web and food chain analysis to assess the adverse effect of environmental stressors on ecosystem health and function depends largely on the thorough understanding of all the components of the community and their complex interrelationship. The variety of organisms able to perform similar tasks will also impact the resiliency of the ecosystem to withstand environmental perturbations (Perry et al. 1989). The loss of species diversity, superficially unimportant species or apparently functionally redundant individual species may also reduce genetic potential and long-term functional capacity of the ecosystem with additional or future environmental stressors in the longer term. Food webs and food chains may also assist in addressing the indirect effects of contaminants as evidenced by other relationships between species, such as predator-prey, host parasite, and plant mycorrhiza, as well as more complex tri-trophic relations, such as plant-phytophage-parasite (Eijsackers 1994).

In conclusion, at this time, no single site on earth has been inventoried for all species present (Hawkesworth and Ritchie 1993). This accomplishment may in fact be unachievable for microbial and invertebrate groups and would not represent the genetic variation within the morphological species. However methods are available through appropriate extensive or intensive sampling to assess comparative community structures between sites as a bioassessment of environmental quality in relation to the presence of environmental stressors.

FUNCTIONAL ASSESSMENTS

Overview

In soil systems, a large number of soil processes depend upon biological activity (Torstensson 1997). Microorganisms, in particular, are responsible for soil energy and nutrient cycling through the release of CO_2 and conversion of organic nitrogen, phosphorous, sulfur and other nutrients into their inorganic state. Microorganisms and invertebrates are also responsible for the development of the very soil environment that they occupy, through decomposition of organic matter, soil aeration and texturing of humus and particle aggregates, for example. Soil contaminants can have a significant effect on soil processes through the impact on microorganism and invertebrates.

Measures of Soil Processes

Measures of soil processes are designed to assess the capacity and stability of the functional roles of soil organisms. Many soil organisms play key roles in energy and nutrient cycling through the mineralization of dead organic materials. In this process CO_2 and nutrients such as nitrogen, sulfur, phosphorus and other minerals from dead organic matter are recycled by transforming them from their organic state into their inorganic state and released for use by plants and other soil organisms. In addition to nutrient and energy cycling, soil organisms are also responsible for the detoxification of naturally occurring toxins, such as phytotoxins (Torstensson 1997) and provide the soil ecosystem with a naturally occurring mechanism for detoxifying man-made environmental contaminants. Soil microorganisms, such as bacteria, fungi, and protozoa also act in themselves as a critical reservoir for soil nutrients and are grazed upon by other soil organisms (Eijsackers 1994). For these reasons the presence of a well functioning soil community is necessary for the soil processes.

Primary Production and Measures of Biomass

Primary producers, such as plants, algae and some microorganisms, are capable of capturing solar energy and building organic biomass through the fixation of atmospheric carbon dioxide through photosynthesis. Primary producers are therefore the primary source of energy input into the community. Primary producers occupy the foundation of the food pyramid described by the food web (see above) by providing the food source for the primary consumers. Primary consumers, in turn, provide the food source for successive levels of trophic levels until ultimately consumed by decomposers, thereby releasing nutrients and completing the energy cycle.

The microbial community in its role in storing and recycling nutrients is critical to maintaining healthy soil processes. One measure of the health of the microbial community is to measure **microbial biomass**, or the dry weight of the microbial community per unit of area. Microbial biomass is a measure of soil fertility and is a sum total of all microorganisms including bacteria, actinomycetes and fungi, dormant or active. Soils with higher biomass have been found to have greater nutrient storage and nutrient cycling capacity (Domsch 1977). Although some individual organisms my increase under contaminated conditions, either due to utilization of the contaminant as a food source or from reduced competition, others will become intoxicated and lyse. Nematodes and protozoa biomass may also change under contaminated conditions. Reduced nematode and protozoa populations may also effect microbial populations by reducing predation pressure.

The most accepted method of measuring total soil microbial biomass is by the **Fumigation Method** (van Beelen and Doelman 1994). In this method the majority (>99%) of soil microorganisms are killed by chloroform fumigation. Chloroform disintegrates the cell walls which allows for the excretion of CO_2 and soluble organic compounds (e.g., nitrogen and phosphorous) which can be measured directly. By itself the microbial biomass is not considered a particularly sensitive parameter, but the ratio between respiration (see carbon transformation test) and the biomass is considered a moderately sensitive indicator of contaminant effect (Brookes and McGrath 1984).

Soil Nutrient Cycling and Geochemical Processes

The ability to cycle key nutrients including carbon, nitrogen, sulfur and phosphorous as well as mediate key geochemical processes is an essential feature of a healthy soil. Almost exclusively very specialized microbial organisms carry out these processes. Many assays have been described to measure these processes, usually in the laboratory, but using site-collected soils (see Section I of this document). The presence of microflora is also indispensable to degrading other organic materials, including many environmental contaminants, and to the subsequent formation of soil aggregates. The degree of specialization with the microbial community to facilitate these reactions is such that many species cannot be readily replaced by other species (van Beelen and Doelman 1996).

Table 2-1 summarizes many of the bioassays currently proposed for assessing these critical processes. Many of these assays were described in Part 1 of this document as well but are included here because they can be conducted using contaminated soils and results can be used in multimetric ecological risk assessment. Because many of the processes can be performed a multitude of species with varying tolerances to contaminants, assay sensitivity may vary (van Beelen and Doelman 1994). Generally, the more specialized and limited the bacterial species (e.g., autotrophic nitrification), the more sensitive the contaminant effect assay.

Table 2-1. Nutrient Cycling and Geochemical Process Bioassays

Carbon Transformation (Respiration) Test	
Assay Overview:	This assay measures the effect of chemicals/soils of interest on carbon transformation in aerobic surface soils under laboratory conditions favorable to microbial metabolism. Soils are homogenized and incubated in the dark at room temperature for up to 100 days. Soils are periodically sampled and glucose-induced respiration rates measured as carbon dioxide released or oxygen consumed. Results are compared against control samples or a dose-response is prepared. Changes in respiration reflect changes in size and activity of microbial communities through both chemical stress and carbon starvation.
Goal:	Assess the potential effects of environmental contaminants on carbon transformation activity of soil microorganisms
Assay endpoint:	Glucose-induced respirations rates [mean carbon dioxide released (mg carbon dioxide/kg dry weight soil/h) or mean oxygen consumed (mg oxygen/dry weight soil/h)].
References:	OECD 2000a, Anderson and Domsch 1978
Respiration Field Test	
Assay Overview:	This assay measures the effect of chemicals/soils of interest on respiration in field soils. Given volumes of soil atmosphere are measured by pumping the air from the soil over a period of time or by determining the carbon dioxide released into an enclosed space above the surface of the soil.
Goal:	Assess the potential effects of environmental contaminants on respiration of soil microorganisms
Assay endpoint:	Carbon dioxide released (Problems can occur in determining the volume of soil from which the carbon dioxide came, and in distinguishing between microbial respiration and root respiration)
References:	Gray and Williams 1971
Nitrogen Transformation Test	
Assay Overview:	This assay measures the effect of chemicals/soils of interest on nitrogen transformation and nitrification in aerobic surface soils under laboratory conditions favorable to microbial metabolism. In this assay the production of nitrogenous oxides, nitrate and nitrite from organic nitrogen is assessed as a measure of microbial health. Soils are sieved, amended with

	powdered plant meal or ammonium sulphate (to ensure a nitrogen source) and incubated under conditions to ensure adequate gas exchange and prevent water loss in the dark at room temperature for at least 28 days. Soils are periodically sampled (0. 7, 14, 28 days and up to 12 weeks) and extracted with an appropriate solvent and nitrate measured. Results are compared against control samples or a dose-response is prepared. Nitrate forms following the degradation of the carbon-nitrogen bonds. Therefore, if nitrate production is equal in treated and control samples, major carbon degradation pathways are presumed to be intact and functional. Changes in nitrogen transformation, however, may reflect changes in size and activity of microbial communities through chemical stress.
Goal:	Assess the potential effects of environmental contaminants on nitrogen transformation/nitrification activity of soil microorganisms
Assay endpoint:	Nitrate production (mg nitrate/kg dry weight soil/day), also nitrite and ammonium.
References:	OECD 2000b, Nederlands Normalisatie Instituut 1988
Nitrogen Mineralization or Ammonification	
Assay Overview:	This assay measures the effect of chemicals/soils of interest on nitrogen mineralization, the process by which organic nitrogen is degraded to NH_4^+ in anaerobic laboratory conditions favorable to microbial metabolism. Water is added the soil to create a slurry in order to minimize maintenance of optimal water content and to assist in substrate diffusion. Samples are incubated under anaerobic conditions to minimize nitrification and uncontrollable nitrogen oxide formation. Ammonium is analyzed on a photometer using the indophenol blue method. Net mineralization is calculated as the difference in ammonium at the start and after 10 days of incubation at 37^oC.
Goal:	Assess the potential effects of environmental contaminants on nitrogen mineralization activity of soil microorganisms
Assay endpoint:	Ammonium (NH_4^+)
References:	Pell et al. 1998

Ammonium Oxidation (Nitrification) Assay	
Assay Overview:	This assay measures the effect of chemicals/soils of interest on ammonium oxidation, the process by which NH_4^+ is first transformed to NO_2^- and then secondly transformed to NO_3^-. This process is carried out by a limited number of autotrophic bacteria all in the family Nitrobacteriaceae, providing energy for growth and reducing capacity to fix carbon dioxide for these bacterial species. In this assay, laboratory conditions are adjusted to optimize ammonium-oxidizing bacteria. Soils are amended with excess NH_4^+, buffered to pH 7.2 and maintained as aerated slurries. Chlorate is added to the slurry to inhibit the second transformation step to NO_3^-. Therefore the final measure is NO_2^- as measured colorimetrically on a flow injection analyzer.
Goal:	Assess the potential effects of environmental contaminants on ammonium oxidation activity of soil microorganisms
Assay endpoint:	Nitrate (NO_2^-), the product rate is constant over the assay and can be calculated as the slope by linear regression
References:	Pell et al. 1998, Tortensson 1993
Denitrification Assay	
Assay Overview:	This assay measures the effect of chemicals/soils of interest on denitrification, the process by which nitrogenous oxides, mainly NO_2^- and NO_3^- are reduced to gases NO, N_2O and N_2. This process is carried out under anaerobic conditions by soil bacteria representing almost all taxonomical and physiological groups. Anaerobic conditions are necessary because almost all denitrifiers prefer O_2 as the terminal electron acceptor and therefore will reduce nitrogenous oxides only under anaerobic conditions. In this assay, laboratory conditions are adjusted to optimize denitrification enzymes. Soils are amended with excess glucose and nitrate and maintained as anaerobic slurries. Acetylene is added to the slurry to limit the endpoint to N_2O, which can be measured by gas chromatography.
Goal:	Assess the potential effects of environmental contaminants on denitrification activity of soil microorganisms
Assay endpoint:	Nitrous oxide (N_2O), a specific growth rate constant and the initial production rate can be determined from the growth data
References:	Pell et al. 1993

Cyanobacterial Nitrogen Fixation

Assay Overview:	This assay measures the Cyanobacteria nitrogen fixing capacity in contaminated or treated soils. Soils are incubated in 10% acetylene and 0.5% CO at 22 ^{o}C for up to 3-4 months and the production of ethylene measured is measured to assess cyanobacterial nitrogen fixation. Under these conditions, the normal reduction of acetylene to N_2 by nitrogenase is inhibited by the presence of 10% acetylene and instead acetylene is reduced to ethylene. CO is added to inhibit the spontaneous formation of ethylene by soil bacteria from endogenous methionine.
Goal:	To determine the effects of contaminated soil on cyanobacterial nitrogen fixation
Assay endpoint:	Ethylene production
References:	Mårtensson 1993a and 1993b

Heterotrophic Nitrogen Fixation

Assay Overview:	This assay measures the microbial (e.g., *Clostridium* and *Azotobacter*) nitrogen fixing capacity in contaminated or treated soils. Soils are incubated in 10% acetylene and 0.5% CO at 22 ^{o}C for up to 3-4 months and the production of ethylene measured as an assessment of heterotrophic bacterial nitrogen fixation. Under these conditions, the normal reduction of acetylene to N_2 by nitrogenase is inhibited by the presence of 10% acetylene and instead acetylene is reduced to ethylene. CO is added to inhibit the spontaneous formation of ethylene by soil bacteria from endogenous methionine.
Goal:	To determine the effects of contaminated soil on heterotrophic bacterial nitrogen fixation
Assay endpoint:	Ethylene production
References:	Mårtensson 1993c and 1993d

Acid Phosphate Assay

Assay Overview:	This assay measures the effect of chemicals/soils of interest on phosphorous cycling, in particular the process by which ortophosphate ($H_2PO_4^-$) is enzymatically released from organic compounds. Phosphatases are enzymes that catalyze the hydrolysis of phosphoric monoesters. Both acid and alkaline phosphatases exist with optimal reactions at low and high pH, respectively. Acid phosphatases are thought to exist primarily extracellularly, adsorbed to cell surfaces, or humic and mineral surfaces. Alkaline phosphatases occur intracellularly in the periplasmic space and can also be

	stabilized extracellularly. In this assay, laboratory conditions are adjusted to optimize acid phosphatase enzymes. The process is carried out under aerobic conditions in soil buffered to 6.5 pH and maintained at 15 ^{o}C at specific water content level. Soils are amended with nitrophenyl phosphate and preincubated for 6 weeks, thoroughly mixing the soil every second week. The rate of product formation (ortophosphate ($H_2PO_4^-$)) is measured and is dependent on the amount of active enzymes in the soil, usually following Michaelis-Menten kinetics (sigmoidal).
Goal:	Assess the potential effects of environmental contaminants on acid phosphotases activity in the soil.
Assay endpoint:	Ortophosphate ($H_2PO_4^-$), the rate of product formation is dependent on the amount of active enzymes in the soil.
References:	Sjoquist (undated)

Microbial Detoxification of Chemically Contaminated Water and Soil Using a Toxicity Test with a Luminescent Marine Bacterium (Microtox® Assay System)

Assay Overview:	This test method is design as a rapid evaluation of the toxicity of wastewaters or aqueous extracts from contaminated soils and sediments to a luminescent marine bacterium. In this study the bioluminescent marine bacteria *Photobacteria phosoreum* is exposed to the test wastewater or aqueous soil/sediment extract and the inhibition of light output measured over a specified time. The reduction in light output, compared to control, indicates the toxicity of the test material to this specific microbe and has implications on the biodegradation of the test material and is often used to assess samples resulting from biotreatability studies and procedures.
Goal:	A rapid assessment of acute toxicity of a soil extract, and its associated contaminant(s), to the bioluminescent marine bacteria *Photobacteria phosoreum* as a measure of toxicity through toxicity inhibition analysis.
Assay endpoint:	Quantitative reduction in light output of luminescent marine bacteria (i.e., IC20 or the calculated concentration of sample that would produce a 20% reduction in the light output of exposed bacteria over a specified time)
References:	ASTM 1996

Soil Microbial Community Toxicity Test	
Assay Overview:	This guideline is used to assess the toxicity of chemical substances and mixtures using natural soils to microbial populations indigenous to the soil. Surface soil is sieved and supplemented with ground, dry alfalfa. The test substance, if soluble, is added as a solution to moisten the soil, or is added in a manner that best simulates its anticipated mode of entry in nature. All soil samples are then incubated in darkness at approximately 22°C. Soils are then sampled on days 5 and 28 and analyzed for NH_3 and NO_3 concentrations and CO_2 efflux rate to determine microbial health through measuring ammonification, nitrification and respiration, respectively, as a measure of the soil microbial community to decompose organic matter and release plant nutrients.
Goal:	This test is used to assess the toxicity of chemical substances and mixtures using natural soils to microbial populations indigenous to the soil.
Assay endpoint:	NH_3, and NO_3 concentrations (concentrations per gram of soil) and CO_2 efflux rate
References:	EPA 1996d
Terrestrial Soil-Core Microcosm Tests	
Assay Overview:	In this battery of tests, an intact soil-core containing the natural assemblages of biota surrounded by the boundary material is collected from the site or region of interest. Contaminated soils are selected to include vegetation representative of the site or region. Soil cores are collected and maintained for 12 or more weeks under laboratory or green house conditions of light, temperature and moisture simulating those of the site or region of interest. Microcosms are then monitored for ecological effects and contaminant fate, including transformation products.
Goal:	This guide is intended to define the requirements and procedures for using microcosms to test the environmental fate, ecological effects and environmental transport of chemicals that may enter the terrestrial ecosystem at either a site-specific or possibly regional level. Specifically these tests are intended to assess the potential ecological impacts and environmental transport and fate of chemicals applied to agricultural soils or accidental chemical spills in natural soils through the measurement of plant growth and reproduction, nutrient uptake and cycling within the soil/plant.
Assay endpoint:	Ecological effect measures include plant growth (e.g., primary

	productivity, crop yield, and total biomass) and health (e.g., plant stress or lesions), and nutrient loss or uptake (e.g., leachate measures of primary nutrients). Environmental fate measures include chemical analysis of leachate, soil or plants for the parent compound and appropriate transformed by-products.
References:	ASTM 1987, EPA 1996e

Glutamic Acid Mineralization

Assay Overview:	This assay measures the effect of chemicals/soils of interest on glutamic acid mineralization, the process by which glutamic acid is degraded to CO_2 in aerobic laboratory conditions favorable to microbial metabolism. Soils are amended with sodium glutamate is added in a non-growth-limiting concentration and talcum and incubated for approximately 3 days. CO_2 is measured for at least 50 hours.
Goal:	Assess the potential effects of environmental contaminants on glutamic acid mineralization activity of soil microorganisms
Assay endpoint:	CO_2 production
References:	Vonk and Matla 1993

Soil Litterbag Test

Assay Overview:	This assay measures the decomposition potential of the litter heterotrophic microbial and invertebrate community. Plant material is put in a mesh-walled bag to permit gases, water, solubles and different organisms, depending upon mesh size, to move in and out of the bag when placed in contaminated soils.
Goal:	Assess the potential effects of environmental contaminants on nutrient mineralization through decomposition by soil organisms.
Assay endpoint:	Weight loss kinetics, nutrient release, changes in chemical composition, organism growth
References:	Wessén 1983 (as found in Torstesson 1997)

MEASURES OF SOIL PROCESSES

Chemical Degradation Processes

Microbial community health has also been assessed through a number of microbial substrate biodegradation assays (Nannipieri et al 1990, Dobbins et al. 1992). In these assays easily measured or radiolabelled substrates are incubated in test soils and degradation by microbial communities is determined as a measure of microbial health and activity.

Other Measure of Soil Process

In addition, there are a large number of miscellaneous microbial activity assays, such as enzyme inhibition, adenosine triphosphate (ATP) content, adenylate energy charge (AEC), incorporation of radiolabelled nucleic acids, and calorimetry that, with time, may become more standardized and usable to environmental effect assessments and regulatory decision making.

The most well studied of these assays are the measurements of enzyme inhibition (Rossel et al 1997). Microorganisms, especially fungi, and plants commonly excrete enzymes into the soil in order to facilitate the extracellular digestion of high molecular weight macromolecules, such as lignins, cellulose, and large proteins, which cannot pass the through the cell membranes. These enzymes can accumulate in the soil by being protected from biodegradation binding to clay or humic substances. The measurement of extracellular enzymes, such as dehydrogenase, catalase, aryl sulfatase, saccharase, amylase, glucosidase, urease, sulphatase, and phosphodiesterase, among others, have been used as measures of macro-nutrient cycling by the microorganism community. In these assays, a defined amount of soil is amended with a specific enzyme substrate, and soils are incubated as an aqueous slurry under conditions which optimize the kinetics of the reaction under consideration. The enzyme transforms the substrate to a specific by product, which is then analyzed. Although an indirect approach to contaminant effects, it is an easy measure of enzyme inhibition once optimal conditions can be established. The relative sensitivity of these assays has been evaluated only in a few soils and contaminants. For a review see van Beelen and Doelman (1994), Domnsch et al. (1979), and Fairbrother et al. (2000).

Another assay described by Rossel et al (1997) is the Direct Solid-Phase Toxicity Testing Procedure (DSTTP). This assay utilizes the Toxi-Chromotest kit which allows for the measurement of the *de novo* biosynthesis of β-galactosidase. The inhibition of β-galactosidase induction is determined after exposure of the test bacteria (e.g., *Escherichia coli*) to the test soil in an exposure scenerio similar to the Micro-Tox assay utilizing a bioluminescent bacteria.

The ATP assay measures the cellular concentration of ATP, the key form of energy storage in the cell. Viable vs. dead or dying cells can be identified with this test (Fairbrothers et al. 2000). It has the advantage of having available relatively inexpensive and simple commercial kits.

Many other functional measures, such as rates of colonization and succession rates, have not been studied well but hold potential value as future functional bioassays. Community functions, such as predation, mutualism, and commensalisms, also need additional study and assay standardization in order to be a valuable bioassessment method.

Genetic Shifts and Resistance

Biodiversity measures would appear to be a sensitive and useful parameter to measure contaminant effects in the soil ecosystem. However, the eradication of species in exchange for increases in resistant species is often difficult to measure, especially within microbial communities. Yet, the development of resistant species is a clear community indicator of ecological deterioration. As summarized in van Beelen and Doelman (1994), resistant communities have demonstrated decreased competitive capacity, decreased mineralization, decreased biodegradation capacity, decreased cold resistance, decreased gene pool available for genetic exchange and decreased metabolic diversity. Plant growth has also been shown to be negatively effected when mycorrhizal fungi became resistant to heavy metals. This effect on plants has been demonstrated repeatedly in contaminant (primarily metals) resistant nitrogen-fixing *Rhizobia* bacteria. It has also been shown that resistant bacteria accumulate contaminants which in turn can have adverse effects on higher levels of the food chain.

Organisms become resistant in four ways (van Beelen and Doelman 1994):

1. Organisms obtain resistance by mechanisms which allow them to withstand stress when exposed to that stress by limiting uptake, maximizing excretion, or by detoxifying the stressful contaminant.
2. Organisms obtain phenotypic physiological resistance by becoming acclimated to the contaminant for longer periods of time
3. Organisms obtain genetic resistance when individuals most adapted to the contaminant gain a competitive advantage over less adapted individuals and become dominant over many generations
4. Communities become resistant as resistant species replace non-resistant ones which may or may not perform the same function, or perform under the same conditions.

Methods for measuring resistance are generally only available for microbes. The most utilized method requires the isolation of soil microbes grown on agar plates in the laboratory, which are then exposed to high threshold concentrations of the contaminants of interest. The percentage of resistant strains from contaminated soils is compared to that of clean soils. Other methods take advantage of visual detection mechanisms and incorporation of traceable materials to identify resistant strains.

Limitations and Advantages of Functional Assays

The value of functional measure assays lie are their relative ease of conduct and quantified data which facilitates interpretation. Results are available relatively quickly and specialized equipment is generally not needed which assist in keeping cost low. As with many bioassays, functional assays provide only a snap-shot in time of the status of the ecosystem. Populations are therefore subject to large natural fluctuations that might mask any real contaminant effect. The heterogeneous nature of soils also requires careful selection and definition of samples and sites.

BIOINDICATORS AND BIOMONITORING

While toxicity tests are designed to assist in predicting the possible impact of a contaminant(s) should it be released into the environment, biomonitoring methods are designed to assess overtime and with changing conditions the impact of stressors or contaminants actually in the environment. Biomonitoring is the assessment of change in an environmental parameter(s) over time when compared to baseline data (Butterworth et al. 1995, van Straalen and Krivolutsky 1996). Biomonitoring methods are designed to determine if disturbances in soil processes and communities are occurring and their possible effect on the soil ecosystem.

Use of Environmental Bioindicators

An environmental indicator is a metric which reflects the status of or trends in an environmental parameter (Cal/EPA 2002; NCR, 2000) . A bioindicators is one type of environmental indicator that measures a change in an individual, species, population, community, or ecosystem level in response to a change in some essential environmental parameter (Jeffrey and Madden 1991, Butterworth et al. 1995, Straalen and Krivolutsky 1996). Because it is not possible to measure all aspects of an environment, biotic indicators are necessary to providing timely and cost-effective insight into the health of the environment. The US EPA's Science Advisory Board (SAB) prepared a report on the use of environmental indicators in 2002. They identified biotic conditions as one of the 'essential ecological attributes' that describe an ecosystem. They defined an environmental indicator reporting on biotic conditions (bioindicator) in a similar fashion as others; a measure of the structure and composition of biota including organisms, population, and ecological communities.

The ideal bioindicator should (modified from Hawkesworth 1992 and NCR 2000):

1. Demonstrate a prompt and accurate response to a particular discrete cause of environmental stress
2. Be representative of relevant ecosystem function
3. Represent lower parts of food webs and food chains in order to allow early detection and correction
4. Be amenable to cost effective and have resource-friendly sampling methods and standardization
5. Be easily understandable and interpreted, especially in relevance to environmental goals and policies, i.e., the convey meaningful information that reflect key features of the environment
6. Have widespread utilization
7. Be quantifiable and credible. Although the choice of which indicator to use may be subjective, a good indicator will be repeatable and in a numerical form that can be compared to other sites or times in anon-subjective manner.
8. Be sensitive to different degrees of environmental perturbation

While soil bioindicators have not been developed and applied to the level of sophistication found in aquatic systems (van Straalen and Krivolutsky 1996), several approaches have been taken to

use biological indicators for the assessment of soil. The following section reviews some of the apporaches currently being used.

In the late 1990s, the US EPA asked the National Research Council (NCR) to assist the agency in reevaluating its approach to environmental monitoring by critically evaluating the use of indicators to monitor ecological changes from either natural or anthropomorphic causes. In response, NCR established the Committee to Evaluate Terrestrial Environments (hereafter referred to as the "Indicators Committee"). The Indicators Committee reviewed the EPA's Environmental Monitoring and Assessment Program (EMAP), as well as, relevant NRC previous reports, other agency and published documents and peer-reviewed literature. In concert with other NRC committees and boards, the Indicators Committee developed and published a list of ecological indicators for the nation (NRC 2000). These indicators were based upon careful consideration of desirable characteristics including: *general importance, conceptual bias, reliability, temporal and special scales, statistical properties, data requirements, skills required, data quality, data archiving, robustness, international compatibility, and costs, benefits and cost-effectiveness.* In order to be useful to policy makers, the indicators need to be understandable, clearly quantifiable and broadly applicable.

The final NCR recommended indicators were placed in three broad categories as follows:

Indicators of the nation's ecosystems
- Land Cover
- Land Use

Indicators of the nation's ecological capitol
- Total species diversity
- Native species diversity
- Nutrient runoff
- Soil organic matter

Indictors of ecological function or performance
- Carbon storage
- Production capacity
- Net primary production
- Lake trophic status
- Stream oxygen
- Nutrient-use efficiency (agricultural ecosystems)
- Nutrient balance (agricultural ecosystems)

Many of these indicators can be used to evaluate the health of soils on a regional or site-specific scale.

Keystone Species Assessment

Keystone species are species within the environmental that have a greater role in maintaining ecosystem function than would be predicted based on its abundance; they exercise a disproportionate effect on other species or ecosystem function (Hawkesworth and Ritchie 1993). They are important, if not essential to maintaining and preserving ecological balance. For

example, certain parasitic wasps that regulate one or more prolific plant-eating host species can serve as a keystone species. The loss of control by such wasps has been shown to have devastating effects on the environment (LaSalle 1993). When such species are rare or particularly sensitive to environmental stressors, their identification and preservation may be critical to maintaining ecological health. Many other examples of keystone species, such as pollinating bee/wasp species, mycorrhizal associations, mutualistic gut bacteria and fungi, key resources species (e.g., microbiotrophic nematodes) and pathogens, have been documented (see Hawkesworth and Ritchie 1993). The loss of such keystone species can have a cascading effect, which can be both detrimental and unrecoverable for the ecosystem. Consequently, measuring the population of keystone species in the soil is can be helpful in an evaluation or assessment process.

Critical Body Concentrations

Critical body concentration or residue (CBR) is the concentration of a contaminant that is associated with a toxic endpoint, such as mortality or a physiological dysfunction. The residue is that fraction of the environmental contaminant that is bioavailable, as evidenced by its uptake and assimilation by the organisms. Evaluating the body burden of contaminants in soil dwelling organisms is another way to evaluate the biotic condition of the soil. In particular, the measurement of body concentrations of contaminants can provide insight into the bioavailability of environmental contaminants.

CBR becomes a measure of bioavailability when certain conditions are met (van Straalen 1996):

1. The chemical of interest is persistent in the body, i.e., the rate of elimination and metabolism should be of the same order of magnitude as the rate of uptake, resulting in measurable residue.
2. The chemical is not well regulated by the organism so that the body's internal concentration is dependent on the external bioavailability.

This measure is therefore useful for persistent chemicals, such as organohalogen compounds and non-essential metals. Critical body concentrations can be used to assess the retention of the chemical in the body of the organism proportionate to the environmental concentration of the environmental contaminant and are the sum of the body's assimilation and excretion of the chemical. In the case of chemicals which can be metabolized (e.g., non-persistent pesticides), are heavily regulated or excreted by the body, or are otherwise transient in the body, critical body concentrations may not give an accurate measure of body uptake. Macro-invertebrate species with their close contact with the soil environment and measurable size have been assessed at several sites (van Straalen 1996). Newer micro-techniques have allowed assessment of organisms as small as an individual oribatid mite (20 μg dried body weight) (van Straalen 1996). Vertebrate species may also be used when their utilization of the site can be assessed. The larger home range of vertebrate species may limit the utilization of this method unless use of the site under examination can be relatively accurately ascertained. *In situ* pen studies using small mammals or other vertebrate or invertebrate species have been used to assess on-site bioavailability, by can be limited in terms of accurately reflecting actual site use.

In order to effectively use this data to assess ecological risk, the results must be assessed in relation to a reference system that ties the results to actual risk of harm. The highest body concentration may not necessarily be the best indicator of the greatest risk if it is not associated with some adverse effect. The Lethal Body Concentration (LBC), the concentration above which physiological functions are irreversibly impaired for the test animal, or the Internal Threshold Concentrations (ITC) for sublethal endpoints (e.g., growth, reproduction, etc.), which ever has the higher ecological relevance, have been proposed as a means to estimate actual ecological risk from body residue measurements. Van Straalen (1996) describes the Bioindicator Index for body residues as the critical body residue divided by the LBC or ITC for a particular species in the field. Comparisons between species can indicate relative ecological risks between species. Long-term exposure may subject to genotype selection of tolerant individuals, which may effect data interpretation, as well as, the presence of multiple contaminants that may have synergistic or other effects.

Changes in Populations as an Indicator

Microbial biomass in soil is a useful indicator of the health of soil. As noted in the previous section, healthy soil contains a diverse community of organisms that serve a variety functions, including fixing nitrogen, recycle plant nutrients, and controlling plant diseases (Arias, 2005). Direct counting of biomass can be done with fluorescent microscopy or by measuring soil respiration, the oxidation of organic matter to CO_2 by aerobic organisms. A number of molecular techniques can be used to estimate biomass, including the quantification of DNA extracted from soil.

Invertebrate species offer promising use as indicators of disturbances in soil ecosystems, including beetles, spiders, earthworms, termites, myriapods, mites, free-living nemotodes and springtails to mention just a few (Lee 1991, Nestel et al. 1993, Paoletti et al. 1991 and van der Wal and de Geode 1988). Limitations in use stem chiefly from the lack of baseline data in undisturbed environments and the inability to tie changes in numbers to specific changes in the environmental contamination or perturbations. Other limitations in sampling methods, size and species identification may also limit widespread use in some cases.

Soil dwelling organisms can also serve as a useful indicator of the health of non-soil ecosystems, such as forests. For example, the frequency of ectomycorrhizal fungal fruit bodies in spruce forests is an excellent measure of the health of these forests. Had they been assessed at the time, they would have indicated the adverse effect of pollution on the central European spruce forests 10-15 years prior to the actual forest impact. (Arnolds 1991).

Biomarkers

Biomarkers are biotic indicators at the sub-organism level. They are molecular, biochemical, physiological, or histological measures that reflect a change in response to exposure to a stressor. They are measured in individuals or their excretion products and have been widely used as indicators of environmental stress and contamination when compared non-stressed individuals (van Straalen and Krivolutsky 1996). The number and variety of potential biochemical or physiological biomarkers is extremely large but not well explored in soil organisms in

comparison to aquatic organisms. One example is the measurement of phospholipid fatty acids (PLFA) in soil. PLFA are an essential memebrane component, the measurement of which can be used to characterize microbial communities in soil (Arias, 2005).

Biosensors

One of the newest bioassay technologies currently in development is the use of transducer technology to enable pollution monitoring through the use of bacterial biosensors (Atkinson and Rawson 1994). Biosensors transducer technology directly monitors cellular activity, exploiting the fact that biochemically generated signals can be converted into quantifiable electronic signals using a transducer. Biosensors were originally developed for the health care industry with specific analyte detectors, but by monitoring more generalized biological components, such as multireceptor cellular biocatalysts, broader band detectors have been developed for pollution monitoring, primarily in aquatic environments.

Whole cell biomolecular and cellular biosensors have been developed utilizing respiratory and photosynthetic bacteria as the biocatalyst. In this assay, whole cells are immobilized generally using physical methods (adsorption, supportive medium) rather than chemical methods, such as covalently binding, to optimize the stability, sensitivity and operational life of the biocatalyst. Gel membranes and filters have often been employed in this capacity allowing for the necessary intimate contact between biocatalyst and the transducer. The immobilized cells are then held against the transducer elements, which transform the biological signal into an electrochemical signal when the cells are stimulated. Soil samples, as opposed to aquatic samples, require a phase change pretreatment that may limit the usefulness of this technology. Like other bioassays, effective biosensors must respond to a wide range or chemicals, and ideally to both single chemicals as well as contaminant mixtures. Catalytic reactions found to be useful include substrate consumption, product formation, photosynthesis and respiration

Biosensors have the potential to be rapid, low cost, and sensitive. The necessary phase change and the current inability to utilize anaerobic bacteria may limit this assay at this time, but the number of species yet to be explores may offer a new battery of assays for environmental assessment and monitoring in the future.

UTILIZATION OF ORGANISMS IN BIOASSESSMENT

Terrestrial Microorganisms

That soil microbial communities are essential to soil ecosystem structure and function where they perform required mineralization and geochemical reactions is well understood. Microorganisms also maintain critical symbiotic and pathogenic relationships between microorganisms and higher organisms (van Beelen and Doelman 1996). Not only do many plant species require very specialized symbiotic root fungi or mycorrhiza to survive, but every plant lives in association with specialized rhizosphere microflora which are vital to the competitive ability of the plant. In addition, many invertebrates, such as *Collembola* and *Enchtraeidae*, graze heavily on microorganisms as a major or primary food source. Changes in the microorganism community may result in the inability of plants, other microorganism, and higher organisms to

survive in their absence.

The critical and encompassing role of microorganisms in the environment and their intimate contact with the soil, suggest that they would make ideal indicators of contaminant effects and that changes in the genetic diversity of the microbial community would have an inherently negative impact on the long term quality of the soil ecosystem. Doelman and Vonk (1994) suggest that microorganisms are particularly suited to providing an early warning of environmental impacts because of their ubiquity, size, versatility and important role in foodwebs and element recycling. Their very small size, in comparison to invertebrate and vertebrate species and constant soil contact provides a very large surface area-to-volume ratio in contact with potential contaminants.

Torstensson (1997) lists four functional levels at which microorganisms can be used to assess environmental contaminants (Table 2-2).

Table 2-2. Microorganism Indicators of Environmental Change within Functional Levels (modified from Torstensson 1997)

Function Level	Examples of Indicators
Organisms	• Genetic change • Enzyme activity changes • Physiological changes (e.g., growth)
Populations	• Biomass • Number of populations (e.g., actinomycetes, bacteria, fungi) • Specific organisms (e.g., ammonifiers, cellulose degraders, cyanobacteria, denitrifiers, ligninolytic organisms, mycorrhiza, nitrifiers (ammonium oxidizers, nitrite oxidizers), proteolytic organisms, *Rhizobium* spp.
Activities	• ATP-measurement, CO_2 production, heat production, O_2 consumption, • Ammonification, cellulose decomposition, denitrification, litter decomposition, nitrogen fixation (*Rhizobium*, heterotrophs, cyanobacteria), straw decomposition, sulfur oxidation • Combination of activity and biomass data
Interactions	• Mycorrhyza (ecto, arbuscular), pathogens, physiological changes, *Rhizobium*, rhizosphere organisms (associate nitrogen-fixers, producers of growth stimulating or inhibiting substance) • Soil aggregate stabilization (bacteria, fungi)

Microorganisms are generally studied at the community level primarily through functional responses of "microbial consortia" (Eijsackers 1994) rather than at the single species level. There are numerous functional responses that can be studied but most focus on the soil respiration (carbon mineralization) or more specific mineralizations, such as nitrogen, phosphorous or sulfur, as a measure of nutrient cycling. Using these techniques, the Most Probable Number (MPN) is estimated as the numbers of strains or species capable of breaking down the specified substrate. Because the majority of soil microorganisms in soil are in the inactive state, these measures are critical to assessing contaminant effects on potential soil capability. In these studies, the biodegradation potential, through estimating redox conditions such as methanogenic, sulfate reducing, denitrifying, and aerobic conditions, can be used to define the electron acceptor involved in the breakdown of the contaminant of concern (Eijsackers 1994).

Terrestrial Invertebrates

Invertebrate toxicity tests were reviewed in some detail in Part 1 of this document. Use of these assays in bioassessment has been a relatively recent event. Although their diversity is not as high as the microbial community, there are a tremendous number and variety of multicellular soil organisms, with protozoans and nematodes being the most abundant (van Gestel and van Straalen 1994). Although nematodes and other small organisms, such a protozoans and tardigrades, inhabit the soil environment with minor influence on soil particles, other organisms

interact with soil through burrowing or ingestion, such as mites (Acarina), springtails (Collembolla), woodlice (Isopoda), snail and slugs (Mollusca), millipedes (Diplopoda), and earthworms (Lumbricidae). Organisms in the litter layer, such as spiders and arthropods, live on the soil surface but play an important role in the soil ecosystem.

With the standardization of the earthworm toxicity test (OECD 1984) and earthworm subchronic test (EPA 1996a), invertebrate toxicity endpoints are increasingly being used in bioassessment procedures. Overall, soil invertebrates are used to assess the effects of contaminants on the biochemical/physical, individual, population and community level (van Gestel and van Straalen 1994, Eijsackers 1994). As described in Phase I, soil invertebrates can be used to assess contaminated soils brought into the laboratory or in artificially created soils with specific contaminant levels made in the lab where lethality and reproduction are the primary endpoints assessed. A wide range of *in situ* protocols are also increasingly being used in site bioassessment, including assessment of presence or absence of species, relative diversity and abundance, uptake, accumulation and excretion mechanisms and effects, behaviors, such as avoidance, biomarkers, such as biochemical and physiological responses, species composition and population ecology characteristics, however few of these assays have been standardized (Eijsackers 1994). Invertebrates contribute significantly to decomposition and nutrient cycling, particularly nitrogen mobilization, which can be useful bioassessment endpoints. Changes in community structure and species interactions , e.g., predator-prey relationships, offer other possible endpoints. The selection of the most appropriate invertebrate species is also under much investigation, especially in correlation to the life history pattern of the species and exposure scenario (dermal, oral or respiratory). Bioassay species, such as earthworms, often range on the higher end of invertebrate reproduction rates which facilitates laboratory utilization but may not provide the most sensitive life history pattern for the contaminant(s) of concern.

Honey bees are a terrestrial species that have been routinely advocated and used as a test species for pesticide registration assessment (Urban and Cook 1986). Test procedures include the Honey Bee Acute Contact Toxicity (EPA 1996b) and Honey Bee Toxicity of Residue on Foliage (EPA 1996c) tests. While valuable in comparing the relative toxicity of chemicals to an extremely important and sensitive species, the life history of honey bees do not bring them in contact with soil to an appreciable amount, limiting the value of these tests in the arsenal of soil bioassessment methods.

Terrestrial Vertebrates

Terrestrial vertebrate assessment procedures have by and large been limited to standardized lethality, reproduction and limited bioaccumulation tests (Suter 1993). And within that limited scope, data is further generally limited to traditional human health laboratory test species and to the effect of pesticides. Terrestrial species testing has been guided by the need for comparing the relative toxicity of pesticides and hazardous chemicals as required under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA; EPA 1972) and Toxic Substances Control Act (TSCA; EPA 1985, 1987, 1988), respectively. Exposure routes of pesticides, in particular, were predicted to be direct exposure (e.g., granular consumption, coated seed consumption, direct dermal/respiratory exposure during application, plumage preening) and most testing has

consequently been focused on short-term laboratory studies, to determine acute oral and dietary median lethal (LD_{50}) toxicities.

Beginning in the early 1980s, representative wildlife species have been included in routine testing. Bobwhite quail (*Colinus virginianus*) and deer mice (*Perimyscus maniculatus)* have become the acceptable representative terrestrial wildlife avian and mammal species (Urban and Cook 1986 and EPA 1982), respectively. Several authors have compiled relative toxicity data for wildlife species for pesticides and repellents of concern including Hill et al. 1975, Schafer et al. 1982, 1983, Hudson et al. 1984, Schafer and Bowles 1985, and Hill and Camardese 1986). Much additional information has been generated, but is often unavailable due to the proprietary nature of chemical manufacturer's data. Data for non-pesticides is generally unavailable although some metal data has been generated and compiled (See, for example, Venugopal and Lucky 1978)

Toxicity tests for reptiles and amphibians are rare (Suter 1983). Standardized test method for aquatic amphibian larval are available but do not provide extrapolation to soil systems (Hudson et al. 1984, Mayer and Ellersieck 1986, Birge et al. 1981). Very few vertebrate species live essential parts of their life stages in the soil.

The utilization of many vertebrate species is limited by their relatively large home ranges, fluctuating use of the contaminant site or unmeasurable exposure characteristics. There are, however, representative soil dwelling mammals (e.g., badgers, moles, shrews), amphibians (e.g., salamanders, some frogs) and reptiles (e.g., some lizards and snakes), which spend a substantial amount of time in direct contact with the soil environment for either extended periods of time or during critical life stages (e.g., feeding, reproduction, hibernation, aestivation). Under these conditions, assessment of bioaccumulation, physical and biochemical effects, behavioral effects, and community structure in soil-dwelling vertebrates can be useful characteristics in a multimetric site assessment. Birds and other mammals, reptiles and amphibians, which may access the site on a regular basis in the course of normal activities, feeding, bathing, nesting, providing both direct and indirect exposure to site contaminants may also provide critical bioassessment information. The usefulness of bioassessment tools for these vertebrate species will depend upon the size of the site, the extent of the use of the site by individuals or populations and the nature of the contaminants of concern. Utilization of the site can be enhanced through the use of *in situ* pen studies, nest box establishment (Kendall et al. 1989), or other mechanisms that improve, control, or accurately assess site utilization by the those species.

Several bioassays utilizing small mammals have been proposed (Ma 1994). Small mammals have been proposed as model species because of their close proximity with the soil, their comparatively small home ranges, their small body size and metabolism in relation to their consumption of plants and organisms in direct contact in the soil and their occupation in a wide variety of trophic levels. They are generally free ranging on sites of interest and relatively easy to handle and capture. Alternatively, some species are amenable to being placed on-site in enclosures for more controlled study. A wealth of data is available on laboratory counterparts that can facilitate data interpretation. And finally, small mammals fulfill important roles in the terrestrial ecosystem that makes their presence critical. Some of the more frequently used/proposed endpoints include: target organ analysis, bioaccumulation and bioconcentration

factor, biochemical indicators of exposure and effect, mortality or presence/absence, reproduction, physical parameters of health and growth and population dynamics (e.g., age class distribution).

CONCLUSIONS

It is well accepted that there is a fundamental shortage of data regarding the possible adverse effects of environmental contaminants on biological life in soil (Eijsackers 1997). Recent bioassay development for soil environments has shifted away from the use of higher organisms for toxicity assessment towards microorganisms (Atkinson and Rawson 1994). The role of microorganisms and invertebrates, in particularly, in performing vital ecosystem functions and sustaining stable ecosystems, have been overlooked in assessing and managing ecosystems and yet are critical to ecosystem conservation (Hawkesworth and Ritchie 1993). These newer assays offer rapid and inexpensive results suitable for environmental assessment and for the first time potentially offer mechanisms for real-time, continuous environmental monitoring. Interpretation is sometimes exacerbated by the sheer complexities of soil composition effecting both availability (i.e., physico-chemical and biological) and the biological organization and interactions of possibly many thousands of species and their organizational structure. Research is currently being conducted to develop additional new test methods at both the species and population level, calibrating existing methods and conducting much needed field validation (Eijsackers 1997). This last point is particularly of importance, as ecological validation of the methods was clearly recognized as one of the most important data gaps at the 1994 SERAS Workshop on Ecological Principles for Risk Assessment of Contaminants in Soil held in Papendal, Arnhem, The Netherlands (van Straalen and Løkke 1997).

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