October 19, 2005
**Lecture 16: Behavioral Toxicity**

## I. Integration of Nervous System and Endocrine System

A. Behavior can be thought of as an integration of nervous system control with modulation from the endocrine system.
   1. All exogenous stimuli are perceived through an organism's sensory system that is in turn sending nerve signal to the brain.
   2. Hormone release will influence metabolic functions, but also "prime" an organism for reproduction and associated behaviors.

B. Why the fuss over behavioral toxicity?
   1. The concern over behavior as an adverse toxicological endpoint stems from the neurotoxic potential of a number of synthetic compounds, as well as a few metals (including lead and mercury) that have been intentionally introduced into the environment.
   2. Of all the anthropogenic organic chemicals, the neurotoxic insecticides have been argued to cause effects on neonatal rodents in testing, and therefore they may affect development, growth, and intellect of children.
   3. Some recent evidence suggests that OP insecticides, in particular chlorpyrifos, may have effects on brain development through adverse effects of neuronal growth and patterning that are not due to acetylcholinesterase inhibition directly as is characteristic of acute toxicity.
      1. Review article: Slotkin, T. A. 1999. Developmental Cholinotoxicants: Nicotine and Chlorpyrifos. Environmental Health Perspectives 107(Supplement 1):71-80.
         a. Abstract: "The stimulation of cholinergic receptors in target cells during a critical developmental period provides signals that influence cell replication and differentiation. Accordingly, environmental agents that promote cholinergic activity evoke neurodevelopmental damage because of the inappropriate timing or intensity of stimulation. Nicotine evokes mitotic arrest in brain cells possessing high concentrations of nicotinic cholinergic receptors. In addition, the cholinergic overstimulation programs the expression of genes that evoke apoptosis and delayed cell loss. Effects of cholinesterase inhibitors exhibit many similarities to those of nicotine. Chlorpyrifos administered to developing rats in doses that do not evoke signs of overt toxicity decreased DNA synthesis and caused shortfalls in cell numbers in brain regions enriched in cholinergic innervation. In embryo cultures, chlorpyrifos also evoked apoptosis during neurulation. However, chlorpyrifos also evokes noncholinergic disruption of cell development by interfering with cell signaling via adenylyl cyclase, leading to widespread disruption that is not limited to cholinergic systems. We have tested this hypothesis in vitro with PC12 cells, which lack the enzymes necessary to produce chlorpyrifos oxon, the metabolite that inhibits cholinesterase. Chlorpyrifos inhibited DNA synthesis in undifferentiated PC12 cells, which have relatively few cholinergic receptors. Furthermore, chlorpyrifos was more effective than nicotine and its effects were not blocked by cholinergic antagonists. When cells were allowed to differentiate in the presence of chlorpyrifos, cell replication was inhibited even more profoundly and cell acquisition was arrested. At higher concentrations, chlorpyrifos also inhibited neuritic outgrowth. Thus, chlorpyrifos elicits damage by both noncholinergic and cholinergic mechanisms extending from early stages of neural cell replication through late stages of axonogenesis and terminal differentiation.

> Accordingly, the window of developmental vulnerability to chlorpyrifos is likely to extend from the embryonic period into postnatal life."

4. Note that even if a chemical, for example, PCBs, do not have a distinct neurotoxic mechanism, these have been hypothesized to affect brain function and thus intellectual development of children. (e.g., Jacobson, J. L. and et al. 1990. Effects of in utero exposure to polychlorinated biphenyls and related contaminants on cognitive functioning in young children. Journal of Pediatrics 116:38-45.)
    a. The hypothesis for such an effect would likely be through interaction at the level of the endocrine system, perhaps through an effect on thyroid gland development and hormonal secretions.
        1. This interaction would be classified as an organizational effect because gland development could be altered during embryonic or fetal development, but the functionality of the effect might not be observed until after birth and through childhood.
        2. Another more recent hypothesis is that certain congeners of PCBs, specifically the non-coplanar ortho substituted congeners, may increase uptake of calcium at nerve terminals, thus stimulating either release of inhibitory or excitatory neurotransmitters (see Figure 1 for structures and an explanation of coplanar). (Seegal, R. F. 2003. Effects of polychlorinated biphenyls on neuronal signaling. Chapter 10 in Dioxins and Health, 2nd ed., A. Schecter, T. A. Gasiewicz, Ed. John Wiley & Sons, Inc. New Jersey. Pp. 433-455)

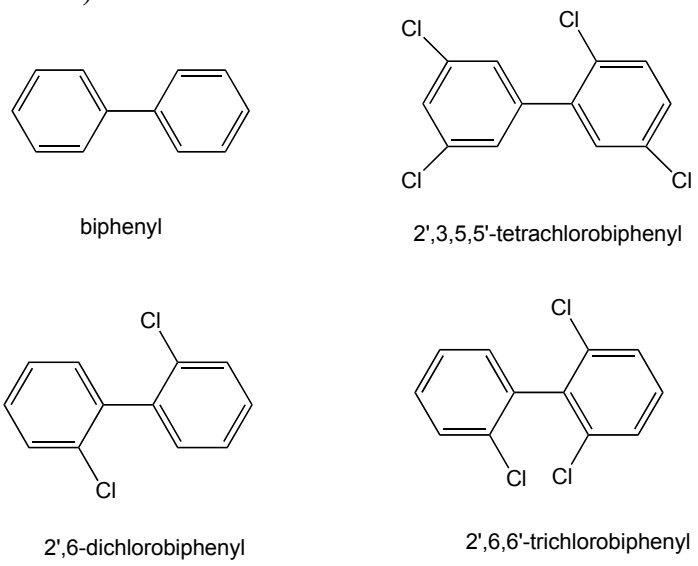



Figure 1. PCBs (polychlorinated biphenyls) are based on chlorine-substituted biphenyl; given all possible combinations of chlorine placement around the ring, 209 congeners are possible. Each ring can rotate freely and the structure can assume a co-planar shape (i.e., both rings lie in the same plane). When chlorine occupies either the 2 or 2' and 6 or 6' position of each ring (a structural form called ortho-substitution) simultaneously, rotation of the rings are inhibited from being in a co-planar position. Thus, in the figure, the 2,'6-dichlorobiphenyl and 2',6,6'-trichlorobiphenyl would be consider non-coplanar. In contrast, each ring of the 2',3,5,5'-tetrachlorobiphenyl is free to

rotate and assume a planar spatial shape. The non-coplanar PCBs appear able to stimulate opening of the calcium channels in nerve cells.

C. Neurotoxicity testing of organophosphorus insecticides and other chemicals that are suspected of causing neurotoxicity (based on observations of characteristic signs of nervous system effects during acute or subchronic testing) includes not only an examination of effects on acetylcholinesterase enzyme activity, but also functional observation batteries in which neonatal and juvenile rat behavior is examined.
   1. Thus behavioral toxicology is applied to understanding and characterizing the adverse effects of any chemical suspected of having an effect through interaction (direct or indirect) with the nervous system.

**II. Behavioral toxicology** rests on the premise that it is crucial to evaluate how the whole organism functions when exposed to a chemical. In contrast, in-vitro tests provide mechanistic data toward understanding what processes and structures govern an organism's behavior (Weiss 1999; Chapter 34, p. 650 in General and Applied Toxicology).

A. Behavioral toxicity testing presumes that the myriad functions collectively defining behavior can be isolated for study. Thus, different behaviors can be observed selectively by appropriate investigative techniques.

B. Major types of testing include:
   1. Functional Observation Batteries (FOBs)
      a. The typical behavioral observations associated with rodent FOBs include:
         1. Home-cage and handling
         2. Posture
         3. Ease of handling
         4. Piloerection
         5. Vocalizations
         6. Open field
         7. Time to first step
         8. Urination, defecation
         9. Gait
         10. Bizarre behavior
         11. Rearing behavior
         12. Reflex and physiologies
         13. Approach response
         14. Touch response
         15. Finger snap response
         16. Righting reflex
         17. Grip strength
         18. Catalepsy
         19. Forelimb grip strength
      b. Caveats:
         1. FOBs are not designed to elucidate underlying toxicological mechanisms.

2. FOBs are designed to determine the potential of a toxicant for producing neurotoxicity and to suggest more specific follow-up tests (for ex., cognitive function tests).
3. FOBs are not intended to set exposure standards.
4. FOBs are prone to reliability problems unless observers are well trained in objective and consistent scoring of behavioral measures.
    a. Thus, scoring must be made blind.

c. An example of where FOBs were able to distinguish the subtleties of differences in toxicological response within a chemical family was the testing of Type I and II pyrethroids.
    1. Permethrin (Type I) elicited rodent responses of hyperthermia and aggressive sparring. Cypermethrin (Type II) elicited responses of hypothermia, pawing, and burrowing. (McDaniel, KI. L. and V. C. Moser. 1993. Utility of a neurobehavioral screening battery for differentiating the effects of two pyrethroids, permethrin and cypermethrin. Neurotoxicology & Teratology 15:71-83.)
        a. Abstract: "The ability of a neurobehavioral screening battery to differentiate the effects of two pyrethroids, permethrin and cypermethrin, was assessed in this experiment. Although the structures of these pesticides differ only in the α-cyano group, the behavioral syndromes associated with the Type I and II pyrethroids are quite different. The tests included a functional observational battery, which is a series of subjective and quantitative measures of neurological function and behavior, and an automated measure of motor activity. Our results verified previous reports in the literature describing these different syndromes, i.e., aggressive sparring behavior, fine to whole-body tremor, hyperthermia, and decreased motor activity for the Type I pyrethroid permethrin, and pawing, burrowing, salivation, whole body tremor to choreoathetosis, hypothermia, and lowered motor activity for the Type II pyrethroid cypermethrin. In addition, we report that permethrin produced decreased grip strengths, increased resistance to capture, increased reactivity to a click stimulus, and induced head and forelimb shaking and agitated behaviors, whereas cypermethrin produced pronounced neuromuscular weakness and equilibrium changes, retropulsion, lateral head movements, alterations in responses to various stimuli, and increased urination. Although there were similarities in some effects (e.g., decreased motor activity), the pesticides differed sufficiently in their overall behavioral profiles, and severity and time course of effects, to discriminate these two compounds. Thus, this type of screening approach is sensitive enough to differentiate these pyrethroids for hazard identification purposes."

2. Motor Activity
    a. Takes advantage of rodents tendency to explore their environments;
    b. Observing activity levels of animals as they move within a space (for example, mazes, running wheels, open or unencumbered fields);
    c. Must be careful in interpretation: what does it mean if activity is depressed or stimulated relative to the control. Thus, motor activity tests are not mechanistic.
    d. Must be cognizant of cyclical variations in activity and changes in activity with age.
    e. Activity measures do not necessarily apply only to neurotoxicity; they can also indicate other systems effects.

f. Motor activity tests can be automated with sensors so that the activity levels are recorded automatically.

3. Naturalistic Behaviors
   a. Includes other behaviors not typically measured by FOBs or specific motor activity tests.
      1. Aggressive behavior
         a. Attack, defensive, submissive responses
      2. Mating behavior
   b. Both mating and aggressive behavior in rodents, especially if it is gender specific, can be used to assess organizational effects on the endocrine system (i.e., these tests are not necessarily only for measures of neurotoxicity). The following is an abstract from a research paper in which endocrine system affects were manifested in behavioral responses (Hotchkiss, A. K., J. S. Ostby, J. G. Vandenbergh, and L. E. Gray, Jr.. 2002. Androgens and Environmental Antiandrogens Affect Reproductive Development and Play Behavior in the Sprague-Dawley Rat. Environmental Health Perspectives 110(Suppl. 3):435-439.)
      1. Abstract: "In mammals, exposure to androgens early in development is essential for masculinization of the male reproductive phenotype. Male fetuses exposed to antiandrogens during perinatal life are permanently demasculinized in their morphology and physiology, whereas exposure to exogenous androgens permanently masculinized females. In some litter-bearing species, proximity in utero of females to males can partially masculinized female siblings and alter their responsiveness to endocrine-disrupting compounds. However, in our strain of rat (CD-SD Charles River), intrauterine position does not significantly influence testosterone concentrations and anogenital distance of fetuses. In comparison, administration of testosterone propionate to pregnant females, at doses that doubled fetal female testosterone levels, did masculinize the reproductive system. Discovery of androgen-active chemicals in the environment has placed increased emphasis on describing the reproductive and behavioral effects of both natural and environmental androgens and antiandrogens. Recently, the effects of an antiandrogen, vinclozolin, on the brain and behavior were cited as a special concern by the U.S. Environmental Protection Agency in its risk assessment of this pesticide. In rats, one such behavior that is perinatally organized by androgens is social play. Males play more than females, and administration of exogenous androgens during the neonatal period alters the juvenile expression of this sexually dimorphic behavior. Vinclozolin is an androgen receptor antagonist that inhibits androgen-dependent tissue growth in vivo. We were interested in whether developmental exposure to vinclozolin could also alter androgen-dependent behaviors such as play. Neonatal male rats were injected on postnatal days (PNDs) 2 and 3 with corn oil, the pharmacologic antiandrogen flutamide (50 mg/kg), or vinclozolin (200 mg/kg). On PNDs 36-37 animals were observed for social play. Behaviors associated with general social activity such as sniffing and dorsal contact were unaffected by treatment. However, play behavior in males treated with flutamide or vinclozolin was significantly reduced, resembling levels of play characteristic of females rather than untreated males. Therefore, this study demonstrates that perinatal exposure to vinclozolin, an environmental antiandrogen, can alter androgen-dependent play behavior in the male rat."

4. Ethological Analysis
   a. Ethology is conventionally the study of animal behavior. In the context of toxicology testing, ethological analysis is applied to observing animals in their natural environment. For experimental purposes the setting might be "quasi-natural" for more control over environmental variables.

1. Thus, behavior is studied in the context of the animal's total environment.

C. Specific Functional Tests (described in Weiss 1999):
   1. Motor function and coordination
      a. Foot splay
         1. Hind feet of a rat or mouse are inked.
         2. Animal is dropped from a specified height (for example, 30 cm);
         3. The distance between the marks made by the feet is a measure of hindlimb splay resulting from neuropathy.
      b. Rotarod
         1. The rat or mouse is placed on a grooved rotating rod;
         2. The rotation speed is increased;
         3. Adversely affected animals will not be able to hold on as well as control animals.
            a. The measurement is how long the animal can stay on the rod.
   2. Sensory function (for ex., acoustic startle response)
      a. Describes the neuromuscular reaction to a brief loud sound.
   3. Cognitive Function
      a. Avoidance behavior
      b. Maze learning (for spatial learning)
   4. Operant Behavior Tests
      a. Measure learned or acquired behavior (includes avoidance and maze learning);
      b. Scheduled learning behavior, wherein mice are trained to respond to one schedule of activity to be rewarded, but then the requirements (schedule) is changed to require a different level of activity (such as pressing a lever a pre-defined number of times before food is awarded)

**III. Behavioral Toxicity in Fish**

A. Categories of fish behavior include: (partly based on Heath 1995)
   1. Schooling
   2. Feeding (including predation)
   3. Migration
   4. Aggression
   5. Fear
   6. Learning
   7. Rheotropism
   8. Attraction or Avoidance
   9. Breathing frequency (may really be indicative of a metabolic effect)
   10. Swimming activity (spontaneous locomotory behavior)
   11. Reproductive behaviors

B. Measuring Behavior
   1. Fish placed in tanks that may be divided into chambers
      a. Fish entry into different chambers is recorded
   2. Tank may be outfitted with electrodes or other types of detectors/transmitters that detect change in motion of water to record swimming activity

3. Visual observation:
    a. Can divide a chamber in half, separated by a barrier with holes in it that allow fish to pass through
        1. Confine fish to one side, expose them to a stressor or contaminant, and then determine the number of fish moving through the barrier per unit of time.
    b. Use of video camera and computers placed above a tank for continuous real time monitoring of activity

C. Case Study: Swimming and ammonia toxicity in salmonids: the effect of sub lethal ammonia exposure on the swimming performance of coho salmon and the acute toxicity of ammonia in swimming and resting rainbow trout. (Re: Wicks, B. J., R. Joensen, Q. Tang, and D. G. Randall. 2002. Aquatic Toxicology 59:55-69.)
    1. Note that ammonia represents a naturally occurring nutrient that at high concentrations is lethal to aquatic organisms, especially fish.
        a. Ammonia concentrations increase beyond acceptable natural levels owing to runoff of untreated sewage (such as from a cattle waste lagoon), feedlot runoff, or industrial discharges.
    2. Abstract: This study tested the hypothesis that swimming exacerbates ammonia toxicity in fish. Both sub-lethal and acute toxicity testing was conducted in a swim tunnel on swimming and resting coho salmon and rainbow trout, respectively. The sub lethal tests on coho salmon also considered the compartmentalization of ammonia within the fish. Coho salmon showed a significant linear decrease in *U*crit [critical swimming velocity] both with increasing water ammonia (0, 0.02, 0.04 and 0.08 mg per l NH3) and increasing plasma ammonia. Data collected included plasma pH and ammonia, muscle pH and ammonia and muscle membrane potential. Based on results found in these experiments it was concluded that the reduction in swimming performance was due to both metabolic challenges as well as depolarization of white muscle. Acute toxicity testing on swimming and resting rainbow trout revealed that swimming at (60% *U*crit or approximately 2.2 body lengths/s) decreased the LC50 level from 207 21.99 mg N per l in resting fish to 32.38 10.81. The LC50 for resting fish was significantly higher than that for swimming fish. The acute value set forth by the US EPA at the same pH is 36.1 mg N per l and may not protect swimming fish. In addition the effect of water hardness on ammonia toxicity was considered. It was found that increased water calcium ameliorates ammonia toxicity in fish living in high pH water.

D. Case Study: Predatory behavior in fish exposed to ppb levels of the OP insecticide diazinon. (re: Scholz, N. L., N. K. Truelove, B. L. French, B. A. Berejikian, Quinn T. P., E. Casillas, and T. K. Collier. 2000. Diazinon disrupts antipredator and homing behaviors in chinook salmon (Oncorhynchus tshawytscha). Can. J. Fish Aquat. Sci. 57:1911-1918.)
    1. Salmon react to an alarm pheromone given off by other members of its population when a predator is nearby.
        a. When the pheromone is detected through a sensory gland in the nose, known as a rosette bulb, the salmon stop swimming and cease striking behavior used in food gathering.
        b. Chinook salmon were exposed to increasing levels of diazinon.
            1. Scholz et al. (2000) observed that both swimming behavior and striking behavior continued at greater rates than in unexposed control fish in the presence of the insecticide (Figures 2 and 3).

2. Additionally, in a preliminary experiment, Scholz et al. observed that homing behavior of salmon released from a pen might have been adversely affected by diazinon exposure (Figure 4).

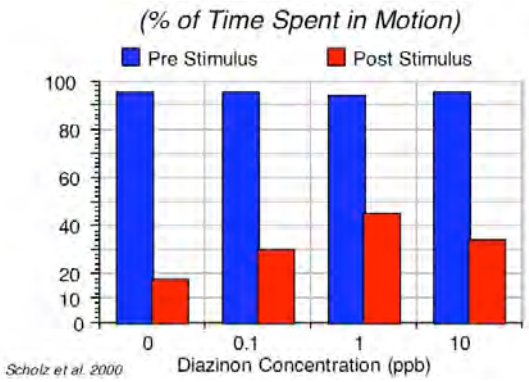



Figure 2. Effect of diazinon on swimming behavior in the absence and presence of alarm pheromone stimulation (Scholz et al. 2000).

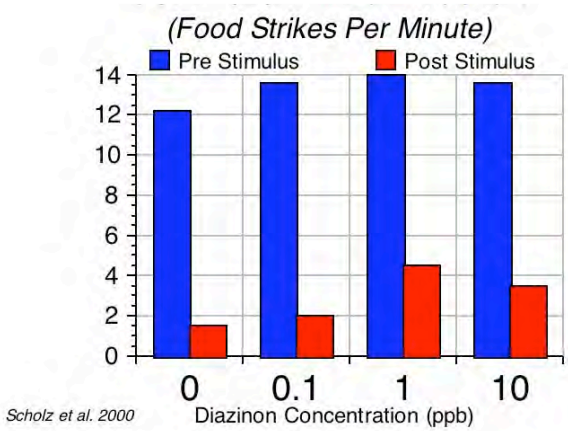



Figure 3. Effect of diazinon on food strikes by salmon in the absence and presence of alarm pheromone (Scholz et al. 2000).

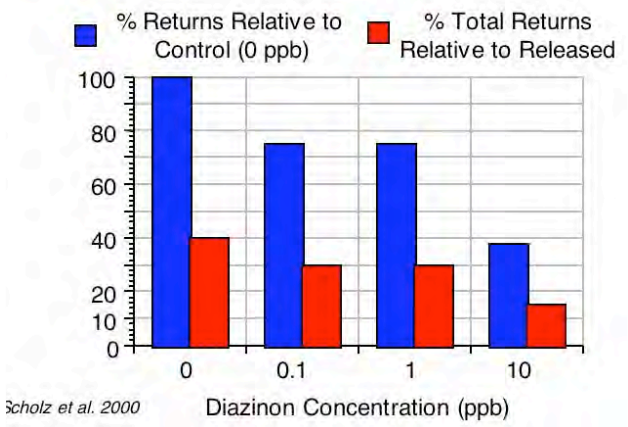


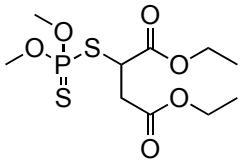

Figure 4. Effect of diazinon on homing behavior of salmon exposed to diazinon (Scholz et al. 2000)

3. Recall from the discussion about FOBs that behavioral toxicity studies are not ideally suited to making regulatory decisions regarding standards for exposure. The results from Scholz show a potential hazard from exposure of salmon to diazinon, but note there is not a clear dose-response effect. Part of the problem is just the low numbers of fish used to do these time and resource intensive studies, and thus the robustness of the experimental outcome is uncertain. Another reason for the lack of a clear dose-response is that the magnitude of the effect is low to begin with. However, as we will discuss later risk characterization is highly uncertain without a clear dose-response relationship.

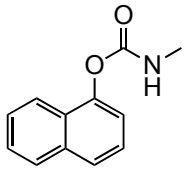
## IV. Amphibian Behavior

A. Recent research suggests that certain pesticides can be more toxic to tadpoles when in the presence of a predator, probably owing to the imposition of additional stress. The pesticides thus far involved include glyphosate (a herbicide), malathion (OP insecticide), carbaryl (methyl carbamate insecticide), and fenpropimorph (a fungicide).

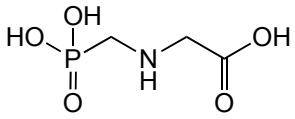
1. Relyea, R. A. and N. Mills. 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles. Proc. Natl. Acad. Sci. **98**(5): 2491-2496.
2. Relyea, R. A. 2005. The lethal impacts of Roundup and predatory stress on six species of North American tadpoles. Arch. Environ. Contam. Toxicol. **48**: 351-357.
3. Relyea, R. A. 2004. Synergistic impacts of malathion and predatory stress on six species of North American tadpoles. Environmental Toxicology and Chemistry **23**(4): 1080-1084.
4. Teplitsky, C., H. Piha, A. Laurila and J. Merila. 2005. Common pesticide increases cost of antipredator defenses in Rana temporaria tadpoles. Environ. Sci. Technol. **39**: 6079-6085.



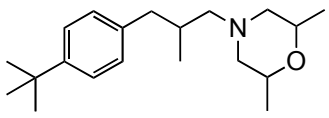

malathion




carbaryl




glyphosate




fenpropimorph

B. In the studies with carbaryl (Relyea and Mills 2001) and fenpropimorph (Teplitsky et al. 2005), activity of the tadpoles in the presence of both the pesticide and the predator (a caged larval salamander and dragonfly nymph, respectively) were observed. The data suggested a reduction in activity the tadpoles in the combined presence of the predator and carbaryl. In the study with fenpropimorph, predators reduced activity of the tadpoles, but the reduction was

not enhanced by pesticide. In the other studies (malathion and glyphosate), the activity of the tadpoles was not observed, but mortality was greater in the presence of the predator and insecticide than in the presence of the insecticide alone.

## V. Avian Behavior

A. Birds are another well-studied wildlife group for effects of contaminants on behavior. In particular the effect of OP insecticides have been most studied, owing to incidences of bird kills reported to the EPA.
   1. As with fish, bird locomotory behavior and food searching capabilities can be affected by sublethal exposure to OP insecticides.
   2. Other behavior that may be affected includes the ability to avoid predators.
   3. The ability of birds to withstand cold temperatures can be affected by sublethal OP insecticide exposure.
   4. On the other hand, birds are also known to avoid insecticide treated granules, which had been an important formulation for delivery of insecticides to control corn rootworms in the Corn Belt (insecticides were applied directly to soil). However, the avoidance is not 100% perfect.

B. Case Study from the Literature:
   1. Hawkes, A. W., L. W. Brewer, J. F. Hobson, M. J. Hooper, and R. J. Kendall. 1996. Survival and cover-seeking response of northern bobwhites and mourning doves doses with aldicarb. Environmental Toxicology and Chemistry 15(9):1538-1543.
      a. Abstract: Survival and cover-seeking responses of wild northern bobwhites (Colinus virginianus) and mourning doves (*Zenaida macroura*) dosed with aldicarb were investigated. Acute toxicity range-finding tests were conducted to establish optimum dose levels for subsequent dose-release activities. Estimated median lethal dose values derived for doves and bobwhites were 0.82 and 1.48 mg/kg body weight, respectively. Wild mourning doves and northern bobwhites were orally administered aldicarb or a vehicle blank and monitored for 7 d post-treatment to determine survival and cover-seeking response. Doves receiving the highest aldicarb concentration (1.07 mg/kg bodyweight) had a significantly lower survival rate than did controls. However, when mortalities attributable to acute toxicity were removed from the analysis, there were no differences in daily survival rates among treatment groups. All bobwhite treatment groups had lower survival rates than controls. When acute mortalities were removed from the survival analysis, the 1.36-mg/kg bodyweight treatment group still had a significantly lower survival rate than controls. Post-dose observations of behavior indicate that mourning doves and northern bobwhites receiving a lethal dose of aldicarb are limited in their cover-seeking ability due to the rapid onset of physical impairment associated with this compound.