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Association mechanism between a series of rodenticide and humic acid: A frontal analysis to support the biological data

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Abstract

The binding constants (*K*) of a series of anticoagulant rodenticides with the main soil organic component, humic acid (HA), were determined using frontal analysis approach. The order of the binding constants was identical as the one obtained in a previous paper [J. Chromatogr. B 813 (2004) 295], i.e. bromadiolone > brodifacoum > difenacoum > chlorophacinone > diphacinone, confirming the power of this frontal analysis approach for the determination of binding constants. Moreover, and for the first time, the concentration of unbound rodenticide to HAs could be determined. Thanks this approach, we could clearly demonstrate that HA acid protected the human hepatoma cell line HepG2 against the cytotoxicity of all the rodenticides tested and that the toxicity of rodenticides was directly linked to the free rodenticide fraction in the medium (i.e. unbound rodenticide to HA).

Keywords: Frontal analysis; Anticoagulant rodenticides; Humic acid; Binding constant; Cells viability

1. Introduction

The anticoagulant rodenticides are used for commensal rodent control. Rodents poisoned with anticoagulants die from internal bleeding, the result of loss of blood's clotting ability and damage to the capillaries [1–4]. Since the 1980s, the involvement of the humic substances in the mobilization and the transport of hydrophobic compounds in the environment (water, soil) have been recognized [5–7]. Then, solute–humic acid (HA) complex in water and soil can cause indirectly toxic effects to humans. However, the association of pesticide to humic acid are poorly understood. In many biological models, the toxicity of the solute or the metal ions is related to the concentration of the free forms (i.e. uncomplexed) in the culture medium [8–10]. According to this theory, addition of humic acid to the cellular culture medium led to an increase of stable rodenticide humate chelates which cannot enter into cells and consequently the solute toxicity decreased [8]. However, several papers showed a solute toxicity increase with presence of HA in the culture medium [11]. Therefore, determination of the association constant of drugs on HA is important for cytotoxicity studies [8–11]. In general, hydrophobic drugs such as rodenticides are bound to HA with high affinity. Conventional methods for binding evaluation such as ultrafiltration and equilibrium dialysis are very often difficult to apply because of technical difficulties such as undesirable drug adsorption onto the membrane and leakage of bound drug through the membrane [12]. For this reason, the high performance frontal analysis (HPFA) was developed to overcome these difficulties. This technique consumes very small sample volume, does not suffer from drug adsorption

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onto the membrane, requires no special pre-treatment of the analytical system and is particularly convenient for binding analysis of hydrophobic drugs. The reliability of HPFA was further confirmed by a comparison with a conventional ultrafiltration method using several drugs such as indometacin, salycilate, acetazolamide, carbamazepine, ketoprofen and diclofenac [13–16]. In the light of previous result of our group [17], the first aim of the present study was a better understanding of the rodenticide–HA association using a frontal analysis method. The second aim was to evaluate, in relation to these physico-chemical data, the potential role of HA on the toxicity of rodenticides on human hepatocyte cell line HepG2.

2. Experimental and method

2.1. Reagents

Water was obtained from an Elgastat water purification system (Odil Talant, France) fitted with a reverse-osmosis cartridge. All the anticoagulant rodenticides were obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France). Rodenticide chemical structures are given in Fig. 1. Sodium phosphate monobasic dihydrate and sodium phosphate dibasic heptahydrate were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Humic acids are generic words and could describe various materials, according to their aquatic or terrestrial origin [18]. Thus, in this work, a commercial and standardized humic acid was used (Fluka, Saint-Quentin Fallavier, France). The HepG2 cell line was derived from a human hepatoblastoma and was obtained from Dr. J.P. Beck (Strasbourg). Foetal calf serum and culture plastic plates were, respectively, from Eurobio (Les Ulis, France) and Costar (Dutscher, France). Phosphate-buffered saline (PBS) and trypsine were from VWR International (Cergy-Pontoise, France). Unless further specified, all reagents used for biological assay were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France).

2.2. Apparatus

The HPLC system for these measurements consisted of a Merck Hitachi Pump L-7100 (Nogent sur Marne, France), a Rheodyne injection valve with a 5 mL sample loop (Montluçon, France) and a Merck L-4500 diode array detector (Nogent sur Marne, France).

The diol-silica column (Develosil 100Diol5, 100 mm × 4.6 mm) was purchased from Phenomenex (Japan). The column temperature was controlled at 37 °C with an Interchim Oven (TM no. 701).

2.3. Chromatographic operating conditions

A 6.7×10^{-3} M sodium phosphate buffer (pH 7) was used as the mobile phase without addition of an organic modifier so as not to disturb the drug–protein binding equilibrium. The solutions with rodenticide (i.e. 3, 5, 8, 10, 13 and 16 μM) and HA (i.e. 100 μM) were injected into the HPFA column after incubation of the solution at 37 °C for 4 h.

2.4. HepG2 cell culture

The HepG2 cells were routinely grown in 25 cm^2 flasks in William's medium containing 10% foetal calf serum, 2 mM glutamine and 50 μg/mL gentamycin. Cells were grown in a humidified atmosphere of 5% CO_2 in air. The medium was

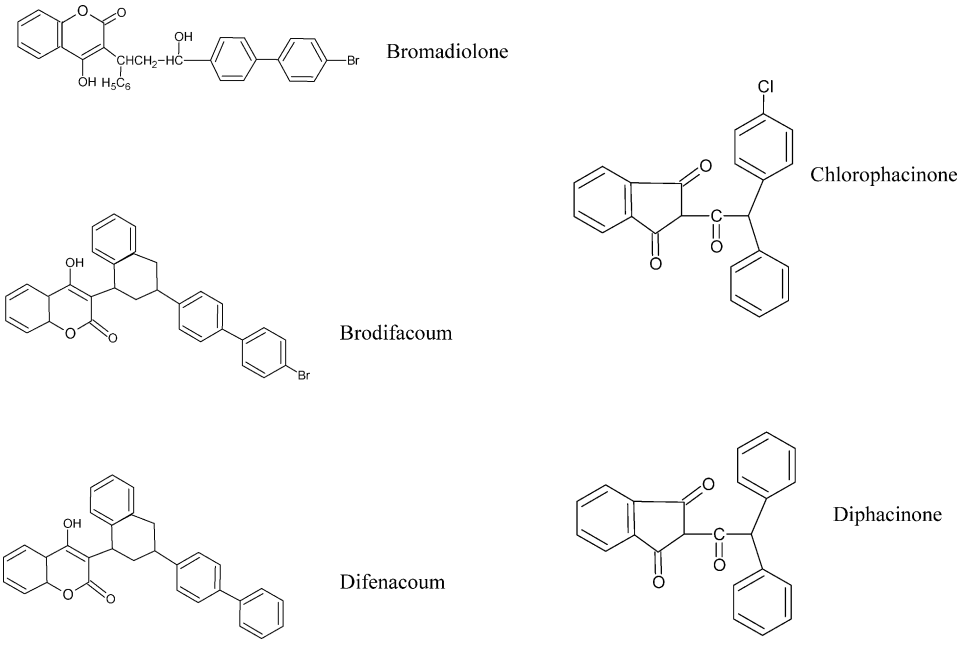

Fig. 1. Rodenticide molecular structures.

renewed every 48 h and cells subcultured every 7 days. Cells were used for experiments within 10 passages to ensure cell line stability.

2.5. Cytotoxicity assay of rodenticides

To study the effect of the various tested compounds, HepG2 cells were plated in 96-multiwell culture plates at a concentration of 0.6×10^{-4} cells/well in 100 μL medium and were allowed to attach for 24 h before treatment. Twenty-four hours after plating, the medium was discarded and fresh medium containing the rodenticide at different concentrations were added to cell cultures. Control cells were not exposed to drugs (anticoagulant rodenticide or HA). After 24 h, cell survival was assayed by measuring mitochondrial activity with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [19]. This assay is based on the reduction of the yellow tetrazolium salt MTT by the mitochondrial succinate dehydrogenase to form an insoluble formazan blue product. Only viable cells with active mitochondria reduce significant amounts of MTT. Briefly, after the 24 h treatment period, the medium was removed and the cells monolayer was washed with phosphate-buffered saline. One hundred microlitres of MTT solution (0.5 g/L in medium) was added to the culture cells for a 4 h incubation. Cell material and formazepam blue are dissolved in 100 μL of DMSO and the latter was quantified at 570 nm. Values of absorbance were converted into percentage of residual viability (Y). When possible, the concentration inducing a 50% decrease in MTT reduction (IC_{50}) was chosen as the best biological marker of cytotoxicity. The effect of each tested substance on cell viability was evaluated by carrying out at least three independent experiments with treated cell cultures.

2.6. Assay of HA protection against rodenticide cytotoxicity

First, the HA toxicity profile was evaluated by using the above-mentioned cytotoxicity assay repeated three times. Fig. 2 presents the average curve obtained, and shows that HA did not reduce HepG2 cell viability up to 250 μM. Then, the potential protective effect of HA was evaluated by co-incubating for 4 h HA at a given concentration raging from 0 to 250 μM with a given tested rodenticide at various concentrations. The comparison of the cytotoxic profiles of rodenticide in the presence and absence of HA was performed by one-way analysis of variance (ANOVA) [20]. When the ANOVA yielded a significant result ($P < 0.05$), the groups differing from one another were identified by Scheffé test [20].

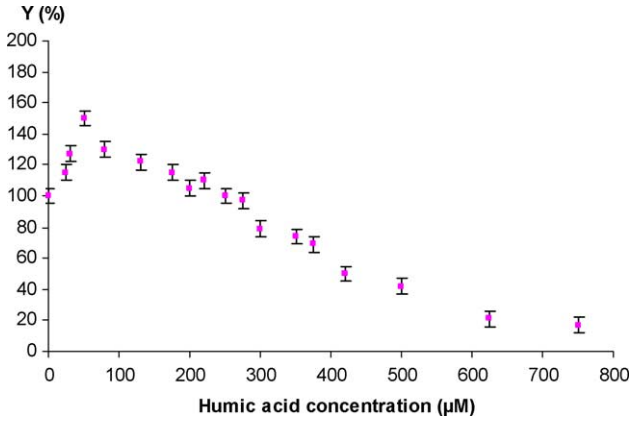

Fig. 2. Plot of the percentage of cell viability, Y (%), vs. humic acid concentration (μM) ($n = 3$).

3. Results and discussion

It is well known that when an equilibrated solution of drug–protein is injected onto a diol-silica column, drug is released from protein in the mobile phase. However, the release of bound drug is apparently suppressed by the introduction of a large volume of sample solution and an equilibrium zone is generated in the interstices of the packing material near the top of the column. In this zone, two different equilibrium states can be established simultaneously. One is the chromatographic partition equilibrium inside the micropores and the other is drug binding equilibrium in the interstices (outside the micropores). The drug concentration in the stagnant flow of mobile phase in the micropores is equal to the unbound drug concentration in the bulk mobile phase in the interstices. After the continuous sample loading is over, the protein is separated from the drug. Therefore, a zone of unbound drugs appears in the column. Finally, this zone was eluted from the column as a zonal peak having a plateau zone. The plateau region extends with increasing injection volume, but the peak height remains constant, which is dependent on the free drug concentration in the original sample solution [14].

It is necessary to prevent the diffusion of the sample solution into the injector loop because otherwise the binding equilibrium may be disturbed and the plateau region may disappear. The injector reswitching technique is useful for overcoming this problem. In this study, the 5 mL volume injector loop was fully loaded with the drug–HA solution and connected to the mobile phase for a certain period by switching the injector valve, which resulted in a sample injection of desired volume. The injector valve was then reswitched to the load position and the loop was detached from the mobile phase. Using this technique, the diffused tail of the sample was cut-off and the sample input could be regarded as an ideal rectangular shape [14].

It is also essential that the injection volume should be large enough to obtain the plateau drug zone. Therefore, the suitable injection volume was optimized in advance. It can be seen that when the injection volume is below 600 μL, rodenticide can only be eluted as ordinary HPLC peak, a zonal profile with an obvious plateau appears when injection volume is above 700 μL. Then, the injection volume of 900 μL was chosen. Further increase

Table 1
Slopes and intercepts of the calibration curves for the five rodenticides

Rodenticide	Slope	Intercept	r^2
Bromadiolone	0.2614	≈0	0.9997
Brodifacoum	0.2756	≈0	0.9992
Chlorophacinone	0.2356	≈0	0.9997
Difenacoum	0.2611	≈0	0.9991
Diphacinone	0.2245	≈0	0.9993

in the injection volume did not raise the plateau height level.

3.1. Determination of the unbound rodenticide

For quantitative determinations, calibration curves were carried out under the same chromatographic conditions as in HPFA but standard samples (i.e. rodenticides made up in phosphate buffer at different concentrations) with no HA were injected. When the injection volumes were large enough (900 μL), it was eluted as a zonal peak, and the peak height was proportional to rodenticide concentration. By plotting of peak height versus concentrations, the regression equations are obtained and given in Table 1. Then, different concentration of rodenticide (i.e. C_{tot} = 3, 5, 8, 10, 13 and 16 μM, respectively) with HA concentration maintained at 100 μM were injected to the chromatographic system. All the experiments were repeated three times and the chromatograms present similar shapes for all the rodenticides. An example was given for bromadiolone–HA association (Fig. 3). From the heights of the peak plateaus and the calibration curves, the unbound drugs could be determined (Table 2).

3.2. Determination of binding parameters

From the unbound drug concentration (C_{unb}) and the number of moles of bound drug per mole of HA (r), the binding constant (K) can be calculated by fitting the experimental data

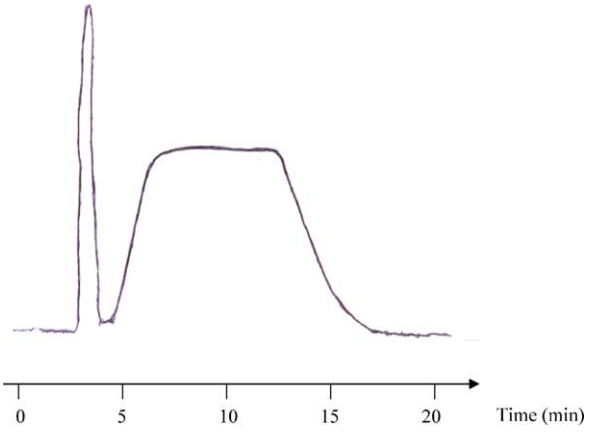

Fig. 3. Chromatogram of bromadiolone (C_{tot} = 3 μM) and HA concentration equal to 100 μM. The injection volume was in each case 900 μL.

Table 2
Determination of rodenticide unbound (C_{unb}) and bound (C_{bind}) to HA for different rodenticide total concentration (C_{tot})

Rodenticide	C_{tot} (μM)	C_{unb} (μM)	C_{bind} (μM)
Bromadiolone	3	1.234	1.766
	5	2.122	2.879
	8	3.552	4.448
	10	4.571	5.423
	13	6.201	6.799
	16	7.947	8.053
Brodifacoum	3	1.094	1.906
	5	1.870	3.130
	8	3.106	4.894
	10	3.978	6.022
	13	5.362	7.628
	16	6.837	9.163
Chlorophacinone	3	1.258	1.742
	5	2.139	2.861
	8	3.526	4.474
	10	4.494	5.506
	13	6.013	6.987
	16	7.611	8.389
Difenacoum	3	1.092	1.908
	5	1.868	3.132
	8	3.096	4.904
	10	3.958	6.041
	13	5.319	7.681
	16	6.760	9.240
Diphacinone	3	1.331	1.669
	5	2.269	2.731
	8	3.750	4.250
	10	4.789	5.211
	13	6.421	6.579
	16	8.143	7.857

Standard deviation <0.09 (n = 3).

to the Scatchard equation:

$$\frac{r}{C_{unb}} = -Kr + \theta$$

where θ is a constant.

Fig. 4 illustrates the Scatchard plots obtained for bromadiolone and chlorophacinone. For all tested rodenticides, the regression coefficients were higher than 0.976, which indicates a good agreement of the experimental data to the theoretical equation. From the slope and intercept of the Scatchard

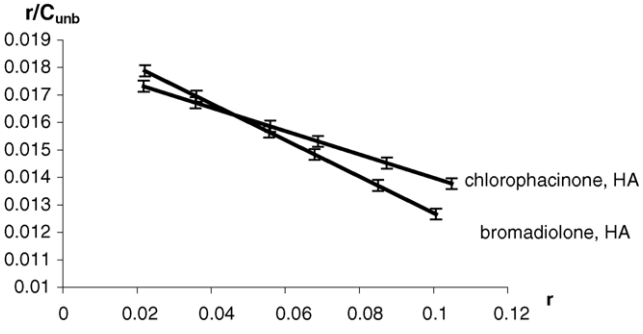

Fig. 4. Scatchard plots for bromadiolone and chlorophacinone compounds.

Table 3
Determination of rodenticide binding constants K with HA

Rodenticide	K ($\times 10^4$)
Bromadiolone	6.63
Brodifacoum	5.52
Chlorophacinone	4.26
Difenacoum	4.89
Diphacinone	4.22

Standard deviation <0.1 ($n = 3$).

plots, the rodenticide–HA binding parameters were calculated and given in Table 3. As previously observed with HA [17] and also with HSA [21], the affinity of the coumarin rodenticide class (i.e. bromadiolone, brodifacoum, difenacoum) for HA were higher than those determined for the indandione anticoagulant class (diphacinone, chlorophacinone). This confirmed the good reproducibility of this frontal analysis approach for the determination of binding constants.

3.3. Rodenticide cytotoxicity in the absence and presence of HA

First, for all anticoagulant rodenticides tested, HepG2 cell viability (Y) was plotted against concentration of test compound. Their $\overline{IC}_{50}$ values were determined (Table 4). It can be observed that the $\overline{IC}_{50}$ of the coumarin rodenticide class (i.e. bromadiolone, brodifacoum, difenacoum) were higher than the one determined for the indandione anticoagulant class (diphacinone, chlorophacinone). Diphacinone displayed the greatest toxicity toward HepG2 cells while brodifacoum did not alter the cell viability at the concentration studied. Second, the cytotoxicity profile of the five cytotoxic anticoagulant rodenticides (i.e. bromadiolone, brodifacoum, difenacoum, diphacinone, chlorophacinone) was determined in the presence of HA at various concentrations. The plots percentage of cell viability (Y) versus rodenticide concentration were drawn for different HA concentrations. As an example, Fig. 5 presents the curves obtained for the bromadiolone compound in the presence of various HA concentrations (0–250 μM). As shown in Figs. 5 and 6, a concentration-dependent protection of HA against bromadiolone cytotoxicity was obtained with IC_{50} being no more reached with 150 and 250 μM HA, displaying a complete protection against bromadiolone cytotoxicity. Fig. 6 shows a similar profile of protection of HA against chlorophacinone cytotoxicity profile. In fact, we found that for all the rodenticides tested, the addition of HA in the medium led to a decrease of the rodenticide toxicity towards the human hepatoma cell line HepG2 (data not shown). This HA protection effect was in contrast to a previous observation of our group demonstrating that the more the rodenticide associated with HA, the more the rodenticide was toxic for the human keratinocytes (HaCat) [17]. This toxicity of rodenticide in the presence of HA toward HaCat suggested that the latter cells may incorporate the rodenticide humate chelate by phagocytosis which facilitated the enzymatic lysis of the rodenticide humate chelates in the food vacuoles and the liberation of rodenticide under a free (unbound) and toxic form into the cells [17,22]. The opposite results observed in the present study when using the human hepatoma cell line HepG2 suggest that the presence of HA culture medium led to an increase of stable rodenticide humate chelates (bound rodenticide) which cannot enter into cells. To further explore this hypothesis, we used the frontal analysis to calculate for a given total rodenticide concentration added to culture medium and for HA concentration of 100 μM, the unbound rodenticide concentration (C_{unb}) present in the culture medium. For all the rodenticides tested, cells viability (Y) was plotted against calculated unbound rodenticide concentrations. The IC_{50unb} values obtained were

Table 4
$\overline{IC}_{50}$ values for four anticoagulant rodenticides on HepG2 cells

Rodenticide	$\overline{IC}_{50}$ (μM)
Bromadiolone	7.2
Brodifacoum	–
Chlorophacinone	3.8
Difenacoum	6.3
Diphacinone	2.6

Standard deviation <0.7 ($n = 3$).

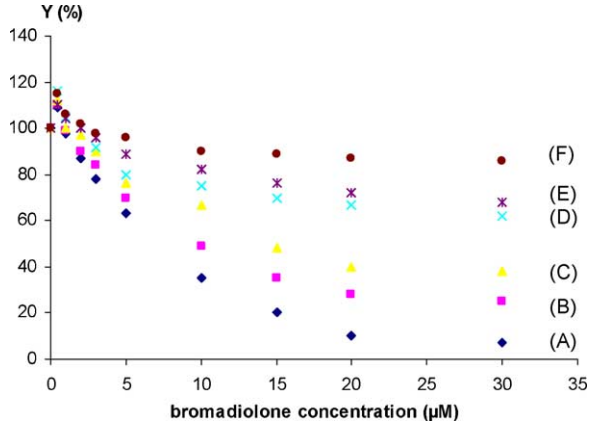

Fig. 5. Plot of the cell viability percentage, Y (%), vs. bromadiolone concentration (μM) for six HA concentrations (A = 0 μM; B = 50 μM; C = 100 μM; D = 175 μM; E = 200 μM; F = 250 μM).

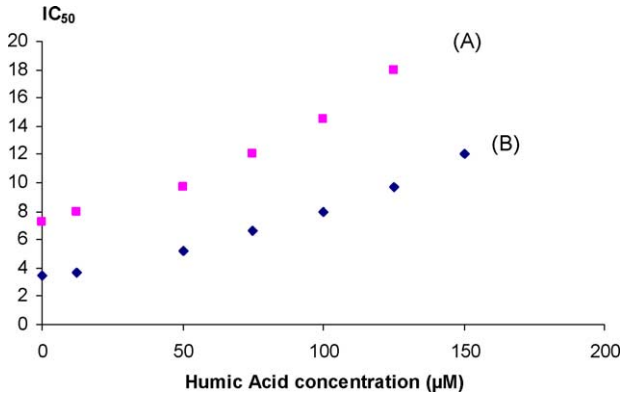

Fig. 6. Plot of the (A) bromadiolone and (B) chlorophacinone IC_{50} values vs. humic acid concentration in the medium.

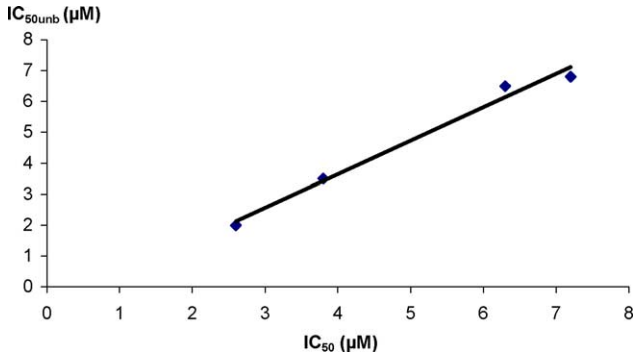

Fig. 7. Correlation between the IC_{50unb} values and experimental $\overline{IC}_{50}$ values.

equivalent to the one obtained in the absence of HA in the culture medium (see $\overline{IC}_{50}$; Table 4). The correlation between the IC_{50unb} values and experimental $\overline{IC}_{50}$ values is evident (Fig. 7). The slope (0.978; ideal is 1.00) and r^2 (0.984) indicate that the toxicity of rodenticides is directly linked to the free rodenticide fraction in the culture medium (i.e. unbound rodenticide to HA).

4. Conclusion

In this paper, frontal analysis method has been successfully applied for the rodenticide binding study with humic acid. First of all, thanks to the Scatchard plots, the association constant of rodenticide with HA can be determined at 37 °C. A comparison with previous data confirmed the power of the frontal analysis approach for the determination of binding constants. The rodenticide toxicity on human hepatocyte cell line (HepG2) was also investigated. Diphacinone displayed the greatest toxicity toward HepG2 cells while brodifacoum did not alter the cell viability at the concentration studied. Moreover, the data clearly demonstrated that rodenticide–HA association plays an important role in the hepatocyte cell protection towards toxicity of rodenticide since the rodenticides toxicity was directly linked to the free rodenticide fraction in the culture medium.

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