Acute Silver Toxicity in Aquatic Animals Is a Function of Sodium Uptake Rate

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On the basis of these facts about freshwater fish and invertebrates: (i) the Na^+ turnover is a physiological process associated with the gill membranes; (ii) the key mechanism of acute silver toxicity consists of reduction in Na^+ uptake by blockade of gill Na^+,K^+-ATPase; (iii) the mass-specific surface area of the gills depends on animal body mass; and (iv) the gill surface is also the major site of Na^+ loss by diffusion, we hypothesized that whole body Na^+ uptake rate (i.e., turnover rate) and secondarily body mass would be good predictors of acute silver toxicity. Results obtained from toxicological (LC_{50} of $AgNO_3$) and physiological (^{22}Na uptake rate) tests performed on juvenile fish (rainbow trout, *Oncorhynchus mykiss*), early juvenile and adult crayfish (*Cambarus diogenes diogenes*), and neonate and adult daphnids (*Daphnia magna*) in moderately hard water of constant quality support the above hypothesis. Therefore, sensitivity to $AgNO_3$, in terms of either total measured silver or free Ag^+, was reliably predicted from the whole body Na^+ uptake rate in animals with body mass ranging over 6 orders of magnitude (from micrograms to grams). A positive log−log correlation between acute $AgNO_3$ toxicity and body mass of the same species was also observed. Furthermore, the whole body Na^+ uptake rate was inversely related to body mass in unexposed animals. The combination of these last two results explains why the small animals in this study were more sensitive to Ag^+ than the larger ones. Taken together, these results clearly point out the possibility of incorporating the Na^+ uptake rate into the current version of the Biotic Ligand Model to improve the predictive capacity of this model. In the absence of information on Na^+ uptake rate, then body mass may serve as a surrogate.

Introduction

Although most silver in surface waters originates from natural sources, elevated concentrations are usually associated with anthropogenic activities such as mining and photographic processing (*1*). Therefore, several different geochemical or biological modeling approaches have been developed in the past few years in an attempt to predict acute silver toxicity in aquatic vertebrate and invertebrate species (*2*). For example, acid-volatile sulfide concentrations and silver interstitial water toxic units have been employed to predict the toxicity of silver to estuarine invertebrates in different sediments (*3*). Other modeling approaches with emphasis on geochemical speciation use the Ag^+ concentration in the water column to directly predict toxicity (e.g., the "free ion activity model"; *4*, *5*).

A modeling approach to predict silver toxicity to freshwater fish that takes into account the geochemistry of the biological ligand has been developed over the past few years (*6*, *7*). According to the Biotic Ligand Model (BLM), the gill is considered a negatively charged ligand (the biotic ligand) to which Ag^+ can bind. Toxic effect is considered a function of the degree of saturation of "toxic sites" on the biotic ligand by Ag^+. The gill modeling approach presents some advantages over simply considering speciation of silver in the water column (*2*). For example, current versions of the BLM (*7*, *8*) take into account the competition between other cations and Ag^+ for toxic binding sites on the gills as well as the influence of different complexing agents on silver speciation and availability.

Parallel to the development of the BLM, evidence elucidating the physiological mechanism of acute silver toxicity in freshwater fish and crustaceans has been accumulating (*2*). In rainbow trout, the most studied freshwater fish species, the Na^+,K^+-ATPase located at the basolateral membrane of the gill seems to be the key site for silver toxicity (*9*). Recently, the same toxic mechanism has been demonstrated in the crayfish (*10*). It is also well-known that the gills are the main site of active transport of Na^+ and Cl^- from the water into the extracellular fluid of the animal and that Na^+ and Cl^- uptake across the gills is directly related to the branchial Na^+,K^+-ATPase activity (*11−14*). This uptake is essential to counteract the diffusive losses of ions through the gills and excretory organs in freshwater fish and crustaceans.

Given that the toxic mechanism of silver involves an interference with ionic uptake at the gill surface, that the gills are the major site of ion loss, and that mass-specific gill area is inversely proportional to body mass in both fish (*15*) and crustaceans (*16*), body size might well influence sensitivity to acute silver toxicity. Specifically, we hypothesized that smaller fish or crustacean species exhibit a higher Na^+ turnover due to the larger mass-specific surface area of the respiratory surface(s) in contact with the hypo-osmotic freshwater. As a consequence, they will also be more sensitive to acute silver toxicity. Herein, we present evidence that whole body Na^+ uptake rate (UR) serves as a good predictor of sensitivity to acute silver toxicity for freshwater fish and crustaceans ranging in size over several orders of magnitude.

Experimental Section

Experimental Animals. All organism weights are wet weights and were expressed as mean $\pm$ 1 SD. Juvenile rainbow trout *Oncorhynchus mykiss* (2.2 ± 0.5 g; $n = 10$) and adult crayfish *Cambarus diogenes diogenes* (18.57 ± 0.51 g; $n = 140$) were obtained from Humber Spring Hatchery (Orangeville, Ontario, Canada) and Boreal Laboratory Supplies (St. Catharines, Ontario, Canada), respectively. Two-week-old juvenile crayfish *C. diogenes diogenes* (11.5 ± 0.45 mg; $n = 65$) were obtained from spawning and hatching in our laboratory. Fish and adult crayfish were held in 400-L fiberglass tanks supplied with a minimum of 2.5 L min^{-1} flow-through of dechlorinated,

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aerated Hamilton City tap water ([Na^+] 0.6 mmol L^{-1}; [Cl^-] 0.7 mmol L^{-1}; [Ca^{2+}] 1.0 mmol L^{-1}; [HCO_3^-] 1.9 mmol L^{-1}; [Mg^{2+}] 0.2 mmol L^{-1}; [DOC] 1.3 mg L^{-1}; pH 8.2) for at least 7 days prior to experimentation. Early juvenile crayfish were held in a 25-L fiberglass tank supplied with a minimum of 100 mL min^{-1} flow-through of the same dechlorinated tap water used for fish and adult crayfish. Temperature and photoperiod were kept at $15 \pm 2\,°C$ and 12L:12D, respectively. The rainbow trout were fed dry trout pellets (Martin's Feed Mills, Ontario) three times a week, and the crayfish (both adults and early juveniles) were fed chopped sole fillets twice a week.

Several colonies of adult gravid *Daphnia magna* (ARO strain, Lot No. 090600 DM) were obtained from Aquatic Research Organisms (Hampton, NH). The brood origination was the U.S. Environmental Protection Agency Laboratory of the State of Ohio (U.S. EPA OH), and the daphnids had been reared in a freshwater static renewal system with water saturated in dissolved oxygen, pH 7.5, hardness ~150 ppm, and 25 °C. Upon arrival at our laboratory, the *D. magna* colonies were gradually acclimated to synthetic water in an incubator for 48 h. Synthetic water used for acclimation and all tests was designed to approximate Hamilton City tap water, except for DOC, and was prepared as a single batch employing 1000 L of reverse osmosis water in a food-grade polyethylene tank. This water was reconstituted to the following composition: [$CaCO_3$] 1.0 mmol L^{-1}; [$MgSO_4$] 0.15 mmol L^{-1}; [NaCl] 0.6 mmol L^{-1}; [DOC] <0.1 mg L^{-1}; pH 8.2. It was bubbled with pure CO_2 for 24 h to ensure that $CaCO_3$ went into solution and then was bubbled with air for 48 h to ensure removal of excess CO_2 and atmospheric equilibration. During the acclimation period, *D. magna* were fed YCT (a slurry of yeast, cerophyll, and trout chow) and the water was not aerated. Temperature and photoperiod were fixed at 20 °C and 14L: 10D, respectively.

After acclimation, *D. magna* reproduction rate was measured to ensure it met established criteria for a healthy population (15−20 neonates every 3−4 days). Provided that the reproductive rate was satisfactory, we proceeded with the particular colony. To collect neonates (0.090 ± 0.009 mg; $n = 240$), adults (1.35 ± 0.078 mg; $n = 150$) were confined in a fine screen mesh net suspended in a 1-L glass aquarium containing synthetic water. During this time feeding was withheld. Neonates passed through the mesh and into the aquarium of synthetic water and were collected within 6−24 h of hatch for toxicological and physiological studies.

AgNO₃ Acute Toxicity Tests. All PVC chambers and glassware used in the toxicity tests were acid-cleaned in 1% HNO_3 (trace metal grade; Merck Chemicals) and rinsed thoroughly with dechlorinated tap water (PVC chamber) or synthetic water (glassware) prior to use.

The 96-h LC_{50} value for juvenile rainbow trout was that determined in previous experiments in our laboratory using the same water quality as the current experiments; methods have been described in detail by Hogstrand et al. (*17*). Acclimated crayfish were transferred from the holding tank and exposed (96 h) to $AgNO_3$ in aerated PVC chambers (volume = 1200 mL) supplied with a flow-through (200 mL min^{-1}) of dechlorinated Hamilton city tap water. Total silver concentrations in water samples (acidified with 1% HNO_3) from the treatments were measured by graphite furnace atomic absorption spectrometry (GF-AAS; Varian AA-1275 with GTA-9 atomizer) over the 96-h period of the test. The mean measured total silver concentrations tested were juvenile fish: see details in ref *17*; early juvenile crayfish: 0, 1.62, 2.86, 4.95, and 8.13 μg of total Ag L^{-1}; adult crayfish: 0, 2.23, 3.21, 6.00, 6.23, 13.07, 27.54, 37.14, 39.52, 43.28, 108.71, 125.24, and 128.46 μg of total Ag L^{-1}.

Daphnid toxicity tests were performed in a semi-static system (24-h water renewal) using borosilicate glass beakers containing 250 mL of the synthetic water pre-equilibrated to 20 °C and without aeration. This high volume to daphnids ratio was employed to minimize accumulation of dissolved organic carbon during the tests, and to maximize the volume-to-wall surface ratio. To accurately measure the very low silver concentrations employed in the daphnid tests, a proportion of radioactive ^{110m}Ag (RISOE National Laboratory, Roskilde, Denmark) together with the appropriate amount of unlabeled $AgNO_3$ was added into the test solutions 3 h prior to introduction of the daphnids. The final specific activity of radiolabeled silver in all test solutions was 0.72 μCi μg^{-1} of total silver. The ^{110m}Ag radioactivity in water samples (2 mL) from daphnid treatments was determined using a γ-counter (MINAXI gamma Auto-gamma 5000 series, Canberra-Packard) and the appropriate windows as outlined by Hansen et al. (*18*). After 24 h, daphnids were transferred to a new set of test solutions prepared 3 h prior to transfer as previously described. During solution changeover, living daphnids were removed from the original beakers and held in plastic pipets. Mean measured concentrations over the 48-h period of test were as follows: 0 (control), 0.038, 0.083, 0.140, 0.323, 0.625, and 1.294 μg of total Ag L^{-1}.

In all cases, final silver concentrations were obtained from $AgNO_3$ stock solutions (SigmaUltra, Sigma Co., St. Louis, MO; 10 and 1 mg L^{-1}) acidified with 1% HNO_3. Three replicates ($n = 8−10$ per replicate) for each silver concentration were provided. Animals were not fed during the tests. Temperature and photoperiod were maintained as in the acclimation period.

At 48 (daphnids) or 96 h (fish and crayfish) of exposure, mortality (%) in each experimental unit was recorded. The death criteria adopted were a change to milky coloration (daphnids) and lack of movement (daphnids, fish and crayfish) even after mild stimulation. LC_{50} values and the respective 95% confidence intervals were estimated based on cumulative mortality data using Probit analysis (*19*). These values were estimated based on mean measured total silver concentrations over the respective period of test. LC_{50} values were also calculated in terms of free Ag^+ concentrations that were calculated under the chemical conditions determined in our experiments using the MINEQL+ geochemical program (*20*). Parameters were entered into the program as follows: pH 8.2; temperature = 15 (fish and crayfish) or 20 °C (daphnids); total silver = mean measured over 48 or 96 h; Na^+, Cl^-, Ca^{2+}, Mg^{2+}, and HCO_3^- = nominal concentrations. DOC (1.3 mg of C L^{-1}) was included in the program only for fish and crayfish water tests since measured concentrations in the synthetic water used in the daphnid tests were very low (<0.1 mg of C L^{-1}). The constants used for DOC were 35 nmol binding sites per mg of C L^{-1} and a log $K_{Ag−DOC} = 9.0$ (*6*). Ionic strength corrections were calculated by the MINEQL+ program.

Na⁺ Uptake Measurement. Ten fish or 10 adult crayfish were transferred from the holding tank to individual aerated PVC chambers (volume ~300 mL) for measurement of Na^+ UR in their acclimation water (silver-free). They were starved for 48 h prior to test start and were allowed to acclimate to the holding chambers for 24 h prior to experimentation.

Unidirectional influx was measured in individual fish and adult crayfish housed in containers with constant aeration for thorough mixing of the water. The water flow to the individual flux chambers was terminated and ^{22}Na (10 μCi L^{-1}, Amersham, specific activity 303 Ci g^{-1} of Na^+) was added to the water of each flux chamber. Two water samples (5 mL) were taken at 0 and 2 h (rainbow trout) or 3 h (adult crayfish) following a 15-min equilibration period. These samples were used for ^{22}Na radioactivity measurement using the same counter employed for ^{110m}Ag radioactivity previously described and total Na measurement using the Varian AA-1275 atomic absorption unit operated in flame emission mode.

TABLE 1. Coefficients, R^2 Values, and P Values for the Log−Log Regressions (log $Y = a + b$ log X) between Whole Body Na^+ Uptake Rate (μmol g^{-1} h^{-1}), LC_{50} for Silver Based on Mean Measured Total Silver (μg of Ag L^{-1}) or Free Silver (μg of Ag^+ L^{-1}), and Body Mass (g WW)

| | coeff | | | |
regression	a	b	R^2	P
Na^+ uptake vs LC_{50}				
LC_{50} based on total silver	1.028	−1.269	0.959	<0.001
LC_{50} based on free silver	0.375	−1.036	0.964	<0.001
body mass vs LC_{50}				
LC_{50} based on total silver	1.201	0.417	0.924	<0.001
LC_{50} based on free silver	0.520	0.346	0.979	<0.001
body mass vs Na^+ uptake	−0.131	−0.328	0.979	<0.001

The whole body Na^+ UR from the water was calculated from the disappearance of ^{22}Na radioactivity from the water in the flux container during the 2-h flux period, the mean specific activity of the ^{22}Na in the water, the container volume, the animal's body weight, and the elapsed time as outlined in detail by Grosell et al. (*21*).

Juvenile crayfish and both life stages of daphnids were assayed in groups. Fifteen early juveniles crayfish or 30 daphnids (neonates or adults) were transferred from the holding tank to glass beakers containing 100 mL of dechlorinated tap water or 50 mL of synthetic water, respectively. ^{22}Na (10 μCi L^{-1}, Amersham, specific activity 303 Ci g^{-1} of Na^+) was added to the water. Water samples for measurement of ^{22}Na radioactivity and total Na (as above) were taken at 0 and 1 h. After the 1-h flux period, animals were collected, washed for 15 s in a concentrated (600 mmol L^{-1}) NaCl solution to displace loosely bound ^{22}Na, dried on filter paper, weighed on an electronic microscale (Mettler UMT2; 0.001 mg accuracy), and transferred to plastic vials. The ^{22}Na radioactivity in the whole body was then measured as described for water samples. Na^+ UR was calculated based on the incorporation of ^{22}Na in the whole body during the 1-h flux period, the mean specific activity of the ^{22}Na in the water, the animal's body weight, and the elapsed time as above. It is important to point out that there was no significant decline in the concentration of ^{22}Na in the water during the experiments. All values were expressed as mean ± one standard error of mean (SEM).

Results and Discussion

Species sensitivity to waterborne Ag toxicity changed in accordance with the whole body Na^+ UR. Acute sensitivity to silver was inversely related to Na^+ UR from the most sensitive neonate daphnids (LC_{50} = 0.26 μg of total Ag L^{-1}; Na^+ uptake = ~20 μmol g^{-1} h^{-1}) to the most tolerant adult crayfish (LC_{50} = 65.85 μg of total Ag L^{-1}; Na^+ uptake = ~0.3 μmol g^{-1} h^{-1}). Comparable log−log relationships were observed when LC_{50} values were estimated using either the mean measured total Ag concentrations or the free Ag^+ concentrations calculated by MINEQL+. Parameters for the regression between log LC_{50} values and log body Na^+ uptake are listed in Table 1. In this case, regression slopes (−1.269 for total Ag and −1.036 for Ag^+) are close to −1, which indicates that Na^+ UR does very clearly matter in terms of silver toxicity, especially when free Ag^+ toxicity is considered (Table 1 and Figure 1A).

LC_{50} values estimated based on either the mean measured total silver concentrations or the free Ag^+ concentrations calculated by MINEQL+ also varied according to the size of the animals tested, independent of the species considered. Log LC_{50} values progressively increased with log body weight over a body mass range of ~90 μg (neonate daphnids) to ~22 g (adult crayfish) (Table 1 and Figure 1B). Furthermore, in the species studied, the whole body Na^+ UR was inversely
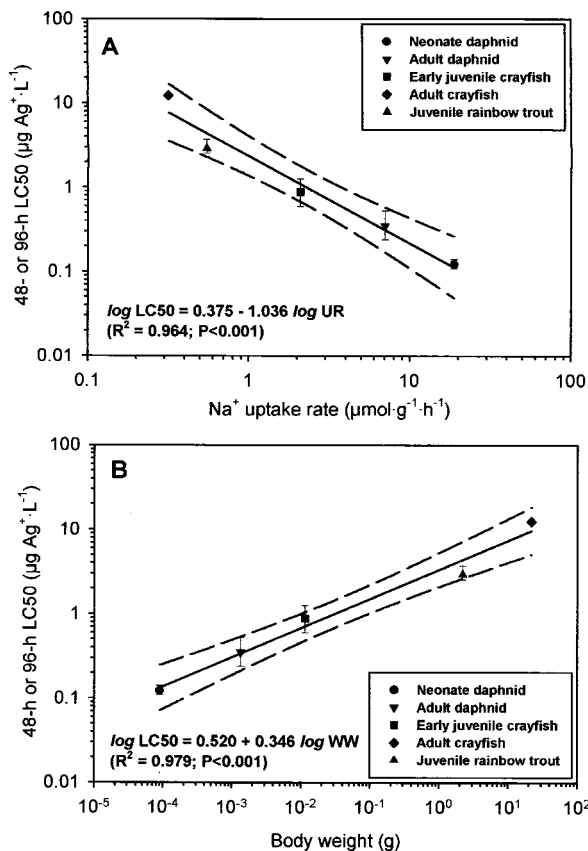
FIGURE 1. Free Ag^+ ($AgNO_3$) 48-h LC_{50} (daphnids) or 96-h LC_{50} (fish and crayfish) with corresponding 95% confidence interval as a function of the whole body Na^+ uptake rate (UR; μmol g^{-1} h^{-1}) (A) or body mass (WW; g) (B) in *Daphnia magna* (n = 30 neonates or 30 adults), the crayfish *Cambarus diogenes diogenes* (n = 15 early juveniles or 10 adults), and the juvenile rainbow trout *Oncorhynchus mykiss* (n = 10) acclimated to moderately hard water.
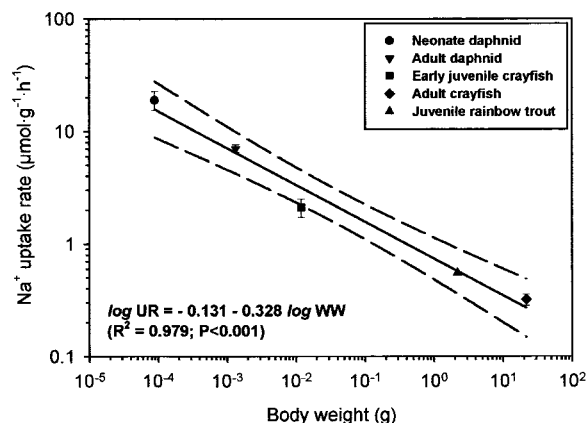
FIGURE 2. Whole body Na^+ uptake rate (UR) as a function of the wet body weight (WW) in *Daphnia magna* (n = 30 neonates or 30 adults), the crayfish *Cambarus diogenes diogenes* (n = 15 early juveniles or 10 adults), and the juvenile rainbow trout *Oncorhynchus mykiss* (n = 10) acclimated to moderately hard water. Data are means ± SEM.

related to the body mass (Figure 2). The parameters for the overall relationship between these two variables using group means for each of the test organisms (neonate daphnids, adult daphnids, early juvenile crayfish, adult crayfish, juvenile rainbow trout) are reported in Table 1. This relationship can be explained by the fact that, in both freshwater fish and crustaceans, the mass-specific gill surface area is inversely related to body mass (*15, 16*) and the gills are the main site

of Na$^+$ uptake to counterbalance the loss of Na$^+$ to the freshwater environment (11−14). Note that the slope (−0.328) obtained from the relationship between the Na$^+$ UR and body mass is very close to that (∼0.3) expected for other surface-dependent physiological process, e.g., respiration (22). Thus, as previously hypothesized, smaller animals presenting a higher mass-specific Na$^+$ turnover exhibited higher sensitivity to acute silver toxicity because a blockade of the active Na$^+$ uptake would lead to more rapid depletion of whole body Na$^+$ in animals with higher Na$^+$ turnover rates.

Taken together, the toxicological and physiological data presented in this study clearly demonstrate that in constant water quality, the whole body Na$^+$ UR can be used to accurately predict the acute sensitivity of freshwater crustacean and fish species to waterborne silver (AgNO$_3$). The predictive capacities of this parameter can be explained by the mechanism responsible for the acute silver toxicity in freshwater fish and crustaceans.

As previously mentioned, the key site of acute silver toxicity is the Na$^+$,K$^+$-ATPase located at the gill basolateral membrane in both freshwater fish and crustaceans (9, 10). In the rainbow trout, silver, probably as Ag$^+$, enters the branchial epithelial cells via the Na$^+$ channel coupled to the proton ATPase in the apical membrane (23). After some hours of exposure, silver reaches the basolateral membrane and blocks the Na$^+$,K$^+$-ATPase, the critical enzyme powering active transport of Na$^+$ and Cl$^-$ across the gills (9, 24, 25). Consequently, blood Na$^+$ and Cl$^-$ levels decline, a phenomenon that initiates a suite of direct and indirect effects that have been well-characterized in the trout. These include blood acidosis, a generalized stress response, increased blood ammonia levels, fluid volume disturbance, and haemo-concentration, which ultimately leads to circulatory collapse and death (24, 26, 27).

Despite the paucity of information regarding the precise mechanisms of silver toxicity in aquatic invertebrates, it is well-known that the gill Na$^+$,K$^+$-ATPase is also the critical enzyme powering active Na$^+$ and Cl$^-$ uptake across the gills to counteract diffusive losses to the hypo-osmotic freshwater environment in crustaceans (13, 14). Recently, we have demonstrated that the acute toxic mechanism of waterborne silver exposure in the freshwater crayfish *C. diogenes diogenes* greatly resembles that of freshwater teleost fish, i.e., Na$^+$,K$^+$-ATPase inhibition, decrease of whole body Na$^+$ uptake, and decrease of hemolymph Na$^+$ concentration (10).

So, the capacity of the whole body Na$^+$ UR to predict the sensitivity of freshwater fish and crustaceans to silver as demonstrated in the present study can be reasonably and directly linked to the physiological basis of the acute silver toxicity. Waterborne Ag$^+$ causes an ionoregulatory imbalance by inhibiting gill Na$^+$,K$^+$-ATPase activity and thereby reducing the capacity of the animal to counteract the losses of Na$^+$ and Cl$^-$ to the freshwater environment.

At least in freshwater rainbow trout, Ag$^+$ has been demonstrated to be the main cause of acute silver toxicity. Evidence for this statement comes from experiments with various complexing agents (e.g., Cl$^-$, DOC, and thiosulfate) that markedly decrease toxicity by binding the Ag$^+$, thereby reducing its availability to interact with toxic sites on the gills (2). The fact that whole body Na$^+$ UR is a slightly better predictor (higher R^2) of the sensitivity of freshwater fish and crustaceans to silver, when LC$_{50}$ is estimated based on free Ag$^+$ rather than total Ag concentrations, supports this idea.

In conclusion, the results presented here clearly demonstrate the reliability of whole body Na$^+$ UR (i.e., Na$^+$ turnover) as a predictor of acute silver toxicity to freshwater fish and crustaceans in a constant water quality. Furthermore, they suggest that, in the absence of information on Na$^+$ UR, body mass might serve as a suitable surrogate. They also suggest the possibility to include these parameters in the current version of the BLM (7) to improve the predictive capacity of this model. Clearly, more work with a wider range of species will be required for this purpose.

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