November 3, 2003

Lecture 16 Microbial and Plant Toxicity

I. Overview of Microbial Biochemical Ecology

A. Composition of Soil

1. Soil essentially consists of a solid, water, and air phase (Figure 1).
   a. The types of solids depends on the soil origin
      1. The solid phase consists of inorganic minerals (for example, iron oxides) and "polymers" and organic matter
      2. The solid components are classified by chemical composition and particle size
         a. Clays (sheets of repeating units of Si, O or OH, Mg, Al, and several other cations)
            1. Particle sizes less than 2 μm
         b. Silts (particles >2 μm – 50 μm)
         c. Sand (particles >50 μm – 2 mm),

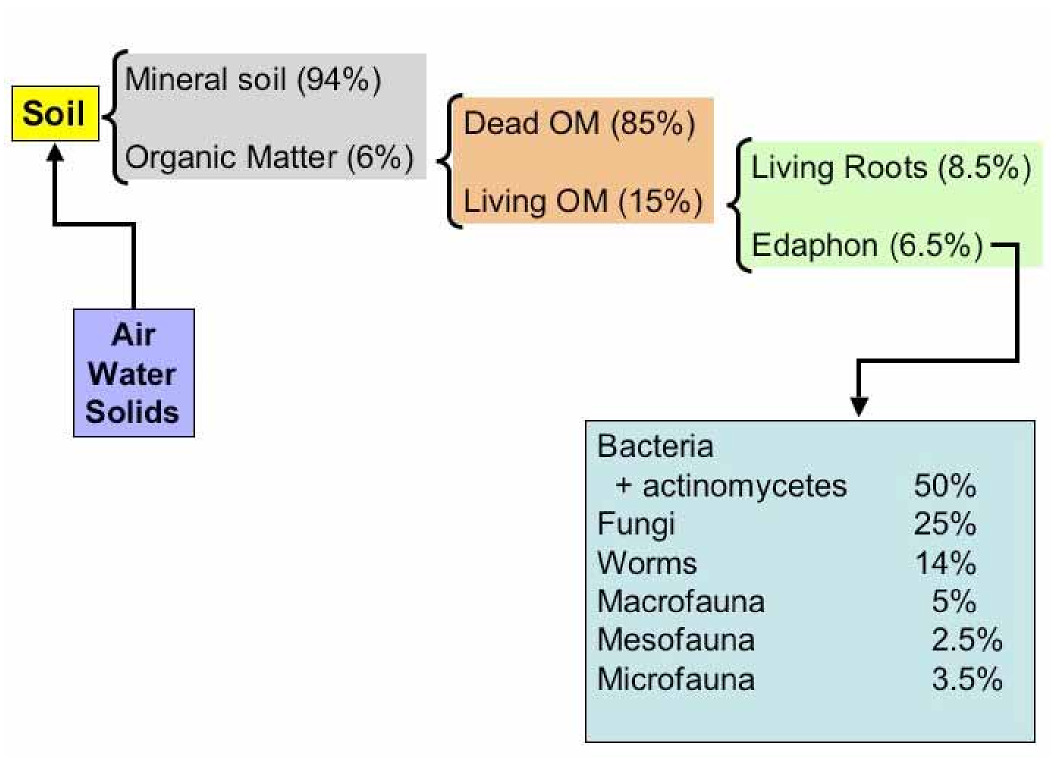

Figure 1. Composition of soil. Model is a grassland soil and percentage represent the dry weight proportion of each component. (Adapted from figure presented in Eijsackers 1994)

   b. The proportion of air and water depends on the texture (mechanical composition) of the materials and the ambient climatic conditions (i.e., has it just rained or is it droughty).

1. The solids are organized so that spaces are left around each particle (Figure 2).

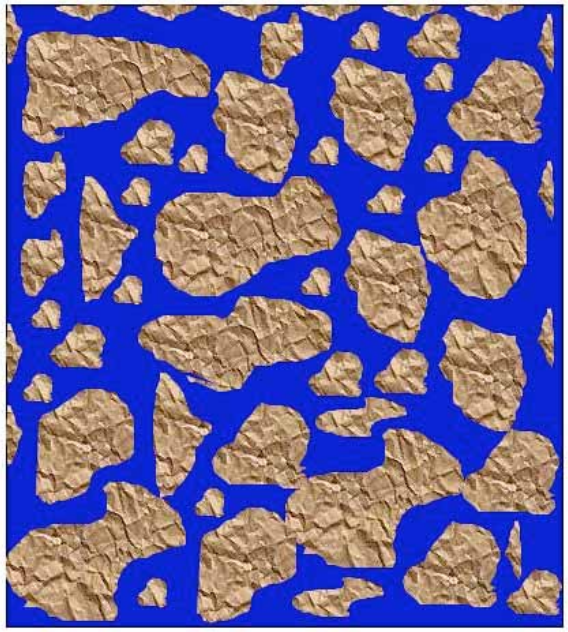

Figure 2. Packing of soil particles determines the amount of pore space and thus the amount of water the soil can hold when all the spaces are filled. Spaces partially filled with water are filled with air.

   a. These spaces form a network of continuous and discontinuous pores that are filled with either water or air
2. The maximum amount of water that the soil can hold depends on the texture of the soil (percentage composition of clay, silt, and sand) as well as the type of solids and amount of organic matter.
3. Whatever

2. Organic matter (OM) content is normally small by proportion to the inorganic components (for example clay fraction);
   a. A soil with 6% organic matter (OM), for example, would be considered to have a moderately high content of OM.
   b. Soils of east central WA tend to have <2% OM and very little clay.
   c. Organic matter can be further divided into dead and living OM (Figure 1).

B. Concerns over microbial toxicity stem from the central role of soil microorganisms in biogeochemical cycling and soil fertility
   1. Biogeochemical cycling mainly involves the cycling of carbon, nitrogen, phosphorus, and sulfur from inorganic to organic forms, and back again to inorganic forms. (See Figures 3, 4, and 5)
   2. All plant life depends on bioavailability of nutrients, especially nitrogen.

a. Microorganisms decompose organic matter and recycle C, N, P, and S by turning it from organic species into bioavailable inorganic forms that plants can absorb and metabolize.
b. Thus adverse effects on microbial functional diversity can reduce soil fertility and thus plant biomass.

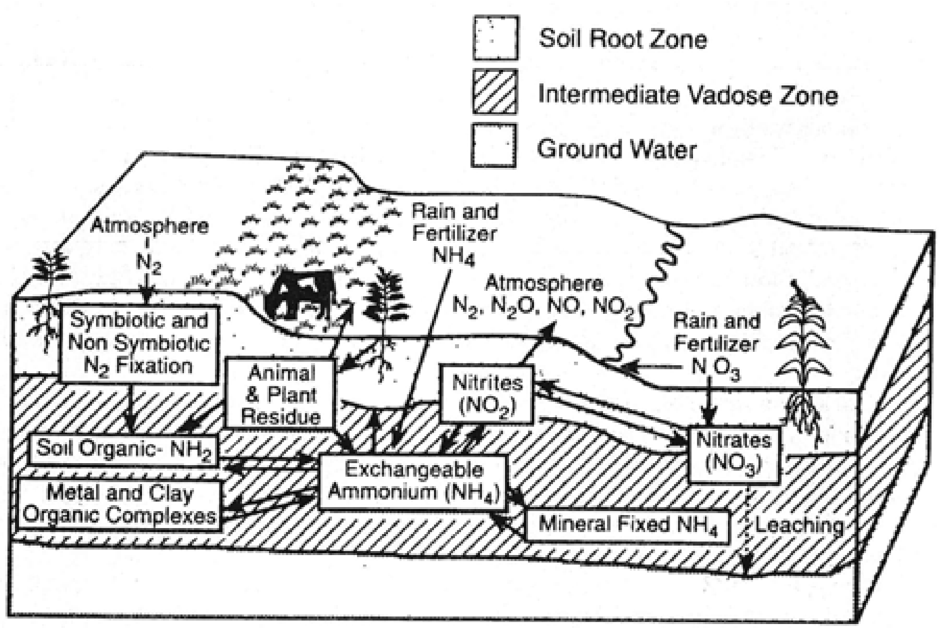

Figure 3. Cycling of nitrogen in the environment (figure from Rechcigl, 1995, Soil Amendments & Environmental Quality, CRC Press). Note that nitrogen goes through a series of oxidations and reductions. Nitrogen species are found in air, water, and soil.

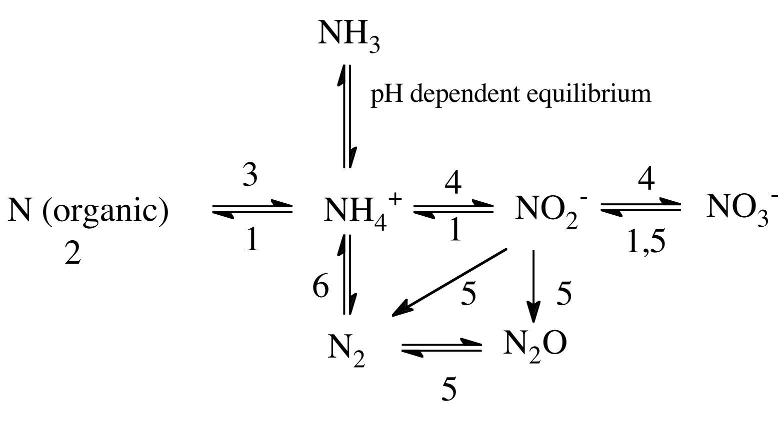

Figure 4. Nitrogen reactivity in the biogeochemical cycle. Numbers refer to specific oxidation or reduction reactions. **(1) <u>Assimilation of inorganic N</u>** (mainly NH_4^+, which is in a pH dependent equilibrium with NH_3, and NO_3^-) by microorganisms and plants to form organic N such as amino acids (which become proteins) and nucleotide bases (which become "polymerized" into DNA, RNA). Also known as immobilization. The metabolic pathway is called the glutamine synthase-

synthetase pathway; **(2)** **Heterotrophic conversions** involving the transfer of organic N among organisms; **(3)** **Mineralization (or ammonification)** involves degradation of organic matter containing N to NH_4^+ by bacteria and fungi; **(4)** **Nitrification** involves conversion of NH_4^+ (ammonium ion) to NO_2^- (nitrite) and NO_3^- (nitrate); the reaction is microbial-mediated oxidation (by nitrifying bacteria); **(5)** **Denitrification** involves reduction of nitrate to nitrite; nitrite can be further reduced to gaseous N_2O and N_2 **(6)** **Biological Nitrogen Fixation** involves direct absorption of atmospheric N_2 to form NH_4^+

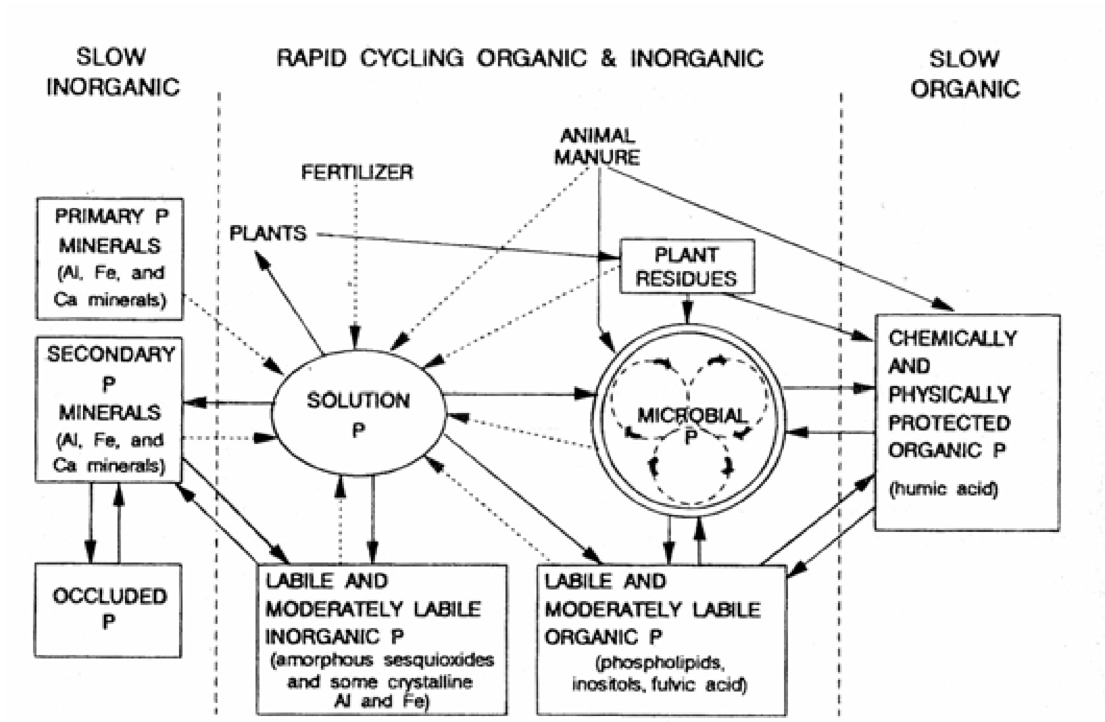

Figure 5. Phosphorus cycling in the environment. Note that there is not air phase in the cycle as there is for nitrogen cycling.

C. Distribution of Microorganisms in the Soil (Table 1)
   1. Most microorganisms, and thus microbial activity most important in biogeochemical cycling, are near the surface of the soil.

Table 1. Distribution of microbial flora in soil (numbers x 10^3/gram soil) (Eijsackers 1994)

Depth (cm)	Aerobic Bacteria	Anaerobic Bacteria	Actinomycetes	Fungi	Algae
3-8	7800	1950	2080	119	25
20-25	1800	379	245	50	5
35-40	472	98	49	14	0.5
65- 5	10	1	5	6	0.1
135–145	1	0.4	--	3	--

D. Attention to microbial toxicity has been focused on essentially two activities:
   1. Disposal of wastes;
      a. Waste disposal activities, whether legally in a landfill, or inadvertently through spills of material, create very high concentrations.
         1. Given "dose makes the poison", the probability of causing microbial toxicity and thereby reducing microbial activity faces a high probability of occurrence.
   2. Pesticide application;
      a. The application process will always produce residues on soil regardless of the intended target.
         1. Often, the intended target may be soil directly:
            a. Weed control had conventionally relied on pre-emergent use of herbicides
               1. Modern weed control mostly use post-emergent herbicides that are applied to growing weeds in the crop
         2. Control of soil insects relies on the use of soil insecticides
            a. Soil insecticides used in corn production, the biggest consumer of insecticides from the viewpoint of total poundage, are actually applied in 18-cm bands over the seed row rather than broadcast throughout the field
         3. Fumigation with methyl bromide, 1,3-D (dichloropropene), and metam-sodium (which quickly hydrolyzes to the toxicant, methyl isothiocyanate)
         4. A number of fungicides are used to treat foliar pathogens, many of which are fungi.
            a. Thus, inadvertent deposition of fungicides on soil could adversely affect soil fungi

II. Measuring Microbial Toxicity

A. "In Vitro" Tests
   1. Microorganisms can be cultured in liquid broth or on agar.
      a. Population size can be established as turbidity units (if in liquid culture) or as colony forming units (in on agar plates)
      b. Can add toxin to culture and determine concentration related to effect on microbial numbers
   2. Can examine rates of respiration or other specific enzyme reaction

B. "In Vivo" Tests
   1. Whole soils can be tested for microbial activity, including
      a. Respiration (overall measurement for microbial activity); measured as the activity of dehydrogenase enzyme.
      b. Evolution of carbon dioxide (as a surrogate for microbial biomass and potential for mineralization of organic carbon)
      c. Enzyme specific reactions for processing nitrogen
         1. Nitrification
         2. Nitrogenase (nitrogen fixation)

        3. Urease
    d. Phosphatase activity (decomposition of organic phosphorus to phosphate)
  2. Specific functional groups can be enumerated using Most Probable Number techniques
C. "Ecological" Tests
  1. Decomposition of litter
    a. Leaf disks or material placed in mesh bags and buried in soil;
    b. Monitor disappearance of biomass over time (by visual inspection and by weighing
    c. Some discrepancy between field studies and lab studies have been noted; the lab studies do not seem predictive of the field studies
      1. de Jong (1998); Abstract:
        a. "A method has been developed for assessing the side effects of fungicides on decomposition using litterbags. Twenty dried leaf disks of Chinese cabbage, ***Brassica oleracea***, were placed in litterbags (20x20 cm) made of nylon fabric (25 μm mesh). The litterbags were laid in a treated plot and 5, 10, 20, and >200 m from the treated plot and covered with 1 cm of standard soil. After 1 week, the dry weight of the leaf disks was determined. In an iterative procedure the method was optimized and significant differences of 5% in decomposition rate between litterbags were found using the final method presented here. In and around agriculturally managed plots, negative effects on decomposition of two fungicides could be traced. Effects on decomposition were found after application of captan (in fruit, 1.5 kg a.i./ha) and maneb (in potatoes, 3 kg a.i./ha) up to 10 m from the treated plot, at moderate wind speeds (35 m/s). In one case the concentration of captan in the soil covering the litterbags was measured. A negative correlation was established between captan concentration and decomposition. These experiments were repeated in an experimental plot under more controlled conditions with respect to the amount and time of application. The results of the field experiments could not be reproduced on the experimental plot. Additional experiments with soil fungi in agar again demonstrated a significant effe3ct of exposure to captan, however. Given the results, the method using litterbags requires further development before a standard field trial can be designed. Given the knowledge gained with respect to the key factors involved a bioassay using soil fungi seems to have high potential, however."

III. Case Studies for Microbial Toxicity

A. Effects of Pesticide Applications on Microbial Activity
  1. Studies of Tu (1981a; 1981b) show that at normal application rates and doubled rates, any changes in various measures of microbial activity (when they occur) are temporary and the soil recovers quickly.
B. There has been concern about the long term use of pesticides and its effect on soil microbial activity and therefore fertility.
  1. Research at the Rothamsted Research Station in the U.K. has been conducted since the late 1800's and represents the longest running controlled experiments of agriculture worldwide.
  2. A study started in the early 1970's has been assessed for the effect of pesticides on soil fertility and crop health. No long term effects were noted as concluded in the abstract below (Hart and Brookes 1996). Nor was there any effect on crop productivity (Bromilow et al. 1996).
    a. Hart and Brookes (1996) Abstract:

1. "The effects of 19 years of cumulative annual field application of five pesticides (benomyl, chlorfenvinphos, aldicarb, triadimefon and glyphosate), applied at, or slightly above, the recommended rates in 25 combinations, on soil microbial biomass and the mineralization of soil organic matter were investigated. Soil samples were taken 1 month after the application of benomyl, chlorfenvinphos and aldicarb in April 1992, and again in October 1992, 1 month after the application of triadimefon and glyphosate. The addition of aldicarb caused a significant increase of 7-16% in soil microbial biomass carbon (biomass C), an effect which appeared to be persistent. This effect of aldicarb was not reflected in the mineralization rate of soil organic C, possibly because the measurements of CO2 evolution showed a greater variation than those of biomass C. Measurement of microbial biomass activity by the substrate-induced respiration method also gave much less precise results than measurements of biomass C by fumigation-extraction. The mineralization of soil organic N to ammonium and then nitrate was mostly unaffected by the pesticide treatments. In the autumn-sampled soil, there was significantly less NH4-N in the aldicarb-treated soil. It is possible that this was due to immobilization by the increased microbial biomass in these treatments, and did not represent a loss to the soil system. The continuous use of these pesticides, either singly or in combination, therefore had no measurable long-term harmful effects on the soil microbial biomass or its activity, as assessed by C or N mineralization."
2. Note that in the experiment by Hart and Brookes, microbial biomass and bioactivity were measured in the 19th year in the separate experimental plots. Each plot was kept separate and treated either singly or with a combination of herbicides/insecticides. In the spring and autumn of the last year of treatment, measurements were made. Figure 6 shows a typical result for microbial biomass measurements in the plots that received each of the denoted herbicides.

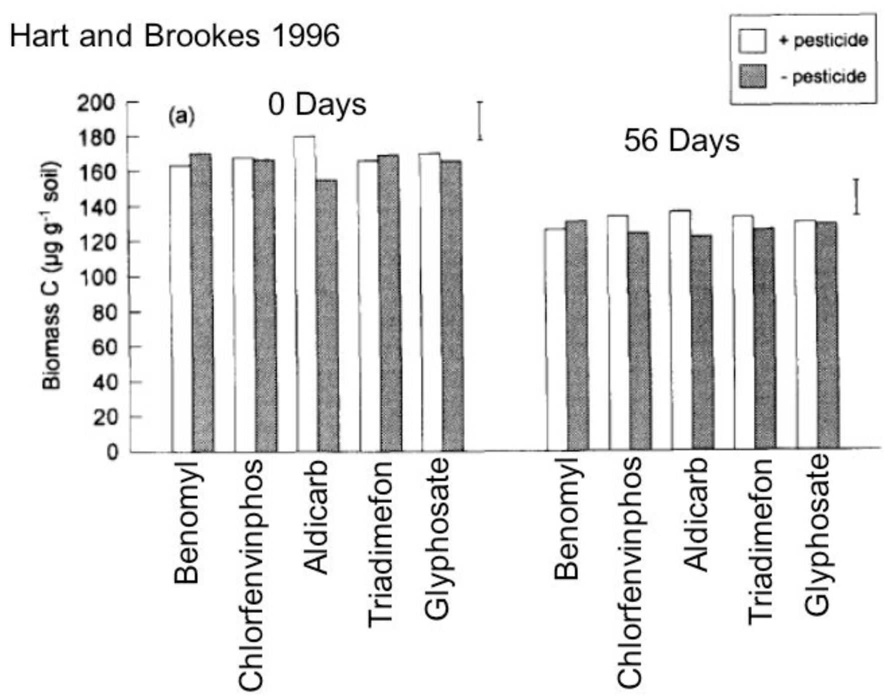

Figure 6. Effect of herbicide application on microbial biomass in long-term pesticide-treated plots at Rothamsted Experiment Station in the U.K. (Hart and Brookes 1996).

C. Effects of Waste Spills
   1. Dzantor and Felsot (1991)
      a. Simulated spills of the herbicide alachlor significantly reduced microbial biomass and bioactivity as measured by the Most Probable Number method and dehydrogenase activity.
      b. At normal rates of application, no effect on microbial biomass or activity were observed.

IV. Plant Toxicity

A. Similar to concerns about microbial toxicity. agricultural activities leading to exposure by pesticides (especially herbicides) at the field margins or alternatively due to longer distance movement in drift have been noted of concern for productivity on nontarget plants.
   1. Another concern has been the re-establishment of plant populations on former waste sites.

B. With regard to agricultural chemicals, herbicides are of most concern.
   1. Intended to kill plants, herbicides can drift during spraying and potentially affect nontarget plants around field margins.
   2. Under certain conditions, herbicide residues can move much farther distances than the field margin and cause morphological effects that could be economically injurious
      a. A good example is the extraordinary sensitivity of grapes to 2,4-D
      b. 2,4-D has been historically used to kill broadleaf weeds in cereal crops.
      c. Off-target movement of 2,4-D, either as direct drift and movement in wind, or as volatilized residues and movement in inversions, can cause injury to grapes that are in the path of the moving air masses.
         1. Many times, the injury is not damaging to yield, but rather causes notable morphological symptomology.
   3. In addition to off-target movement during spraying, some herbicides can persist through the growing season through the beginning of the next season.
      a. When crops are rotated, these new crops may be more very sensitive to the herbicide at low levels, and thus suffer injury leading to productivity loss.

C. Selected Examples of Herbicides and Mode of Action (those that have been most studied from an environmental toxicology perspective)
   1. The vast majority of herbicides have mechanisms of toxic action that is specific to plants.
      a. However, note that for some herbicides, the mechanism could potentially affect bacteria.
   2. Mode of action definitively known; likely related to one enzyme or receptor interaction
      a. Sulfonylurea (SU) herbicides and imidazolinone herbicides inhibit acetolactate synthase (ALS), an enzyme only occurring in bacteria and plants
         1. ALS is a key enzyme on the metabolic pathway for synthesis of branched chain amino acids (valine, leucine, isoleucine).

2. These herbicides are effective at use rates of grams per hectare (ounces per acre).
   a. Show biological activity when taken up from soil and by direct contact with foliage.
3. Examples:
   a. SU herbicides
      1. chlorsulfuron
      2. nicosulfuron
      3. thifensulfuron
      4. metsulfuron-methyl
      5. sulfometuron
   b. Imidazolinone herbicides
      1. imazapyr
      2. imazethapyr
      3. imazaquin

b. Glyphosate
   1. Inhibits the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS) in the shikimate acid biosynthetic pathway for synthesis of aromatic amino acids (tryptophan, phenylalanine, tyrosine)
      a. Only bacteria and plants have this enzyme (i.e., the shikimate acid pathway)
   2. Glyphosate is only active when it contacts the leaf surface.
      a. It is a true systemic, meaning that it can move in the phloem down to the roots.
      b. No activity when residues contact soil; due to lack of bioavailability to plant from soil.
   3. Rates of glyphosate application are in kg/ha (pounds per acre).

c. Auxin mimics (includes phenoxyacetic acids, such as 2,4-D and mecoprop; the benzoic acid dicamba; and the picolinic acids picloram, triclopyr, and clopyralid)
   1. Mimic the natural plant growth hormone indole-3-acetic acid
   2. Rates of application tend to be fractions of a kg/ha.

d. Photosynthesis inhibitors
   1. Includes a large number of herbicides, but the most studied one is atrazine
   2. Atrazine, which is a symmetrical triazine compound, has become the subject of an enormous number of papers because it is widespread in the environment, especially in aquatic habitats.
   3. Atrazine was one of the first broadleaf herbicides that was useful in corn production.
   4. Rates of application tend to be kg/ha.

3. Non-specific mechanisms
   a. Acylanilide (a.k.a. chloroacetamides): includes alachlor and metolachlor
      1. These herbicides, which have been used quite heavily in corn production, are also frequent contaminants of water (but not to the same degree as atrazine)

2. The specific of causing phytotoxicity remains unknown, but this group seems to affect lipid metabolism.
3. Applied directly to soil.
4. Rates of application tend to be kg/ha

b. Bipyridinilium Herbicides (Paraquat and Diquat)
   1. These herbicides, which are cationic (positively charged), cause a reaction that enhances free radical generation in the cell. Free radicals react with all other biochemicals and cause destruction of the cell.
   2. Only can effect toxicity when in direct contact with the plant surface.
   3. Rates of application tend to be kg/ha.

c. Dinitroanilines (trifluralin; oryzalin; pendimethalin)
   1. Adversely affect root elongation by inhibiting mitotic spindle formation; thus, cells cannot divide.
   2. Applied at rates of sub kg/ha.

V. Assessing Plant Toxicity

A. Presently, EPA requires that tests for effects of pesticides on plants be conducted on a few surrogate species including alfalfa and soybeans. Basically there are three types of tests that all pesticides are subject to regardless of their type (i.e., herbicide insecticide, etc.).
   1. Germination tests
      a. Seed are treated with the pesticides and then examined for germination potential
   2. Root elongation tests
      a. Seeds are treated with the pesticide or the newly sprouted hypocotyl is treated.
      b. Roots are examined for growth (elongation) potential
   3. Growth response
      a. Plants are potted and then sprayed with a pesticide
      b. Growth is measured both as biomass (just clip the plant above the soil surface, dry it at a specified temperature, and weigh it) or as height above the soil surface
      c. Plot concentration relative to biomass and estimate concentration causing 25% reduction in growth (called the GR25) (Figure 7).

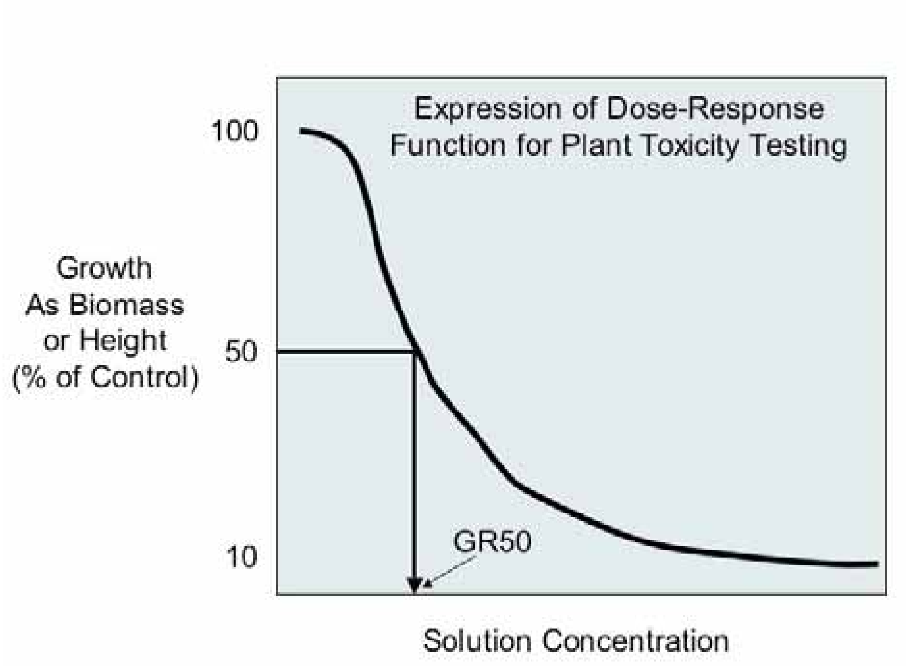

Figure 7. Expression of dose-response function for plant toxicity testing.

B. Ecological Observations
  1. Field observations of phytotoxic effects of pesticides are possible but the problem is the effects are likely to be localized.
  2. However, productivity as measured by chlorophyll A concentration in algal cells, or alternatively, gross photosynthetic activity can be measured under laboratory conditions.
     a. Photosynthesis can be measured in the field using portable instrumentation.
  3. Effects on productivity of aquatic plants can be viewed as a surrogate for higher level ecological effects.
     a. Recruitment in to aquatic habitats is likely to be more restricted than recruitment of plant species on the land.
     b. An example of using productivity of algae (or phytoplankton) as a regulatory endpoint is seen in how the EPA is determining the most sensitive endpoint for the herbicide atrazine in its ecological risk assessment as part of the pesticide re-registration process. (The web site URL for registration eligibility decision documents, known as REDs, can be found at http://www.epa.gov/pesticides/reregistration/status.htm; just click on the atrazine link and you will be taken to a number of documents regarding EPA assessment of atrazine risk. You'll be looking for the document known as EFED (Ecological Fate and Effects Division).
        1. The most sensitive toxicological endpoint is an adverse effect on phytoplankton productivity at a NOEL of 2.3 μg/L.

C. EPA is considering whether to revise its plant toxicity testing requirements to include effects on plant reproduction

1. The concern over plant reproduction came out of research with the sulfonylurea (SU) herbicides, essentially conducted by one group of researchers at the EPA Corvallis Lab and Oklahoma State University (Fletcher et al. 1993, 1995, 1996).
   a. This research was motivated by complaints of herbicide drift from wheat field on the Horse Heaven Hills above the Badger Canyon in Benton Co., WA.
      1. The herbicides involved were sulfonylureas, and at the time of the complaints they were applied by aerial spraying
      2. A couple (literally) of residents in the Badger Canyon complained to both the Washington Dept. of Agriculture and the EPA that the productivity of their crops (generally alfalfa and cherries) were being adversely effected.
      3. One of the tell-tale signs of low levels of SU exposure is an isolated diffuse yellow spot on the foliage (Felsot et al. 1996a).
         a. Cherry leaves, which unfold in April (i.e., comparatively early in the growing season) and have a very shiny waxy surface, are particularly good bioindicators of exposure to low levels of SU herbicide residues.
   b. Working with cherry trees, pea plants, and soybeans, Fletcher et al. reported they saw injurious effects on plant reproduction (for example, increases incidence of flower abortion) from low levels of SU herbicides without seeing an concomitant injury on leaves.
      1. Fletcher et al. claimed that the effect on reproduction would only occur if the plant was in a specific growth stage.
2. In contrast to Fletcher et al. observations, Bhatti et al. (1995) observed no effect of chlorsulfuron on cherry fruit production in the absence of easily observable leaf injury.
   a. Furthermore, comparatively high concentrations of chlorsulfuron were required to affect reproduction (as measured by fruit abortion).
3. To determine the likelihood that spray applications in the Horse Heaven Hills might be directly affecting plants in the Badger Canyon, sentinel plants were set up at different locations and exchanged on a weekly basis.
   a. Plants were returned to the WSU-Prosser research station (Irrigated Research and Extension Center, a.k.a. WSU IAREC) and examined for tell-tale signs of morphological changes indicative of exposure to SU herbicides, 2,4-D (or similar auxin agonist herbicides), paraquat, and glyphosate.
   b. To make a long story short (see Felsot et al. 1996b for the full picture), the appearance of leaf symptomology characteristic of exposure to either SU or 2,4-D residues was not correlated with times of spraying.
      1. A general atmospheric circulation and deposition of residues, perhaps associated with early morning very light drizzles, was more likely the route of exposure and not direct spray drift.
      2. Furthermore, given the nature of the morphological phenomenon on leaf surfaces, productivity of plants was unlikely to be affected.

D. Plants of "conservation" interest (i.e., native plant species) adjacent to farm fields have been studied in the U.K. (Marrs et al. 1991a,b).

1. Marrs et al. simulated drift of herbicides adjacent to an agricultural field. He would set out potted plants of interest and determine the effects on growth, appearance, and fecundity.
2. The age of plants definitively affected susceptibility.
3. Depending on the species saw varied affects, including fecundity
   a. Made observations at a distance of 8 m from the spray source.

References

Bhatti, M. A., K. Al-Khatib, A. S. Felsot, R. Parker, and S. Kadir. 1995. Effects of simulated chlorsulfuron drift on fruit set and quality of sweet cherries (Prunus avianum L.). Environmental Toxicology & Chemistry 14:537-544.

Bromilow, R. H., A. A. Evans, P. H. Nicholls, A. D. Todd, and G. G. Briggs. 1996. The effect on soil fertility of repeated applications of pesticides over 20 years. Pesticide Science 48(1):63-72.

de Jong, F. M. W. 1998. Development of a field bioassay for the side effects of pesticides on decomposition. Ecotoxicology and Environmental Safety 40:103-114.

Dzantor, E. K. and A. S. Felsot. 1991. Microbial responses to large concentrations of herbicides in soil. Environmental Toxicology and Chemistry 10:649-655.

Eijsackers, H. 1994. Ecotoxicology of soil organisms: seeking the way in a pitch-dark labyrinth. pp. 3-31 in "Ecotoxicology of Soil Organisms", Donker, M. H.; Eijsackers, H.;Heimbach, F. (ed.). Lewis Publishers, Boca Raton, FL

Felsot, A. S., M. A. Bhatti, and G. I. Mink. 1996a. Using sentinel plants as biomonitors of herbicide drift and deposition. J. Environmental Science & Health, B31 (4):831-845.

Felsot, A. S., M. A. Bhatti, G. I. Mink, and G. Reisenauer. 1996b. Biomonitoring with sentinel plants to assess exposure of nontarget crops to atmospheric deposition of herbicide residues. Environmental Toxicology & Chemistry 15(4): 452-459.

Fletcher, J. S. T. G. Pfleeger H. C. Ratsch. 1993. Potential environmental risks associated with the new sulfonylurea herbicides. Environ. Sci. Technol. 27:2250-2252.

Fletcher, J. S., T G. Pfleeger, and H. C. Ratsch. 1995. Chlorsulfuron influence on garden pea reproduction. Physiologia Plantarum 94:261-267.

Fletcher, J. S., T. G. Pfleeger, H. C. Ratsch, and R. Hayes. 1996. Potential impact of low levels of chlorsulfuron and other herbicides on growth and yield of nontarget plants. Environ. Toxicol. Chem. 15(7):1189-1196.

Hart, M. R. and P. C. Brookes. 1996. Soil microbial biomass and mineralization of soil organic matter after 19 years of cumulative field applications of pesticides. Soil Biology and Biochemistry 28(12):1641-1649.

Marrs, R. H., A. J. Frost, and R. A. Plant. 1991a. Effects of herbicide spray drift on selected species of nature conservation interest: The effects of plant age and surrounding vegetation structure. Environmental Pollution 69:223-235.

Marrs, R. H., A. J. Frost, and R. A. Plant. 1991b. Effect of mecoprop drift on some plant species of conservation interest when grown in standardized mixtures in microcosms. Environmental Pollution 73:25-42

Tu, C. M. 1981a. Effects of some pesticides on enzyme activities in an organic soil. Bull. Environ. Contam. Toxicol. 27:109-114.

Tu, C. M. 1981b. Effects of pesticides on activities of enzymes and microorganisms in a clay soil. J. Environ. Sci. Health. B16:179-191.