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Cerium chloride heptahydrate ($CeCl_3 \cdot 7H_2O$) induces muscle paralysis in the generalist herbivore, *Melanoplus sanguinipes* (Fabricius) (Orthoptera: Acrididae), fed contaminated plant tissues

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HIGHLIGHTS

- Toxicity of cerium was tested against a herbivorous insect, *Melanoplus sanguinipes*.
- Insects were fed on contaminated plant shoots to establish dose–response.
- Accumulation occurred at all doses in frass, exoskeleton and intestines.
- Paralysis of appendages found in highest doses.
- Increasing pollution may put herbivores at risk from contaminated food sources.

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ABSTRACT

Of increasing economic importance are the rare earth elements (REEs). Pollution from mining and processing activity is expected to rise with industrial demand. Plants are known to accumulate REEs, although levels vary with species and soil content. However, the effect on wildlife of ingesting REE contaminated vegetation is not well understood. Here we examined the effect of consuming vegetation with elevated levels of cerium on the generalist grasshopper, *Melanoplus sanguinipes* (Fabricius).

Adults excreted a substantial portion of ingested contamination. However, after only four-days of feeding, accumulation in the body occurred at all doses and paralysis of appendages resulted at the highest doses.

Short-term toxicity studies may underestimate the impact of ingesting REE contamination. Metals tend to be low in toxicity; however, their persistence in the environment may be better represented by exposure over longer portions of the life cycle.

1. Introduction

Increasingly, rare earth elements (hereafter REEs) are mined and processed for use in emerging technologies. Although commonly found in the earth's crust, REEs are termed "rare" because they do not concentrate in pure ore deposits (Hu et al., 2006).

Natural crustal levels of the most abundant of the REEs, cerium (Ce), vary by location and soil type. Average estimates are approximately 66 mg kg^{-1}, comparable to levels of copper (68 mg kg^{-1}) and zinc (76 mg kg^{-1}), two of the most widely studied metals (Greenwood and Earnshaw, 1997; Tyler, 2004). Far less attention has been paid to REEs, possibly because they are not considered to be essential to life nor strongly toxic (Tyler, 2004). As a result of their low toxicity, threshold limits and maximum permissible concentrations are poorly established in the literature.

Increased use of REEs in emerging technologies may result in elevated environmental levels. For example, concentrations of Ce in polluted soils from industrial locations in The Netherlands run as high as 900 mg kg^{-1} (Slooff et al., 1993). Elevated Ce levels were found up to 6 km from a processing plant in China and were correlated with distance from the source (Li et al., 2010).

Plants are known to accumulate REEs, including Ce, through contaminated soils (Carpenter and Boutin, 2013; Thomas et al., 2014). Accumulation is thought to be linked to the similarity in

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ionic radii between REEs and calcium (Pickard, 1970). Herbivores feeding directly on plant tissues with elevated REE levels may likewise accrue these metals in their bodies. For example, Cowgill (1973) observed uptake of several REEs, including Ce, by water-lilies (*Nymphaea odorata* Ait.) and subsequently found these metals in the aphids (*Rhopalosiphum nymphaeae* (L.)) feeding on the lilies.

The effects of consuming an REE contaminated diet on insects are currently unknown. Fed upon by other arthropods, amphibians, reptiles, birds and small mammals, insects represent a substantial portion of the biomass available in wildlife food webs. In this study, we assessed the toxicity of Ce to herbivorous insects fed on contaminated leaf material. We used the generalist grasshopper, *Melanoplus sanguinipes* (Fabricius) as a representative species. Biomass gains, consumption, deterrence and accumulation in tissues and in frass were evaluated.

2. Materials and methods

2.1. Rearing

A colony of *M. sanguinipes* was established using eggs from a laboratory-reared colony maintained at Agriculture and Agri-Food Canada's Saskatoon Research Centre (Saskatoon, SK). Eggs were maintained in sifted sand in 500 mL plastic containers refrigerated at 4–6 °C until moved to a rearing cage for incubation. Each rearing cage was fitted with a 60-W light-bulb to provide both warmth and a 16:8 day:night cycle. Average temperature in the cages was 30.8 ± 0.3 °C. All grasshoppers were fed on a mixed diet of leaf lettuce, fresh cut wheat treated with a sulfonamide antibiotic (as a prophylaxis against fungal infection) and wheat bran prior to testing. Within 24-h after metamorphosis to the adult stage, individuals were sexed based on external genitalia and assigned haphazardly to a feeding treatment.

2.2. Cerium doses

Cerium chloride heptahydrate ($CeCl_3{\cdot}7H_2O$ – Sigma–Aldrich, Oakville, ON, CA; CAS: 18618-55-8) was selected for use due to its high solubility in water. The lowest dose, 30 mg L^{-1} dH_2O, was selected to be representative of the highest accumulation of Ce found in shoot material (including leaves) in previous greenhouse studies by Thomas et al. (2014) (i.e., *Solanum lycopersicum* shoot (including leaves) concentration of 39.4 mg Ce kg^{-1} dry biomass when grown in a soil concentration of 978 mg Ce kg^{-1}). Two additional doses were selected following a geometric progression of 10 (i.e., 30, 300, 3000 mg Ce L^{-1} dH_2O or nominal concentrations). Cerium comprises approximately 37.6% of the total mass of $CeCl_3{\cdot}7H_2O$ necessitating the use of 80, 800 and 8000 mg of $CeCl_3{\cdot}7H_2O$.

Twelve female and twelve male replicates were used at each dose and controls for a total of 96 insects. Due to space limitations, treatments were run in cohorts consisting of one to four female and male adults per dose.

2.3. No-choice feeding trials

Individuals were weighed to the nearest 0.001 g and assigned to a dose. We compared mean start weight of females and males across doses to ensure an even distribution of larger and smaller individuals (ANOVA; $p > 0.05$ for all gender–dose combinations).

Selected grasshoppers were placed individually in 500 mL glass jars fitted with aluminium screen lids. A 24-h starvation period preceded the start of the trials. Based on consumption in healthy individuals during pre-trials, we chose the daily feeding ration to consist of 2 g of lettuce (*Lactuca sativa* var. "buttercrunch" L.) leaf material, enough to feed each individual *ad libitum* for a 24-h period. All lettuce was grown from seed in our greenhouses.

Cerium solutions were mixed in 1 L batches and dispensed daily into individual glass containers in 100 mL aliquots. Negative controls consisted of deionized water (dH_2O). Each ration was weighed to the nearest 0.001 g and soaked overnight in a jar containing 100 mL of solution. One additional lettuce leaf was added to each container for chemical analysis of Ce content. These lettuce leaves were also dried and used to provide an estimate of water loss.

Once removed, leaves were air dried, reweighed, and presented to the grasshoppers. Each day, we weighed, bagged and oven-dried the remaining food and frass to constant weight at approximately 70 °C. Grasshoppers were given a new food ration each day. After four days, grasshoppers were weighed to determine final biomass and were starved for 24-h to eliminate gut contents. On the final day, remaining frass was weighed and dried and individual grasshoppers were frozen.

2.4. Consumption and growth estimates

Using the extra leaves in each aliquot, we calculated the average water loss and percentage dry matter in the food rations. The percentage dry matter was used to calculate the estimated dry matter available for consumption, following Schmidt and Reese (1986). For each grasshopper, we calculated the dry weight of ingested food:

$$\text{ingested food (g)} = (\text{ration (g)} * \%\ \text{dry matter}) - \text{dry weight remaining food (g)}$$

This value was converted back to fresh weight consumed by dividing by the percentage dry matter content of *L. sativa* leaves.

Biomass gain was calculated as the difference in weight between the beginning and end of the trial and was then compared between doses and compared to consumption.

2.5. Visual assessments (toxicity index)

Visual assessments were made daily on the condition of each grasshopper, their reaction when provoked (lethargic or responsive) and mobility (healthy, slow or paralysed). Assessments of individuals were scored using a semi-quantitative scale for sublethal effects adapted from Isman (1985) to create a toxicity index. Scores ranged from 0 to 2, where:

0 = healthy individuals with normal movements,
1 = lethargic reactions; reluctance to move,
2 = paralysis of limbs/appendages.

We calculated the toxicity at each dose as the mean toxicity index scores for all females or males within a dose.

2.6. Chemical analysis

Frozen grasshoppers were thawed for several minutes for dissection. The dorsal side of the grasshopper was cut open and the tissues surrounding the gut removed. The gut was separated from the body by cuts posterior to the rectum and anterior to the crop.

Samples of soaked leaves, insect frass, body and gut tissues were dried to constant weight at approximately 70 °C. Samples were pooled by gender and dose, bagged and sent to Brooks Rand Labs (Seattle, Washington, USA) for analysis of Ce content. Samples were analysed using inductively coupled plasma–mass spectrometry (ICP–MS) with Dynamic Reaction Cell (DRC™) technology according to a modified EPA draft method 1638 (USEPA, 1996). Briefly, 0.5 mg aliquots of the sample are digested with 10 mL

ultra-pure nitric acid and 100 μL of hydrogen peroxide, and then heated for a minimum of four hours at 100 °C. Digested samples are then analysed using internal standardization, a method which incorporates ionisation of the samples in inductively-coupled radio-frequency plasma, with detection of the resulting ions by mass spectrometer on the basis of their mass-to-charge ratio.

2.7. Choice feeding trials

Choice feeding trials, to detect avoidance of dosed food, followed similar methodology to our no-choice trials. The dose where toxic effects became visible in the no-choice trials, 800 mg Ce kg^{-1} dry biomass, was selected.

Newly emerged adults (10 female and 10 male) were haphazardly selected and placed in cages separated by gender. A 24-h starvation period preceded the start of the trial. The feeding ration was selected to allow 2 g of leaf material per individual per treatment, or 20 g of dosed and 20 g of control food. Lettuce was grown in the same conditions as in our no-choice trials. We mixed Ce solutions in 1 L batches and used dH_2O as a control. Rations were weighed to the nearest 0.001 g and soaked overnight in 900 mL of solution. Two additional lettuce leaves were added to each container to estimate water loss of leaves in the food ration.

Once removed from the soaking solution, leaves were air-dried and reweighed. Each cage received one ration each of dosed and control food for 24-h. Each day, remaining food and frass was collected in bags and oven-dried to constant weight at approximately 70 °C. The process was repeated for four days after which grasshoppers were weighed to determine final biomass, and starved for 24-h to eliminate gut contents. On the final day, remaining frass was weighed and dried and individual grasshoppers were frozen. Each trial was repeated three times to obtain an average consumption estimate.

2.8. Statistical analysis

Data were analysed using Systat 13 for Windows (version 13.00.05; Systat Software Inc., Chicago, IL, USA). Normality assumptions and homogeneity of residuals were verified using Kolmogorov–Smirnov or Shapiro Wilks test, and Levene's test, respectively.

Differences in percent biomass gain and consumption estimates were established using ANOVA with a Dunnett's one-way post hoc to determine which doses were significantly lower than controls. Female consumption estimates did not meet the assumptions of normality thus requiring a Kruskal–Wallis non parametric test with Conover–Inman post hoc analysis. Fresh biomass gains were evaluated using ANCOVA with initial biomass as a covariate. Biomass gains were also adjusted for consumption using ANCOVA with initial biomass and fresh weight consumption as covariates to determine if effects on biomass occurred pre- or post-ingestion.

Three nutritional indices were compared across doses: the approximate digestibility (AD), the efficiency of conversion of ingested food to biomass (ECI) and the efficiency of conversion of digested food to biomass (ECD). The method of ANCOVA (Raubenheimer and Simpson, 1992) was used to compare efficiencies by dose.

The AD measures the portion of ingested food that is digested. An ANCOVA between the dry weight of frass and dose, using the estimated dry weight consumption as a covariate provides an approximation.

The ECI, sometimes referred to as the gross growth efficiency, estimates the portion of ingested food converted to biomass (Slansky, 1985). An ANCOVA between final biomass and dose, using both initial biomass and fresh weight consumption as covariates was used.

The ECD, sometimes called the net growth efficiency, measures the portion of digested food converted to biomass (metabolic efficiency taking into account food portions used for respiration) (Slansky, 1985). The ECD was approximated using ANCOVA between final biomass and dose using initial biomass and digested mass as covariates. Digested mass was determined as the difference between fresh weight consumption and frass produced. For all ANCOVA, Bonferroni post hoc analysis was used to determine which doses differed significantly from controls.

Fresh weight consumption was compared between dosed and control food using a t-test to identify differences for the choice-feeding trials.

3. Results

3.1. Leaf tissue analysis

Cerium concentrations ranged from 15.9 (control leaves soaked in dH_2O) to 13 900 mg kg^{-1} dry biomass corresponding with increasing dosage of the soaking solution (Table 1). All subsequent analyses are based on the measured concentration in leaves.

3.2. Biomass

Percent fresh weight gain of females did not differ with increasing dosage (ANOVA; $F_{3,44} = 0.555$; $p = 0.648$). Males exhibited an increasing trend of percent fresh biomass gain at lower doses, with a decline at the highest dose; however, these trends were not significant (ANOVA; $F_{3,44} = 0.797$; $p = 0.502$).

3.3. Consumption

Fresh weight consumption of dosed food did not differ from controls (Female: Kruskal–Wallis; $\chi^2 = 0.756$; $p = 0.860$; Male: ANOVA; $F_{3,44} = 0.536$; $p = 0.660$). For females, mean daily consumption was 41 ± 0.12% of the daily ration for controls and 46 ± 0.12%, 45 ± 0.13%, 44 ± 0.14% for each of the lowest, mid and high doses respectively. For males, mean daily consumption was 41 ± 0.11% of the daily ration for controls and 40 ± 0.12%, 33 ± 0.15%, 32 ± 0.14% for each of the lowest, mid and high doses respectively. Likewise, fresh weight biomass gains, either unadjusted or adjusted for consumption, did not differ between doses.

3.4. Nutritional indices

Female AD did not change with dose (Table 2), nor were the ECI and ECD altered at any of the doses tested (Table 2).

Adult male data was heteroscedastic due to a slightly higher variation in frass produced at the control dose. In the absence of a viable alternative (non-parametric) analysis, it was decided to accept the violation of this assumption. At the highest dose, AD

Table 1
Comparison of the nominal and measured concentrations of cerium (Ce) based on the average amount of Ce found in *Lactuca sativa* leaf samples. Background levels were measured in the control leaves soaked in deionized water (dH_2O). Average measured concentrations were used in subsequent analyses of effects.

Dose	Solution dose mg L^{-1} dH_2O	Average measured mg kg^{-1} dry biomass
Control	0.0	15.9
2	30.0	2060
3	300.0	4480
4	3000.0	13 900

Table 2
Summary of least squared means (LS Means) for ANCOVA for nutritional indices associated with adult *Melanoplus sanguinipes* exposed to cerium (Ce) contaminated food. See methods for descriptions of the variables used to approximate indices.

		LS Means by Dose (mg Ce kg^{-1} dry biomass)					Doses sig.
Nutrional index		15.9	2060	4480	13900	Adj. R^2	<control (mg Ce kg^{-1})
Approximate Digestibility (AD)	Female	0.096 ± 0.008	0.102 ± 0.008	0.099 ± 0.008	0.096 ± 0.008	0.933	None
	Male	0.086 ± 0.006	0.071 ± 0.006	0.070 ± 0.006	0.060 ± 0.006	0.276	13900
Efficiency of Converting Ingested Food (ECI)	Female	0.365 ± 0.010	0.378 ± 0.010	0.370 ± 0.010	0.379 ± 0.010	0.748	None
	Male	0.305 ± 0.007	0.310 ± 0.007	0.309 ± 0.007	0.302 ± 0.007	0.656	None
Efficiency of Converting Digested Food (ECD)	Female	0.365 ± 0.010	0.378 ± 0.010	0.370 ± 0.010	0.379 ± 0.010	0.746	None
	Male	0.305 ± 0.007	0.310 ± 0.007	0.309 ± 0.007	0.302 ± 0.007	0.656	None

was significantly lower than for control males (Table 2). However, ECI and ECD were unaffected in the range of doses tested (Table 2).

3.5. Visual assessments

Visually, adults exposed to Ce contamination were not more lethargic than controls. However, calculated toxicity index scores increased with dose starting at the third dose for males, and the highest dose for females (Fig. 1). After one day of exposure, one male at the third dose was unable to move his antenna. After three days, two additional males at the highest dose experienced impaired movement in one or both rear legs. The rear legs were held in an extended position behind the body with no bend in the femur/tibia joint. After four days, the rear leg of a female at the highest dose was found in the same extended position. Two additional males at the highest dose were found to have impaired movement of the rear legs and antenna respectively.

3.6. Choice feeding

Choice experiments showed no significant difference in consumption between dosed and control food for females ($t = 0.222$; $p = 0.835$) or males ($t = -0.162$; $p = 0.879$).

3.7. Tissue accumulation

Measured concentrations of Ce in tissues are reported in Table 3. For males, accumulation was greatest in the frass, exceeding the food concentration. For females, frass concentration at the control dose was similar to that of the food but exceeded the food concentration as dose increased.

Although a considerable amount of the ingested contamination was excreted, female and male exoskeletons showed increasing concentration of Ce with dose, indicating accumulation (Table 3). When considering only the dosed samples, the ratio of incorporated versus excreted Ce by males (i.e., frass:exoskeleton) fell as dose increased, while for females there was some variation by dose.

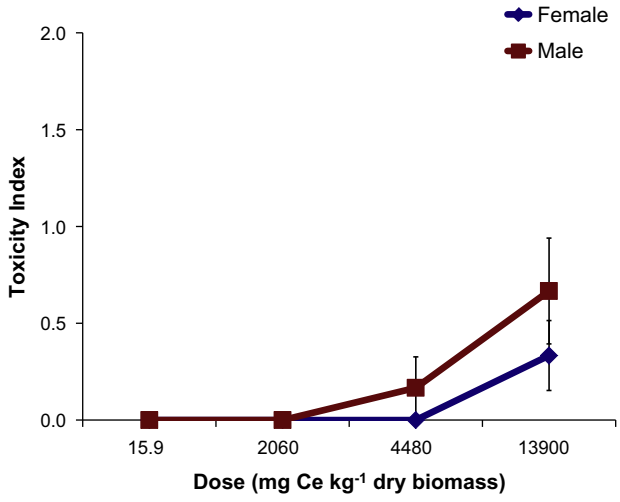

Fig. 1. Toxicity of cerium (Ce) to adult *Melanoplus sanguinipes* after four days of feeding on contaminated vegetation. Toxicity scores (see materials and methods for detailed description) represent the mean score of females and males at each dose, using a semi-quantitative scale ranging from 0 to 2, where 0 = healthy individuals with normal movements, 1 = lethargic reactions, reluctance to move and 2 = paralysis of limbs/appendages. Error bars represent standard error.

4. Discussion

Although REEs are known to accumulate in plants (Tyler and Olsson, 2005; Carpenter and Boutin, 2013; Thomas et al., 2014 among others), little is known about the effect on herbivores feeding on contaminated plant tissues. In this study, we assessed the toxicity of Ce to herbivorous insects when exposed to contaminated vegetation using the generalist grasshopper *M. sanguinipes* as a representative species. Acridids (i.e., grasshoppers) make ideal environmental indicators as they are generally intolerant of pesticide and heavy metal pollution (Schmidt, 1986). In addition, they form an important part of food webs due to their abundance and high individual weight. They are ideal prey items for birds, small mammals, reptiles and amphibians (Schmidt, 1986).

Biomass gains were unaffected in the range of doses tested indicating high levels of pollution would be required to produce an effect on biomass in short-term exposure (four days in this case). Cerium contaminated food did not deter adults from feeding, nor were post-ingestion effects evident in the range of doses tested. Efficiencies of converting food to biomass (i.e., ECI and ECD) were similarly unaffected by dose. However, at the highest dose, there was a drop in the approximate digestibility for males, suggesting less of the ingested food was digested. In contrast, neodymium (Nd) caused a decrease in biomass and consumption as well as a rise in metabolic activity, suggesting an increased metabolic cost of ingesting contaminated food (Allison et al., 2014).

Although reductions in biomass did not occur in Ce treated individuals, the calculated toxicity index increased with dose. Visual assessments during the experiment showed paralysis, predominantly in males, at the two highest doses. Reduced locomotion may affect weight in the long-term due to reduced ability to forage. Paralysed individuals may also find themselves more susceptible to predation, passing toxins to other trophic levels. Similar paralysis was not observed in our experiments with Nd (Allison et al., 2014).

The paralysis may be a result of ingested Ce blocking calcium channels, as has been found with other REEs. Lanthanum is a strong calcium channel blocker due to its similar ionic radius and higher valence (3+) (Craig et al., 1999). Blocking calcium uptake can lead to muscle membrane depolarisation, resulting in sustained muscle contraction or tetanic paralysis. This effect has been observed in cockroach (*Periplaneta americana*) leg muscles

Table 3
Measured concentrations of cerium (Ce) in the frass and tissues (exoskeleton and intestine) of female and male *Melanoplus sanguinipes* adults exposed to Ce contaminated food (*Lactuca sativa* leaves). Numbers represent analyses of pooled tissue samples corresponding to the tissue and dose combination.

Dose	Measured concentrations mg Ce kg^{-1} dry biomass			Tissue ratios		
mg Ce kg^{-1} dry biomass	Exoskeleton	Intestine	Frass	Frass: exoskeleton	Frass: intestine	Intestine: exoskeleton
Female						
15.9	0.22	32.5	15.6	70.91	0.48	147.73
2060	3.75	10.7	5320	1418.67	497.20	2.85
4480	23.6	30.1	12200	516.95	405.32	1.28
13900	45.2	217	40700	900.44	187.56	4.80
Male						
15.9	0.21	1.56	139	661.90	89.10	7.43
2060	2.12	21.3	5750	2712.26	269.95	10.05
4480	7.79	37.5	12800	1643.13	341.33	4.81
13900	51.0	1070	37500	735.29	35.05	20.98

(Washio and Miyamoto, 1983), frog (*Rana temporaria*) Sartorius muscle fibres (Glavinovic et al., 1989) and in snake (*Thamnophis* sp.) muscle fibres (Coniglio et al., 1993) treated with lanthanum. Evidence from the literature suggests the condition is not reversible once exposure ceases (Coniglio et al., 1993). Increasing environmental concentrations of Ce may increase competition for calcium binding sites in exposed organisms. Further study is necessary to determine if blocked calcium channels or some other physiological effect of ingesting cerium is causing this observed paralysis.

Our visual assessments were limited to lethargic reactions when provoked and obvious paralysis of limbs. In a more detailed study of impaired locomotion, Bayley et al. (1995) found reductions in distance travelled by adult female *Pterostichus cupreus* L. (Coleoptera: Carabidae) exposed to elevated levels of copper. Females were active for 51% as much time and moved at 71% the speed of controls (Bayley et al., 1995). Studies of aquatic organisms have found locomotion to be a consistently sensitive measure of toxic stress due to environmental contaminants (Little and Finger, 1990). Detailed studies linking altered locomotion to toxic stress in terrestrial invertebrates are lacking. This link may be important to distinguish actual toxic effects of contaminants, such as metals, from alterations in behaviour (e.g., consumption deterrence).

Adults excreted the majority of ingested Ce contamination, with frass concentration increasing with dose. The elimination process is an essential route for terrestrial invertebrates, including many insects (Dallinger, 1993). Toxic metals are stored in vesicles of digestive cells and eliminated as solid waste (Dallinger, 1993). For example, in addition to high assimilation rates, carabid beetles efficiently excrete metal-containing vesicles (Janssen et al., 1991).

Tissue concentration increased with dose, while at the same time the ratio of excreted to incorporated (i.e., frass:exoskeleton) Ce declined. This suggests the rate of uptake exceeded the excretion rate. Toxic effects occur once the rate of uptake exceeds the rate of excretion and/or detoxification long enough for the metabolically available concentration to exceed the critical threshold of the organism under study (Rainbow, 2007).

The doses used in this study were higher than concentrations reported from plants growing at natural sites, however, there is currently little information regarding plant concentrations at contaminated sites near mining/processing activity. Evaluating acute toxicity at high doses can be useful to identify contaminants that merit further investigation. In this study, toxic effects were most evident at the highest dose, but accumulation in the body tissues occurred at all doses.

Our study was limited to four days of feeding. Short-term studies run the risk of underestimating toxic effects and increased mortality caused by persistent pollutants such as metals. For example, mortality gradually increased over a 22-d period for pea aphids (*Acyrthosiphon pisum* Harris) exposed to cadmium (Laskowski, 2001). Further investigation of REEs using environmentally relevant concentrations over the life cycle of exposed herbivores might shed more light on the levels of REEs that causes toxic effects.

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