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In a Nutshell…

ECOTOXICITY OF NANOMATERIALS TO FISH: CHALLENGES FOR ECOTOXICITY TESTING

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Nanotechnology and the use of nanoscale materials is a relatively new area of science and technology, with estimates of the global market value being around $10 billion in 2006. Nanoparticles and nanomaterials are structures that have 1 or more dimensions within the range of 1–100 nm (Roco 2003). However, this definition perhaps should be applied more broadly in risk assessment to consider aggregates of nanomaterials that may be a few hundred nanometers wide, as well as individual particles. Manufactured nanomaterials have numerous industrial applications, including electronics, optics, textiles, medical devices, biosensors, and in environmental remediation (e.g., Freitas 2005; Aitken et al. 2006). This diversity of materials is perhaps the 1st hurdle to overcome from the viewpoint of identifying materials for regulatory toxicity testing. Attempts are being made to rationalize nanomaterials on the basis of chemical type. For example, broadly classifying materials as 1) carbon-based structures such as carbon nanotubes and carbon fullerenes; 2) metal-containing nanoparticles such as metal oxides or metal particles; or 3) quantum dots. The next tier in chemical identification might be to consider particle size, shape, surface charge, surface area, aggregation characteristics, solubility, and reactivity with other chemicals. However, whatever chemical classification scheme finally is agreed on, this will need to identify clearly the material being tested.

Mammalian studies have raised concerns about respiratory toxicity, inflammation, and the human health effects of nanomaterials (Handy and Shaw 2007). However there are limited ecotoxicity data and, at this early stage in the data collection process, it is worth considering whether or not current ecotoxicity tests are "fit for purpose" and what endpoints we should select to measure the biological effects of nanomaterials. Handy and Shaw (2007) point out that toxicity tests are based on several fundamental assumptions in the dose–response relationship. One of these assumptions is that the concentration of the toxicant at the target (e.g., receptors on cell membranes of the test organism) is related to dose. Nanoparticles do not form simple solutions, may aggregate at high ionic strength, and may adsorb onto surfaces (Lead and Wilkinson 2006). This suggests that some nanomaterials will be trapped in the mucous layer on epithelial surfaces of fish and other organisms, rather than being absorbed in a predictable dose-dependent manner, as we recently demonstrated with carbon nanotube precipitates in the gill mucus of trout (Smith et al. 2007). This chemistry suggests that caution is needed when interpreting data from aqueous toxicity tests using nanoparticles and that adsorption onto other surfaces such as sediment interfaces and food items could lead to exposure via the aquatic food chain if nanomaterials were released into aquatic systems. It also may be necessary to modify current aqueous test methods to account for this chemistry, add an extra uncertainty factor to risk calculations, or include additional tests such as dietary exposure assessments for fish.

Current test methods require some demonstration that target concentrations of the test material were met, and agreement is needed on what measurements should be taken to confirm exposure to nanoparticles. We suggest the following: 1) "total concentration" of the material (e.g., mg/L of nanoparticles); 2) the manufacturer's information on size, shape, surface area, and purity of the product; 3) optical measurements to confirm dispersion of the test material in the aquarium water; and 4) example measurements of particle size in the test solution made on the electron microscope, or similar approaches.

A number of practical problems still must be overcome, such as a lack of certified reference materials and deciding what solvent (if any) should be used. Simple, but sensitive (<1 mg/L) methods for determining nanoparticle concentration in natural water also are needed. Concerns also exist that trace contaminants in the manufacturer's recipe might be toxic to fish. For example, 1% metal contamination in a 1-mg/L carbon nanotube exposure might be a significant µg/L metal exposure for a fish, and the metals should be monitored in the water and tissues during the exposure.

Methods of dispersion and the use of solvents and/or sonnication techniques is a particular dilemma when using fish. Sonication or prolonged stirring can improve dispersion of nanoparticles. However, for many carbon-based nanoparticles, some solvent is needed to maintain dispersion, particularly over the durations that might apply to a fish study. In the case of carbon nanotubes, some of the best dispersants from the viewpoint of chemistry are substances such as N,N′-dimethylformamide, furan derivatives, and chloroform (Ham et al. 2005). Unfortunately, many of these substances are extremely toxic to fish and would be of limited practical use as a solvent in a fish study. However, if the solvent is excluded, then carbon-based nanoparticles tend to aggregate and produce different ecotoxicological effects (Oberdörster et al. 2006). We partly resolved this dilemma for carbon nanotubes by using a solvent that was fairly good at dispersing carbon nanotubes (with some sonnication), but also with a relatively low toxicity to fish (sodium dodecyl sulphate [SDS]; see Smith et al. 2007). Some caution is needed with dispersing agents. Excess solvent can deform nanotubes (Ham et al. 2005) and therefore might change their toxicological properties. Maintaining "dispersion" by chelating nanoparticles with water-soluble products is also problematic, because this could reduce bioavailability (e.g., is the chelated form available to the gill surface?), or change the effective particle size. At least 1 author has attempted to use proteins to disperse carbon nanotubes by chelation (Karajanagi et al. 2006). From the viewpoint of chemistry, this achieves good dispersion, but from a biological perspective the issue of bioavailability and bioreactivity remains. Concerns also exist that proteins and other large macromolecules could have adverse immunogenic properties (Pantarotto et al. 2003), which might result in antibody production and/or inflammation reactions.

The issue of target organs, biological effects, and the selection of suitable test endpoints should not be overlooked. At this early stage, we advocate a body systems approach to logically identify the main physiological and biochemical effects on fish. We have at least demonstrated toxic effects of carbon nanotubes to the respiratory system, central nervous system, and liver in trout. The toxic processes include vascular disturbances, oxidative stress, some very specific disturbances to trace elements, and possibly cell cycle defects (Smith et al. 2007). Some of these effects are apparent without clear evidence of the test material within the body compartment (e.g., toxicity mediated by secondary systemic effects). The point is that we should keep an open mind and be prepared to select endpoints that enable demonstration of biological effect (or not), even if this means modifying test protocols. We also should be prepared to accept observed biological effects, even when techniques are not currently available (and need to be developed) to quantify nanomaterials in the tissues and organs of the exposed animal. For example, we can measure titanium in the tissues of trout following TiO_2 nanoparticle exposure using mass spectrometry techniques, but did the fish absorb titanium metal or titanium nanoparticles? Can the particles be modified inside the organism to release the titanium?

Nanotechnology is probably here to stay, and we have the opportunity to investigate rationally the chemical and biological properties of these new materials so that we can make sensible decisions about toxicity test design, and subsequent interpretation of data for risk assessments. For fish toxicology, and ecotoxicology generally, the practical challenges of working with these new materials are becoming clear, and we must continue with a joined approach to develop the necessary and appropriate analytical chemistry and biological effects portfolio. Collaboration between particle chemists and toxicologists, and an understanding of biological–chemical interphases (Handy and Eddy 2004), is vital to success.

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SELENIUM: DETERRENCE, TOXICITY, AND ADAPTATION

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Introduction

The element selenium (Se), named after the moon (Greek *selene*), has long been known for its toxicity. In 1295, Marco Polo was the 1st to describe the typical symptoms of selenosis in his pack animals. Centuries later, selenosis may have played a role in the demise of General Custer in the battle of Little Big Horn. Still today, chronic or acute Se poisoning is responsible for the loss of thousands of head of livestock every year in the western United States, where Se is prevalent in soils. Due to its chemical similarity to sulfur, Se in excess will nonspecifically replace sulfur in proteins, leading to the observed toxicity. At low levels, however, Se is also an essential trace element for many organisms, including humans, as a component of specific selenoproteins that often play a role in cancer prevention.

Selenosis in animals mostly is due to ingestion of Se hyperaccumulator plants, commonly termed "locoweeds" by ranchers. These plants can accumulate Se to around 1% of their dry weight, 100-fold higher levels than those found in surrounding vegetation, and 1,000-fold higher than soil Se. Selenium hyperaccumulator species mainly occur in the genera *Astragalus* (Fabaceae) and *Stanleya* (Brassicaceae), and predominantly are found on Se-rich soils as seen in the western United States. Several hypotheses exist for the functional significance of the intriguing phenomenon of hyperaccumulation. We currently study the elemental defense hypothesis, which proposes that the accumulated Se protects the plants from herbivory or microbial infection. We also are interested in how accumulated Se in plants affects local ecological interactions (Pilon-Smits and Freeman 2006; Fig. 1A). In this paper, we give an overview of our findings so far, followed by an overall discussion of their implications with respect to the evolution of hyperaccumulator plants and the management of Se-rich plants in natural and agricultural settings.

Research

In our initial laboratory studies, Se accumulation in the nonhyperaccumulator *Brassica juncea* (Indian mustard) was shown to protect plants from herbivory by *Pieris rapae* larvae (white cabbage butterfly) due to both deterrence and toxicity, as well as infection by 2 fungal pathogens (Hanson et al. 2003). Selenium also protected plants from green peach aphid (*Myzus persicae*) colonization at tissue levels 2 orders of magnitude lower than those found in hyperaccumulators in the field (Hanson et al. 2004). In contrast, Se accumulation in Indian mustard promoted feeding by the snail *Mesodon ferrissi* (Hanson et al. 2003). Follow-up field surveys indicated that Se hyperaccumulators harbored fewer invertebrates than nonaccumulators and that Se-hyperaccumulating plants thrive and suffer little herbivory on prairie dog colonies. Subsequent manipulative field studies confirmed that elevated Se levels protects plants from herbivory by prairie dogs and grasshoppers (unpublished results).

Additional fieldwork was conducted to investigate seasonal variation in plant Se accumulation and Se compartmentalization. Selenium hyperaccumulators appear to transfer Se from roots to young leaves and reproductive organs during the growing season and back to the roots during winter dormancy, a pattern not found in related nonhyperaccumulators (Galeas et al. 2007). The spatial distribution of Se in hyperaccumulators also appears to be specialized: Se is accumulated primarily in leaf hairs and epidermal cells of hyperaccumulators, though related nonhyperaccumulators show no localized sequestration (Freeman, Zhang, et al. 2006). Moreover, Se in hyperaccumulators primarily is accumulated as methylselenocysteine (MeSeCys), which is not toxic to the plant, although the predominant form found in nonhyperaccumulators is selenate (Freeman, Zhang, et al. 2006).

Like any plant defense compound, Se hyperaccumulation can lead to the evolution of tolerant herbivores. Indeed, a population of the economically important diamondback moth (*Plutella xylostella*) was found thriving in the field on *Stanleya pinnata* plants that contained extremely toxic levels of Se (2,000 µg/g dry wt; Freeman, Quinn, et al. 2006). Interestingly, the parasitic wasp *Diadegma insulare* was found living on this apparently Se-tolerant diamondback moth. The diamondback moth larvae and adults, as well as the parasitic wasp, contained 200 µg Se g^{-1} dw in their tissues, which is lethal to most organisms. Laboratory studies showed that this population of diamondback moth has no feeding preference for plants with or without Se, and prospers on plants with elevated Se. In contrast, Se deterred feeding and oviposition and was lethal at low levels to another, common population of diamondback moth. The Se-tolerance mechanism was revealed using X-ray absorption spectroscopy. Although the Se-tolerant population of diamondback moth and its parasitic wasp accumulated MeSeCys, the same form found in the hyperaccumulating plants, the Se-sensitive moth accumulated selenocysteine, which is toxic due to its incorporation into proteins. Thus, it appears the tolerant population has lost the ability to demethylate MeSeCys, leading to less Se incorporation into proteins (Freeman, Quinn, et al. 2006). The interactions in this tri-trophic system are summarized in Figure 1B.

Implications for the Evolution of Se Hyperaccumulation and for the Management of Se-Accumulating Crops

Together, these studies offer insight into how the intriguing physiological phenomenon of Se hyperaccumulation may have evolved. The observations that Se protects plants from a variety of vertebrate and invertebrate herbivores, as well as fungal pathogens, support the elemental defense hypothesis. The findings that Se is sequestered in specialized, peripheral structures and that Se is moved seasonally to vulnerable and valuable plant organs support a defensive function as well. The sequestration of Se in the periphery of hyperaccumula-

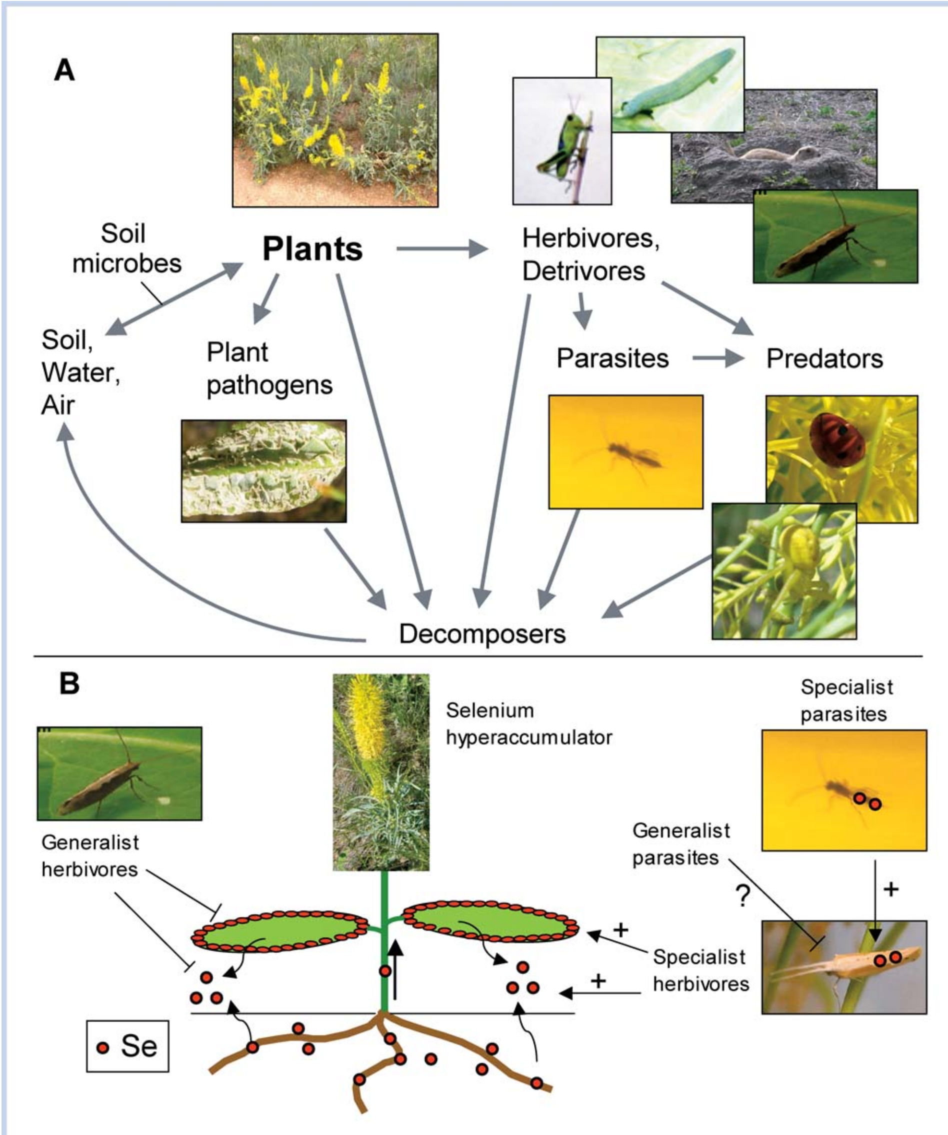

Figure 1. (**A**) Potential flow of selenium (Se) in hyperaccumulator habitat. (**B**) The role of Se in ecological interactions between hyperaccumulator *Stanleya pinnata*, its herbivores, and their predators. Se is accumulated in the leaf periphery and also can be volatilized (Freeman, Zhang, et al. 2006). This plant's Se deters generalist nontolerant herbivores such as the common diamondback moth (**left**). In hyperaccumulator habitat, a Se-tolerant population of the diamondback moth occurs (**right**), which is not deterred by or sensitive to Se. In turn, a Se-tolerant wasp parasitizes this moth (Freeman, Quinn, et al. 2006).

tors also may serve to enhance Se hyperaccumulation and Se tolerance, and the metabolic conversion to MeSeCys further contributes to Se tolerance. If the localized sequestration of Se provides both Se tolerance and protection from herbivores or pathogens, a clear selective advantage would drive the further evolution of this trait.

The characterization of the Se-tolerant, Se-accumulating herbivore that has disabled this plant's elemental defense is evidence of a 2nd level of evolution, and its Se-tolerant parasite presents a 3rd level. The picture that emerges from this study is that, while protecting the plant from generalist herbivores, plant-accumulated Se leads to the evolution of specialist herbivores. This may provide a portal for Se into the local ecosystem, where it may affect ecological interactions at higher trophic levels.

Because Se toxicity and deficiency are problems worldwide, these findings have broad management implications and also may lead to a number of practical uses. For instance, Se-(hyper)accumulating plants may be used for environmental cleanup (phytoremediation). Also, because Se is a nutrient and anticarcinogen, induced or natural Se accumulation in plants may be used to produce Se-fortified foods. Such use may be envisioned for Indian mustard, but perhaps also for the related hyperaccumulator *Stanleya pinnata*. This native of seleniferous soils in the arid western United States accumulates not only the anticarcinogen MeSeCys but also has high levels of other antioxidants like ascorbate (vitamin C). A beneficial side effect of plant Se accumulation is that the Se protects the plants from herbivory and pathogens, alleviating the need for pesticides. The finding that Se protects plants against the common diamondback moth is of particular significance because the diamondback moth is an important pest for many agricultural crops. The discovery of the Se-tolerant population of this herbivore may provide a tool for understanding the evolutionary mechanisms underlying the moth's notorious ability to quickly disarm pesticides. The findings from these studies also may help prevent Se toxicity in livestock. The Se-tolerant diamondback moth may be applicable as a biocontrol for "locoweeds" in areas with high Se. Furthermore, understanding the distribution of Se in hyperaccumulator plants, and their seasonal Se fluctuations, give insight into the times of year these plants are most poisonous. Similarly, knowledge of the distribution of Se in Se-fortified crop plants such as Indian mustard, and of their seasonal Se fluctuations, will enable their harvesting when Se levels are highest. Future studies will be directed at further elucidating the effects of plant Se accumulation on their various ecological partners.

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CADMIUM TRANSFER TO HUMANS FROM SOILS VIA SOYBEANS

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Introduction

Awareness and concern are increasing over trace metal contamination of soils and the adverse effects this may be having on the food chain. Cadmium (Cd) occurs at very low levels in the Earth's crust, but is nonetheless a ubiquitous trace metal, with no known nutritional requirements in humans. Once introduced to the body, Cd can interact with the metabolism of 3 essential metals: Calcium, zinc, and iron (Goyer 1997), particularly calcium metabolism in bone and kidney. This can result in renal dysfunction, osteomalacia, and bone fractures (Goyer 1997; Olsson et al. 2005). Unlike many other trace metals, Cd is accumulated in crops, at concentrations that are not phytotoxic but can be a potential concern to human health (McLaughlin et al. 1999), making it the most significant crop food safety concern of all the trace metals. Excluding tobacco smoking and occupational exposure, the main source of Cd exposure to the general population is through crop consumption (Vahter et al. 2002; Olsson et al. 2005). Accumulation in crops typically is associated with anthropogenic additions via atmospheric deposition and the application of contaminated fertilizers, manures, and biosolids to soil (McLaughlin et al. 1999; Olsson et al. 2005).

Who Is at Risk?

In Canada, where nutritional deficiencies are less common, the young and women are typically at greatest risk for Cd exposure. Women who are pregnant or of childbearing age, typically have greater dietary requirements for iron, resulting in the upregulation of the duodenal metal transporter (DMT1) that is responsible for the uptake of iron, and which has an affinity for Cd (Vahter et al. 2002). As a result, individuals who have low iron stores, prevalent worldwide in women who are of childbearing age, absorb a greater proportion of Cd from their diet (Vahter et al. 2002; Olsson et al. 2005).

Cadmium exposure is also a concern for children. The young consume a greater proportion of food per body weight compared to adults, increasing their exposure to contaminants in food. This is particularly so with Cd, as demonstrated by Health Canada's Total Diet Study from 1993 to 1999 (http://www.hc-sc.gc.ca/fn-an/surveill/total-diet/index_e.html), where children under the age of 4 had an average dietary intake of Cd that ranged from 0.590–0.908 µg/kg body weight(bw)/d compared to 0.339 µg/kg bw/d for all ages. To put this in perspective, the tolerable daily Cd intake estimated from the provisional tolerable weekly intake of 7 µg/kg bw recommended by the Joint Food Agriculture Organization/World Health Organization Expert Committee on Food Additives ([JECFA]; http://jecfa.ilsi.org/evaluation.cfm?chemicalCADMIUM&keyword=CADMIUM) is 1 µg/kg bw/d. With our knowledge of natural distributions around average intakes, there are already children exceeding the estimated tolerable daily Cd intake.

Research Focus

Soybeans and soy-based products have been implicated as 1 mode of Cd intake. Health Canada's Total Diet Study also revealed that soy-based infant formulas had greater than 2× the concentration of Cd as in non–soy-based infant formula. This is a concern because soy-based infant formulas are a popular choice for mothers in North America. Sheehan et al. (2001) reported that 20% of mothers do not initiate breastfeeding, and the proportion of babies fed synthetic

Table 1. Preliminary results of soil properties[a]

Site	Soil type	Cl-	pH	Total Cd in soil mean (ppm)	Available Cd in soil mean (ppm)
Ridgetown	Fine sandy loam	24.96 ± 20.9	5.60 ± 0.050	0.39 ± 0.018	0.017 ± 0.0014
Woodslie	Clay loam	22.96 ± 2.25	6.15 ± 0.044	0.43 ± 0.017	0.0076 ± 0.0011
Elora	Silt loam	20.00 ± 2.93	7.81 ± 0.035	0.28 ± 0.012	<LOD

[a]ppm, parts per million; LOD, limit of detection.

infant formula increases dramatically after 1 month of age, resulting in many babies relying on infant formula for the 1st critical year of life.

In addition, soy is an ingredient that is found in many foods, such as alcohol, processed meats, dairy products, baked goods, cooking oils, margarine, mayonnaise, pastas, peanut butter, salad dressings, etc. Thus, it is of critical importance from a food-safety perspective to assess the on-farm factors that could mediate human exposure to Cd through soy-based products.

Mitigation Strategy

Soybean has the potential to accumulate elevated concentrations of Cd, a trait that is heritable (Arao et al. 2003). The accumulation of Cd in crops is not a straightforward outcome of the Cd concentration in the soil because metals exist in different solution and solid-phase forms that can vary greatly in terms of their bioaccessibility. Typically, low-pH and sandy soils reduce Cd sorption to organic colloids, increasing Cd availability to plant roots; anionic ligands, such as Cl, might form Cd-complexes that also are taken up by plants. In addition, Cd uptake from soil to plants may be reduced by competing cations, such as zinc, at the root uptake sites (Olsson et al. 2005).

Weather also has been demonstrated to play an important role: Crop Cd concentrations tend to be higher with greater precipitation (Olsson et al. 2005). The degree to which each soil characteristic affects Cd accumulation is largely dependent on plant genetics; Cd accumulation may vary widely among plant species and cultivars within species (Olsson et al. 2005).

Predicting Cd accumulation in the edible portion of crops is difficult, because both soil characteristics and plant physiology need to be considered. A mathematical model for predicting toxicity of metals to soil-grown plants (termed the Terrestrial Biotic Ligand Model [TBLM]) will be used as the framework to identify agronomic practices that will make the greatest contribution to reducing Cd accumulation in Ontario-grown soybeans.

Soils from single cultivar plots have been collected and analyzed for pH, $CaCO_3$, Cl, Zn, Mg, $BaCl_2$-extractable Cd and Ca (considered to be "available"), organic matter, and clay content at 3 field sites in Southern Ontario where public variety trials for soybean are conducted: Woodslie, Ridgetown, and Elora. High- and low-Cd–accumulating cultivars (identified from previous work) are being studied at Woodslie and Ridgetown; only low-Cd–accumulating cultivars were grown at Elora in 2006. The results to date demonstrate vast differences in soil properties among the 3 sites that are within 450 km (Table 1); seed Cd from these plots is being determined. The experiment will be repeated in 2007, at which time the TBLM framework will be applied to the data. With this information, recommendations will be provided to soybean producers regarding the agronomic practices that will make the greatest contribution to reducing accumulation of Cd in Ontario-grown soybean to ensure the safe production of Ontario soybeans in relation to Cd contamination.

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IS FOOD GROWN IN URBAN GARDENS SAFE?

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Introduction

Soils in urban areas have received inputs of trace elements from a wide range of human activities. In addition to emissions from industry, transportation, and incineration of garbage, the burning of coal to heat homes and the disposal of ash and other wastes during the last century, have led to the enrichment of urban soils in trace elements compared to soils in rural areas (Ross 1994).

As part of the environmental movement during the past few decades, there has been a growing interest in urban gardening in the back or front yards of private homes and also on land where homes or other buildings have been torn down. Urban gardens provide many benefits to people living in the urban core of large cities. Not only do gardens provide food but also they contribute to a sense of community, participation in an environmentally positive activity and they add beauty to what is sometimes a rather bleak landscape.

This study was initiated to answer 2 fundamental questions: Is the food grown in urban gardens safe? Are urban gardeners exposed to excessive levels of trace element contamination through contact with the soil?

Analytical Methods

During early summer of 2006, 26 samples of soil and leaf lettuce were collected from urban gardens in Montreal, Québec, Canada, and 2 samples from local grocery stores. Lettuce samples were washed with deionized water, dried at 60 °C for 24 h, and ground to a fine powder prior to digestion with HNO_3 in open digestion tubes. Soil samples were analyzed for pH and organic matter content, and trace element content was measured in acid digests (HNO_3/microwave). Soil and leaf tissue digests were analyzed for Cr, Co, Ni, Cu, Zn, As, Cd, Ce, and Pb.

Results

In Quebec, the Ministère du Développement Durable, de l'Environnement et des Parcs has established a 3-tiered system to classify contaminants in soils: The A-level represents the upper limit of normal background concentrations; the B-level represents the upper limit for residential, recreational, or institutional land use; and the C-level represents the upper limit for industrial land use (MDDEP 2007). Trace element concentrations in the garden soils were generally below the A-level; however, 3 gardens had values that exceeded the A-level for Cu of 40 mg/kg, 8 exceeded the A-level for Zn of 110 mg/kg, and 5 exceeded the A-level for Pb of 50 mg/kg (Table 1). In no case were the values as high as the B-level and, hence according to Quebec's soil quality guidelines, none of these soils are considered to be harmful to human health and none of them should cause problems associated with gardening.

Table 1. Soil organic matter content, pH, and trace element contents of soil and lettuce (fresh weight) from 26 urban gardens in Montreal, Quebec, Canada. O.M., Organic Matter; EU, European Union

	O.M.	pH	Cr	Co	Ni	Cu	Zn	As	Cd	Ce	Pb
Soil	%	mg kg^{-1}									
Median	12	7.58	31	6.5	19	26	101	3.2	0.35	32	32
Minimum	6	6.34	16	3.5	11	15	48	0.1	0.22	23	12
Maximum	26	8.00	59	9.3	31	66	326	5.4	0.66	46	142
Lettuce			µg kg^{-1}								
Grocery median			9.00	<[a]	6	185	2,110	14	15	<	9
Garden median			25	3.7	27	441	2,792	19	17	12	44
Minimum			9	1	9	195	990	6	3	2	17
Maximum			60	13	120	898	4,820	43	40	41	133
EU maximum level in fresh lettuce[b]						—	—	—	200	—	300

[a] < indicates below limit of detection.
[b] Commission of the European Communities (2006).

Table 1 also shows the ranges of concentrations of trace elements measured in the lettuce leaves from the urban gardens and from 2 samples purchased in grocery stores. The sample size used in this study, with 26 samples from urban gardens and only 2 samples (of unknown origin) from grocery stores, suggests that interpretations of the results should be done with caution. Although the trace element content of the lettuce leaves was usually higher in the plants from the urban gardens than from the grocery store, the values were well below the norms established by the European Commission for Cd and Pb (the only 2 elements listed; Commission of the European Communities). It is unclear whether the levels of trace elements could present problems for urban gardeners who eat a large amount of urban-garden–grown food or whether other plants in these gardens might be taking up higher concentrations than those measured by lettuce in this study.

Conclusion

Lettuce grown in the urban gardens sampled in Montreal may increase the amount of trace elements being ingested relative to lettuce obtained from grocery stores. Whether or not this poses a health risk depends on how much of the trace elements the people are getting from the rest of their diet as well as from other environmental sources (water, dust, etc.). Garden-grown food is only a part of the total exposure so studies would need to be done to see whether urban gardeners are exceeding recommended maximum daily or weekly uptake levels such as those established by the World Health Organization (Demirezen and Aksoy 2006). The results of this study are not, in themselves, cause for alarm; however, the safety of the food we eat, and especially that grown in trace element–rich environments, such as urban gardens, should not be taken for granted.

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THE SEARCH FOR THE "IDEAL" SOIL TOXICITY TEST REFERENCE SUBSTANCE

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Introduction

Standardized test methods are necessary for the evaluation of adverse consequences of chemicals. The validation of such methods is performed by the Working Group of National Co-ordinators of the Test Guidelines Programme within the Organization of Economic Cooperation and Development (OECD) and by the Technical Committees for Soil and Water Quality within the International Organization for Standardization (ISO). An important step of the validation of any new test method is the selection of a suitable reference substance (positive control; Gourmelon and Ahtiainen 2007). Currently, for each of the existing methods or those currently under development (Römbke and Knacker 2003), different reference substances are used, mainly pesticides (Table 1).

Since these substances have been selected (often more or less by chance), several problems have become evident. For example, benomyl and parathion are no longer available and chloroacetamide has been shown to be carcinogenic and thus not appropriate for routine laboratory usage. Therefore, we

Table 1. Standard soil toxicity tests and reference substances[a]

Test + group	Organism	Guidelines	Reference substance
Mortality (earthworm)	*Eisenia fetida, Eisenia andrei*	OECD 207, ISO 11268-1	Chloroacetamide
Reproduction (earthworm)	*E. fetida, E. andrei*	OECD 220, ISO 11268-2	Benomyl, carbendazim
Reproduction (earthworm)	*Enchytraeus albidus, Enchytraeus* spp.	OECD 222, ISO 16387	Carbendazim
Reproduction (springtail)	*Folsomia candida*	ISO 11267	Phenmedipham, parathion
Reproduction (springtail)	*F. candida, Folsomia fimetaria*	OECD Draft	Dimethoate, copper, boric acid
Reproduction (predatory mite)	*Hypoaspis aculeifer*	OECD Draft	Dimethoate, boric acid
Emergence, growth (plants)	*Brassica napus, Avena sativa*, others	OECD 208, OECD 227	Not specified
Emergence, growth (plants)	18 species	ISO 11269-2	Boric acid, sodium trichloroacetate
Reproduction, growth (plants)	*B. napus, A. sativa*, others	ISO 22030	Zinc sulphate, boric acid, sodium trichloroacetate

[a]OECD, Organization of Economic Cooperation and Development; ISO, International Organization for Standardization.

propose to reassess the use of existing reference substances by applying fixed criteria, trying at the same time to identify 1 reference substance suitable for all ecotoxicological soil tests with invertebrates and plants.

Criteria for Selecting Reference Substances

The following criteria should be used for the selection of a reference substance (Gourmelon and Ahtiainen 2007):

- It is bioavailable during the test and it affects the respective test species and the chosen endpoint in a reproducible way.
- It is not too difficult to obtain (also in the foreseeable future).
- It is feasible to handle in the laboratory (manageable health and environmental risks).
- A practical and affordable analytical method is available (today more relevant for aquatic tests but at least for quality assurance [i.e., test concentration verification] it is also important for soil tests).

When developing new tests, the results of tests with a reference substance also can be used to evaluate the proposed methods in order to identify the most suitable method, thus it would be helpful to use the same reference substance in different tests.

The absolute toxicity of a reference substance is of minor importance. Because any OECD or ISO test will be used for many different compounds, it would be difficult to identify a specific-sensitivity range. However, it would be appropriate to see effects below 1,000 mg/kg dry wt soil because, in practice, tests very rarely are performed at concentrations higher than this (artificial) limit. Finally, the mode-of-action of a reference substance also is not relevant as long as the species and endpoint are affected and the purpose of the method is not to assess this specific mode of action (e.g., endocrine disruption).

The Path Forward

Currently, both OECD and ISO are concerned about the increasing number of different reference substances as well as some problems occurring with existing ones (such as benomyl). Therefore, compilations are being made both for aquatic and soil tests and discussions are being prepared in the respective working groups. For example, within the ISO TC 190 soil group, the available information on the effects of boric acid is summarized and new tests have been encouraged (e.g., Stephenson 2003). At the same time, ring testing for the validation of new test methods, such as the chronic test with the predatory mite *Hypoaspis aculeifer*, are performed not only with the classical reference substance, the insecticide dimethoate, but also with new reference substances such as boric acid. In parallel, some national standardization organizations already have implemented new reference substances (e.g., Environment Canada in their earthworm guidelines; EC 2004).

Based on existing experiences (mainly with pesticides) and the results of recent discussions, we propose for all soil ecotoxicological test guidelines (ISO and OECD) to include 2 reference substances: Besides a test-specific compound (often but not always the existing one [e.g., carbendazim but not chloroacetamide for reasons specified above]) a general toxicant. According to our current knowledge, this could be boric acid. However, we encourage all colleagues interested in the validation of soil test methods to provide their views about potential alternatives. In any case, it is highly recommended to perform sufficient tests with any new reference substance and each individual test species before making a decision.

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IMPROVING REGULATORY RISK ASSESSMENT—USING AQUATIC MACROPHYTES

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Background

Submerged aquatic macrophytes are an important functional and structural element of aquatic ecosystems. Functionally, they are vital to the stability of sediments, flow of nutrients, primary production, and the distribution of other species in the water column. Thus, changes in the macrophyte community and dominance can have major consequences for the aquatic ecosystem. However, despite their ecological relevance, submerged aquatic macrophytes are poorly addressed in the ecological risk-assessment process, a deficiency long recognized, but rarely addressed (Davy et al. 2001).

Currently in North America and Europe, only *Lemna* spp. are tested and used in the 1st tiers of ecological risk assessment (Davy et al. 2001). Under optimum conditions, in the laboratory, *Lemna* species have relatively high growth rates, a short reproduction cycle, and a short recovery time (Hilman 1961). Fast-growing plants seem to be more sensitive than slow-growing plants (Cedergreen et al. 2004). However, this is not always the case. Moreover, high growth rates also imply high recovery potential. Therefore, it has been questioned increasingly whether toxicity tests with *Lemna* species are protective for more slowly growing, submerged macrophytes with different life-history strategies, a longer recovery time, and different exposure potential (Roshon et al. 1999; Cedergreen et al. 2004).

Few standardized tests exist to characterize toxicity in aquatic macrophytes for use in the regulatory ecological risk-assessment process. Only 1 standard submerged aquatic macrophyte test exists: For the dicot *Myriophyllum sibiricum* (ASTM 2004), which also may be applied to *M. spicatum*. In contrast, there are a variety of standard guidance documents for *Lemna* spp. (with ongoing, overlapping, and redundant initiatives, which is an issue in its own right). The *Myriophyllum* spp. assay has not yet been accepted broadly by stakeholders, nor is there a lot of experience in its application.

What Do We Need to Do to Improve Risk Assessment for Aquatic Macrophytes?

In the past several years, work on submerged macrophytes has increased, both in laboratory and field settings, and in the variety of species tested, including: *Egeria densa*, *Elodea canadensis*, *Elodea nuttallii*, *Myriophyllum heterophyllum*, *M. pinnatum*, and *Ceratophyllum demersum* among others. As the body of work on macrophytes begins to increase, we need to address the discipline's shortcomings in a rational and concerted manner. To improve on the use of aquatic macrophytes in risk assessment, we need to do as in the following paragraphs.

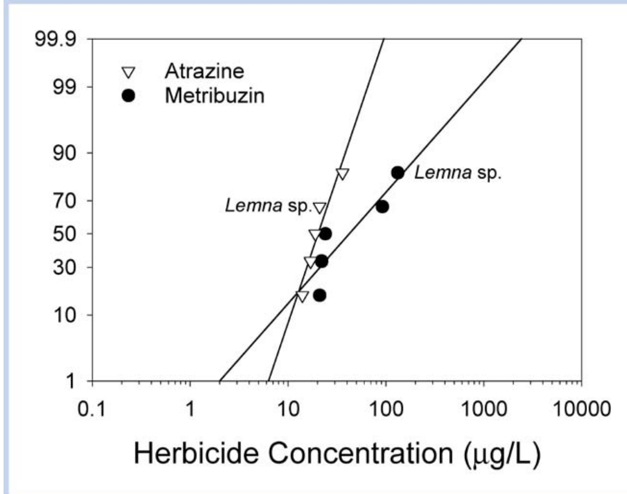

Figure 1. Aquatic macrophyte species-sensitivity distributions for the herbicides atrazine and metribuzin (modified, with permission, from Fairchild et al. 1998).

Create standard test methods

This begins by optimizing conditions for realistic growth rates and reduced variability (both statistical and toxicological) under laboratory conditions. To this end, technical aspects of a proper test design such as the growth solution and (artificial) sediment composition need to be determined. Each species possibly may require different media, light intensities, temperatures, and perhaps test durations. Measurements should focus on the ecologically relevant and sensitive endpoints that easily can be extrapolated to the field, and which may comprise measures beyond growth and biomass (GHP Arts, personal communication). Efforts should be directed to creating assays for plants with varying physiologies (monocots vs dicots), morphologies (rooted vs free floating), and environments (freshwater vs estuarine vs marine). The relative sensitivities among macrophyte species and toxicants then can be established and further efforts directed to those species that may represent a sensitive indicator of toxicity. Currently, no macrophyte species is consistently the most sensitive to a toxicant, including *Lemna* spp., the current model organism (Figure 1).

Validate laboratory assays in the field

Increasing attention is being paid to macrophyte effects at different levels of biological organization (individual, population, and ecosystem level) and extrapolating laboratory results to the field. A few studies now are directed to the understanding of intraspecies and interspecies interactions due to chemical contamination (McGregor, Mabury, et al. 2007; McGregor, Solomon, et al. 2007). However, without a clear demonstration of the link between laboratory toxicity results and field-level effects, the uncertainty associated with the assays and their extrapolation cannot be addressed adequately. Macrophytes in single-species laboratory tests are not consistently more sensitive than single-species tests in mesocosms or in population tests in mesocosms (e.g., monochloroacetic acid; Hanson and Solomon 2004). Guidelines for extrapolation from single-species tests in the laboratory to the macrophyte population level and ultimately to the community level, do not exist and have to be developed. Hand-in-hand with field validation of laboratory data is the development of guideline methods for assessing toxicity in these plants under field conditions. Although a variety of approaches exist, none have been critiqued or evaluated.

How Are We Improving Risk Assessment and Informing Risk Assessors With Our Work?

To begin, we need to elaborate and quantify the uncertainty arising from toxicity tests with *Lemna* species. Are these tests adequately protecting submerged aquatic macrophytes at higher ecological levels? Secondly, after this has been quantified, the scientific questions as defined above have to be addressed. These issues focus on standardization and validation of macrophyte tests at different levels of biological organization. In order to integrate new scientific knowledge into the risk-assessment process, communication among different stakeholder groups is important via publications, meetings, and workshops. The needs of the regulators and managers have to be understood prior to the creation of any new assays. In this way, we will improve the accuracy and effectiveness of our ecological risk assessments.

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