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Evaluation of a Greek Diatomaceous Earth for Stored Product Insect Control and Techniques That Maximize Its Insecticidal Efficacy

Georgia V. Baliota and Christos G. Athanassiou *

Laboratory of Entomology and Agricultural Zoology, Department of Agriculture, Crop Production and Rural Environment, University of Thessaly, Phytokou str., 38446 Nea Ionia, Magnesia, Greece; mpaliota@agr.uth.gr

* Correspondence: athanassiou@agr.uth.gr

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Abstract: Laboratory bioassays were conducted to evaluate the insecticidal efficacy of a diatomaceous earth deposit from Greece, for a wide range of stored product insects. In this context, populations of five different insect species, *Tribolium confusum* Jacquelin DuVal, the confused flour beetle; *Sitophilus oryzae* (L.), the rice weevil; *Rhyzopertha dominica* (F.), the lesser grain borer; *Oryzaephilus surinamensis* (L.), the sawtoothed grain beetle; *Cryptolestes ferrugineus* (Stephens), the rusty grain beetle, which cover a major spectrum of insects species of stored products worldwide, were used in the bioassays. The different treatment of diatomaceous earth (DE) rocks (grinding, diatomaceous enrichment, powder granulometry) led to the creation of five types of formulations (namely DE1, DE2, DE3, DE5 and DE6) that exhibited significant fluctuations in their insecticidal efficacy when applied on wheat. In general, some of the modified formulations were found to be very effective against species such as *R. dominica* and *T. confusum* that may be difficult to control at the current labeled doses of commercial DE formulations. Overall, our data clearly indicate that this specific Greek deposit has considerable insecticidal properties, which can be further utilized in designing commercial formulations for insect control at the postharvest stages of durable agricultural commodities, provided that the deposit will be modified at specific enrichment and granulometry levels.

Keywords: diatomaceous earth; inert dusts; natural insecticides; stored product insects; granulometry

1. Introduction

In 1703, a man looking with his simple microscope a pond-weed's root observed many pretty structures to be attached in the roots. His descriptions and diagrams are the first certain records of a diatom—a unicellular aquatic plant related to the algae [1]. Fossils of such organisms, which contain high percentages (80–93%) of silicon dioxide (SiO_2), an ingredient with insecticidal properties, formed along with other elements such as clay minerals, organic matter, quartz, calcium and magnesium carbonate [2]—the so-called diatomaceous earths (DE). Deposits of this fine white/gray material date back to the Eocene/Miocene era and are scattered world-wide.

For centuries, DE were used as insect repellents by human and animals. However, their mechanism of action was unknown, until Zacher and Kunike [3] attributed the insecticidal properties of DE as a result of dehydration and inability to replenish insect body water. Today, the mode of action of DE is generally accepted to have a desiccating effect on the insects, as diatoms have the ability to absorb the epicuticular lipids of the insect cuticle [4]. This indicates that DE toxicity primarily depends on its physical properties and much less on its chemical composition [5]. DE is safe to use with an extremely low mammalian toxicity, does not react with other substances in the environment, does not affect grain end-use quality, provides long-term protection and is comparable in cost to other methods

of grain protection [6–8]. The pressing need to reduce the use of synthetic pesticides that cause the withdrawal of several active ingredients, the occurrence of resistant populations in several conventional insecticides [9–11] and consumer concerns over insecticide residues have led the grain industry in search for potential alternative substances such as DE [5,12–14]. Drawbacks in DE use, such as their negative effect in the physical properties of grains and particularly in their bulk density [15–17] can be overshadowed with solutions such as combinations of DE with other reduced-risk methods [18–22].

There are several papers describing different factors that contribute in DE insecticide efficacy. Thanks to this extensive research, we now know that the insecticidal properties of DE mined around the world vary, depending on their geological and geographical origin [23–27]. Moreover, according to Round et al. [1], rocks that have been taken from different layers but the same locality, consist only of a selection of the species of the contributing communities that lived in variant conditions over millions of years. This, along with the fact that diatoms' morphological characteristics as well as their quota in the rock largely determine the insecticidal effectiveness of the DE powder, leads to the creation of different formulations around the world, many of which are currently commercially available for use at the postharvest stages of agricultural commodities [5,7,23,28,29].

Ideally, the DE should be a high purity amorphous silica of a uniformly small particle size that contains very little clay and less than 1% crystalline silica [7,22,30]. The DE rocks should be properly milled, the diatoms well-separated and, if possible, physically intact [7]. DE's adsorptive capacity for lipids, and its insecticidal efficacy, is also affected by the size, shape and surface topography of diatom species, uniformity of particles and the purity of the formulation [17,28]; particles with a larger available active surface and of high purity with empty holes, being as dry as possible, have a higher sorption capacity for the lipids [5,7,28]. Furthermore, DE formulations with low tapped densities (<300 g/L), good adherence to the grain (>70%), a SiO_2 content greater than 80%, a pH below 8.5 and can reduce grain bulk density by more than 2.5 kg·hl^{-1} when applied at a concentration of 50 ppm, are considered the most effective [31].

Nowadays, the commercial form of DE is prepared through a basic process of quarrying, drying and milling the mining heterogeneous rocks. When mined, the rocks contain over 50% moisture which is reduced to 3–6% after drying. Furthermore, milling reduces particle size from 0.5 to over 100 microns (μm), with the majority between 10 and 50 μm. Overall, the only changes from rock to the commercially available powder is the reduction in moisture and the adjustment of the particle size. Regarding this, a simple question emerges: is it possible from a given, inefficient DE powder, to create a new "ideal" one with high insecticidal value, by carrying out simple modifications, such as changing the diatom compositions or their diameter?

In the present study, laboratory bioassays were conducted to evaluate the insecticidal efficacy of a DE deposit from Central Greece. Following a procedure of separating the diatoms, four new, enriched and with variations in their particle size DE powders were created by the same initial deposit. Experiments were carried out to identify the insecticidal efficacy of these five DE formulations on five of the most common beetle species in stored agricultural products.

2. Materials and Methods

2.1. DE Deposit Used

The DE deposit was obtained from a single mine located in the Prefecture of Thessaly in Central Greece, in the area of Elassona (Latitude: 39.894293, Longitude: 22.184054). DE sentiments of this mine are characterized as clay diatoms, due to their high clay content, having a discoid and cylindrical structure [32]. Geochemical analysis of these DE sediments was carried out by CTL Group, Construction Technology Laboratories, Skokie, IL, USA. DE components that were qualitatively and quantitatively determined by this analytical procedure were SiO_2, Al_2O_3, Fe_2O_3, K_2O, MgO, CaO, SO_3, Na_2O, TiO_2, P_2O_5, Mn_2O_3, SrO, Cr_2O_3 and ZnO. The sentiments were found to contain 69.49% SiO_2, 13.12% Al_2O_3, 4.88% Fe_2O_3, 1.85% K_2O and 1.52% MgO; all the other elements found were below 1%. Data about

the insecticidal activity of DE deposits of the same wider area are available in the literature [23,26,27]. Regarding the formulations used in these bioassays, a basic process of milling and drying of the heterogeneous rocks firstly occurred, leading to the creation of the first DE powder formulation (DE3) with 90% of particles smaller than 20 μm and 40–60% of totally fractured diatom content (Figure 1).

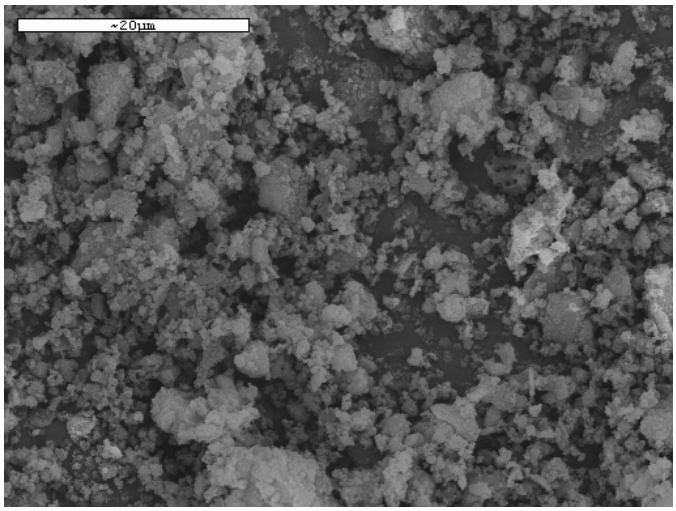
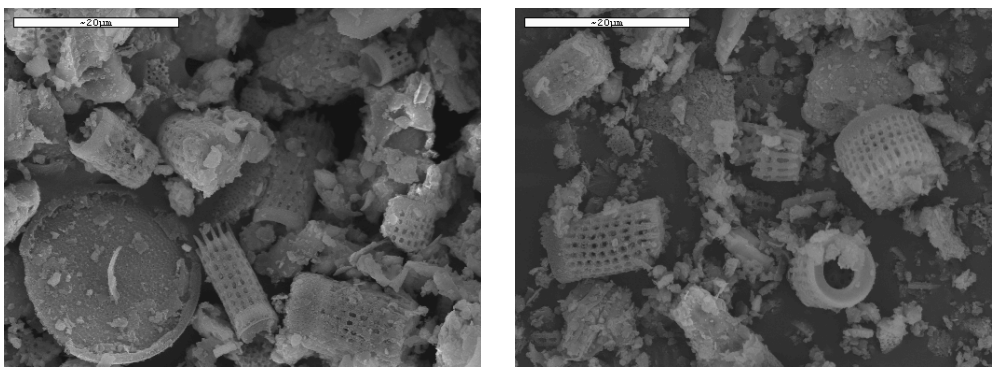


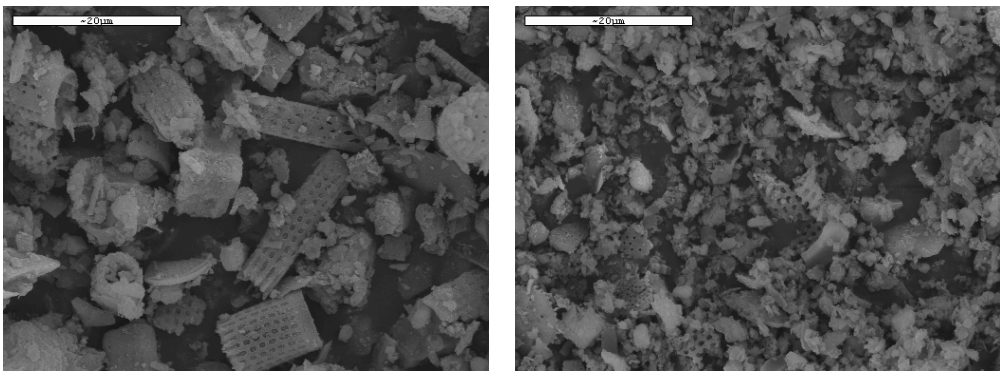
Figure 1. Scanning electron micrographs at 20 μm, showing the diatoms and other materials which constitute the DE3 formulation.

In an attempt to evaluate the magnitude of the powder granulometry, the existence of fractured or intact diatoms and their percentage in the powder in the insecticidal activity of a given DE—a process of separating the diatoms from other elements—was followed, using air separation methods. Therefore, four new DE powders, enriched in diatoms and with variations in their particle size were created. Particularly, DE1 refers to a DE powder with 76% physically intact diatom content and 20% of particles larger than 100 μm (Figure 2a); DE2 with 64% semifractured diatom content and 3.5% of particles larger than 100 μm (Figure 2b); DE5 with 70% of semifractured diatoms and 80% of particles smaller than 45 μm (Figure 2c); DE6 with 68% of totally fractured diatoms and 99% of particles smaller than 45 μm (Figure 2d). The DE formulations were stored in the laboratory at ambient conditions, until the beginning of the experiments. The examination of the formulations under SEM (visually) was conducted by the Centre of Research and Technology Hellas (CERTH), Thermi, Thessaloniki, Greece (Figures 1 and 2). X-ray powder diffraction (XRPD) measurements were carried out by Faculty of Geology and Geoenvironment, School of Science, National and Kapodistrian University of Athens, Greece.



(**a**) (**b**)

Figure 2. *Cont.*



(**c**) (**d**)

Figure 2. Scanning electron micrographs at 20 μm, showing the diatoms and other materials which constitute the formulations (**a**) DE1; (**b**) DE2; (**c**) DE5; (**d**) DE6.

2.2. Insect Species and Commodity Tested

Mixed-sex adults (7–21-d old) of *Tribolium confusum* Jacquelin DuVal (Coleoptera: Tenebrionidae), the confused flour beetle; *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), the rice weevil; *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae), the lesser grain borer; *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae), the sawtoothed grain beetle and *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae), the rusty grain beetle, were used in the bioassays. All insects were obtained from standard laboratory cultures maintained at the Laboratory of Entomology and Agricultural Zoology, Department of Agriculture, Crop Production and Rural Environment, University of Thessaly, N. Ionia, Magnesia, Greece, for more than 10 years at 25 ± 1 °C and 56 ± 5% relative humidity (RH) in continuous darkness. *Tribolium confusum* was reared in whole wheat flour, *O. surinamensis* and *C. ferrugineus* in oat flakes, and *S. oryzae* and *R. dominica* on whole soft wheat kernels. Uninfested organic soft wheat, taken from a local flour mill, was used in all tests. Prior to the initiation of the experiments, the grains were kept for over a week at −20 °C to ensure that no infestations will occur during the experiment. Later, the grains were kept in ambient conditions in order to determine the moisture content by a moisture meter (Multitest, Gode SAS, Le Catelet, France), which was found to be 13.0 ± 0.3%.

2.3. Bioassays

For each trial (species-DE formulation-DE dose), 200 g wheat kernels was placed in glass jars of 1 l capacity (15 cm diameter, 35 cm high, Bormioli Rocco, Fidenza, Italy) and treated with 200, 500 and 1000 mg kg^{-1} (ppm) of each DE. The jars were then tightly sealed with lids and thoroughly shaken by hand for 1 min to achieve a uniform distribution of the DE in the entire grain mass. Subsequently, from each jar, samples of 20 g of the treated grain was placed into cylindrical plastic vials (3 cm in diameter, 8 cm high, Rotilabo Sample tins Snap on lid, Carl Roth, Karlsruhe, Germany). For each combination, there were three samples and vials. A separate series of jars containing untreated wheat was used in the same way as control. Then, twenty adults of each species were introduced into each vial, with different series of vials per species. The whole process was repeated three times, by creating new jars of treated wheat each time. Thus, there were three replicates (jars) with three subreplicates (vials), i.e., 9 vials for each combination (species-DE type-DE dose). Insect mortality was evaluated after 7, 14 and 21 d of exposure to the treated commodity. After the 21 day exposure interval, all adults (dead and alive) were removed, and the vials were left for an additional period of 65 days, after which the vials were opened, and the number of adult progeny was counted. Bioassays were carried out under laboratory conditions at 25 °C and 56% RH in continuous darkness.

2.4. Statistical Analysis

To examine variations in mortality of the tested species among the exposure periods, the data were initially subjected to MANOVA [33] with time interval as the repeated factor, % insect mortality as the response variable and DE and Dose as the main effects. Then, all data were analyzed separately for each species according to the Repeated Measures Analysis [33]. The repeated factor was exposure interval, while the response variable was insect mortality and the main effects were DE formulation and dose. Finally, for each DE formulation and insect species, the mortality data (% of dead insects per vial) were submitted to ANOVA to determine differences among exposure intervals (7, 14 and 21 d). Means of the control groups of each species were not analyzed, as the mortality was low in all cases. For progeny production data, a one-way ANOVA was performed, separately for each species [33]. In this case, the response variable was the mean number of F1 adults per vial, while the main effects were DE formulation and dose. Means were separated using the Tukey–Kramer (HSD) test at the 0.05 level.

3. Results

For all tested species, all main effects and associated interactions were significant, with the exception of Time × DE × Dose interaction in *R. dominica* (Table 1). Moreover, at the 7, 14 and 21 d exposure intervals, all main effects and associated interactions were significant for each species (Table 2). Generally, for all five species, mortality was lower in grain treated with DE1, DE2 and DE3 in all doses and time intervals, in comparison with the respective figures of DE5 and DE6 (Tables 3–7).

Table 1. MANOVA parameters for main effects and associated interactions for mortality levels of *T. confusum*, *S. oryzae*, *R. dominica*, *O. surinamensis* and *C. ferrugineus* adults between or within variables (Error df = 120).

	Species	***T. confusum***		***S. oryzae***		***R. dominica***		***O. surinamensis***		***C. ferrugineus***	
	Effect (Source)	**F**	***p***	**F**	***p***	**F**	***p***	**F**	***p***	**F**	***p***
Between variables	Intercept	457.9	<0.01	1067.5	<0.01	351.0	<0.01	3057.5	<0.01	19,818.6	<0.01
	DE	181.2	<0.01	309.1	<0.01	17.1	<0.01	527.6	<0.01	60.7	<0.01
	Dose	98.0	<0.01	75.5	<0.01	36.8	<0.01	84.0	<0.01	50.1	<0.01
	DE * Dose	64.0	<0.01	20.4	<0.01	12.2	<0.01	6.3	<0.01	16.5	<0.01
Within variables	Time	130.5	<0.01	353.9	<0.01	147.4	<0.01	225.0	<0.01	48.0	<0.01
	Time * DE	38.9 *	<0.01	50.9 *	<0.01	5.5 *	<0.01	20.7 *	<0.01	7.7 *	<0.01
	Time * Dose	16.0 *	<0.01	6.8 *	<0.01	6.1 *	<0.01	20.3 *	<0.01	14.9 *	<0.01
	Time * DE * Dose	9.8 *	<0.01	4.0 *	<0.01	1.5 *	0.07	14.3 *	<0.01	5.1 *	<0.01

Between variables: Intercept df = 1, DE df = 4, Dose df = 2, DE * Dose df = 8; within variables: Time df = 2, Time * DE df = 8, Time * Dose df = 4, Time * DE * Dose df = 16. * Wilks' lambda approximation.

Table 2. ANOVA parameters for main effects and interactions for mortality levels for each species.

Days of Exposure		**7 d**		**14 d**		**21 d**	
Species/Effect (Source)		**F**	***p***	**F**	***p***	**F**	***p***
Tribolium confusum	DE	64.8	<0.01	110.6	<0.01	232.8	<0.01
	Dose	62.6	<0.01	63.3	<0.01	98.2	<0.01
	DE * Dose	39.6	<0.01	42.1	<0.01	65.0	<0.01
Sitophilus oryzae	DE	83.6	<0.01	165.7	<0.01	381.7	<0.01
	Dose	59.9	<0.01	49.9	<0.01	51.1	<0.01
	DE * Dose	20.2	<0.01	16.2	<0.01	11.1	<0.01
Rhyzopertha dominica	DE	7.9	<0.01	13.6	<0.01	19.6	<0.01
	Dose	25.5	<0.01	30.7	<0.01	33.9	<0.01
	DE * Dose	11.3	<0.01	10.5	<0.01	9.3	<0.01
Oryzaephilus surinamensis	DE	267.3	<0.01	579.3	<0.01	191.7	<0.01
	Dose	62.4	<0.01	18.0	<0.01	67.3	<0.01
	DE * Dose	26.7	<0.01	4.1	<0.01	9.8	<0.01
Cryptolestes ferrugineus	DE	62.3	<0.01	35.7	<0.01	35.5	<0.01
	Dose	66.5	<0.01	31.8	<0.01	13.4	<0.01
	DE * Dose	17.5	<0.01	10.5	<0.01	10.6	<0.01

For each species: DE df = 4, Dose df = 2, DE * Dose df = 8; total df = 14,120.

3.1. *Tribolium confusum*

DE1, DE2 and DE3 caused negligible mortality rates in all doses and days of exposure (Table 3). Adult mortality in treatments with DE2 was almost zero, with no significant differences among all intervals. DE6 was the most effective formulation for this species, with mortality rates to increase with increasing exposure time and dose, reaching over 88% in the highest dose (1000 ppm) at the longer exposure period (21 d).

Table 3. Mean (% ± SE) mortality of *T. confusum* adults after exposure for 7, 14 and 21 d on wheat treated with DE at three doses (means within each row followed by the same uppercase letter are not significantly different; means within each column for the same exposure followed by the same lowercase letter are not significantly different; HSD test at 0.05) *.

Dose		200 ppm	500 ppm	1000 ppm
7 d	DE1	0.5 ± 0.5 Bb	2.7 ± 0.8 BCab	5.0 ± 1.6 BCa
	DE2	0.0 ± 0.0 Ba	0.0 ± 0.0 Da	0.0 ± 0.0 Da
	DE3	0.0 ± 0.0 Ba	0.5 ± 0.5 CDa	1.1 ± 0.7 CDa
	DE5	3.8 ± 1.1 Aa	5.5 ± 1.3 Aa	6.6 ± 1.4 Ba
	DE6	3.3 ± 1.1 Ab	3.8 ± 1.3 ABb	35.0 ± 3.1 Aa
14 d	DE1	1.1 ± 0.7 Bb	3.3 ± 0.8 BCab	5.5 ± 1.5 Ca
	DE2	0.0 ± 0.0 Ba	1.1 ± 1.1 BCa	0.0 ± 0.0 Ca
	DE3	1.1 ± 0.7 Ba	0.5 ± 0.5 Ca	1.6 ± 0.8 Ca
	DE5	7.2 ± 2.2 Ab	8.8 ± 2.3 Ba	14.4 ± 1.9 Ba
	DE6	6.1 ± 2.0 Ac	22.2 ± 13.1 Ab	72.2 ± 4.6 Aa
21 d	DE1	1.1 ± 0.7 Bb	3.8 ± 0.7 Cab	5.5 ± 1.5 Ca
	DE2	0.0 ± 0.0 Ba	1.6 ± 1.1 Ca	0.0 ± 0.0 Da
	DE3	1.1 ± 0.7 Ba	0.5 ± 0.5 Ca	1.6 ± 0.8 CDa
	DE5	10.0 ± 1.6 Ab	14.4 ± 2.4 Bab	20.0 ± 1.6 Ba
	DE6	10.5 ± 2.1 Ac	35.5 ± 6.2 Ab	88.8 ± 2.6 Aa

* For ANOVA parameters between doses in the same DE formulation, in all cases df = 14,120. For 7 d: F = 50.1, $p < 0.01$; for 14 d: F = 64.7, $p < 0.01$; 21 d: F = 117.7, $p < 0.01$. For ANOVA parameters between DE in the same Dose and Day, in all cases df = 4,40. At 200 ppm: for 7 d: F = 6.1, $p < 0.01$; for 14 d: F = 5.4, $p < 0.01$; 21 d: F = 16.5, $p < 0.01$. At 500 ppm: for 7 d: F = 5.6, $p < 0.01$; for 14 d: F = 10.2, $p < 0.01$; 21 d: F = 22.7, $p < 0.01$. At 1000 ppm: for 7 d: F = 69.3, $p < 0.01$; for 14 d: F = 162.2, $p < 0.01$; 21 d: F = 559.9, $p < 0.01$.

3.2. *Sitophilus oryzae*

Significant differences were noted among the five DE formulations at all intervals (Table 4). As above, morality was extremely low for DE1, DE2 and DE3 even after 21 d of exposure. After 7 d of exposure, mortality rates for DE5 and DE6 at 200 and 500 ppm were low, with no significant differences among treatments (<16%), but increased at 1000 ppm (>30%). At longer exposure periods, mortality rates continued to rise for all DE and doses. Finally, only with the highest dose (1000 ppm) mortality exceeded 80 and 90% at DE5 and DE6, respectively (Table 4).

Table 4. Mean (% ± SE) mortality of *S. oryzae* adults after exposure for 7, 14 and 21 d on wheat treated with DE at three doses (means within each row followed by the same uppercase letter are not significantly different; means within each column for the same exposure followed by the same lowercase letter are not significantly different; HSD test at 0.05) *.

Dose		200 ppm	500 ppm	1000 ppm
7 d	DE1	0.0 ± 0.0 Ba	0.5 ± 0.5 Ca	1.6 ± 0.8 Ba
	DE2	0.0 ± 0.0 Ba	0.5 ± 0.5 Ca	1.1 ± 0.7 Ba
	DE3	1.1 ± 0.7 Ba	0.5 ± 0.5 Ca	0.5 ± 0.5 Ba
	DE5	5.0 ± 2.2 Ab	8.8 ± 1.6 Bb	33.3 ± 4.2 Aa
	DE6	8.3 ± 1.6 Ab	16.6 ± 2.3 Ab	41.1 ± 4.3 Aa
14 d	DE1	0.5 ± 0.5 Ba	1.6 ± 1.1 Ca	3.3 ± 1.6 Ba
	DE2	0.5 ± 0.5 Ba	2.2 ± 1.4 Ca	3.3 ± 1.1 Ba
	DE3	2.7 ± 1.2 Ba	2.2 ± 1.4 Ca	2.7 ± 1.2 Ba
	DE5	23.3 ± 4.2 Ab	31.1 ± 3.7 Bb	68.3 ± 4.8 Aa
	DE6	25.5 ± 4.7 Ac	48.8 ± 5.7 Ab	74.4 ± 5.3 Aa

Table 4. *Cont.*

Dose		200 ppm	500 ppm	1000 ppm
21 d	DE1	1.1 ± 1.1 Cb	2.2 ± 1.2 Bab	6.1 ± 2.0 Ba
	DE2	2.2 ± 1.6 Cb	6.6 ± 2.0 Bab	8.8 ± 2.6 Ba
	DE3	3.3 ± 1.1 Cab	2.2 ± 1.4 Bb	7.7 ± 2.5 Ba
	DE5	40.5 ± 5.0 Bc	70.5 ± 5.9 Ab	88.3 ± 2.7 Aa
	DE6	54.4 ± 4.5 Ac	72.7 ± 4.4 Ab	92.2 ± 3.4 Aa

* For ANOVA parameters between doses in the same DE formulation, in all cases df = 14,120. For 7 d: F = 44.0, $p < 0.01$; for 14 d: F = 63.7, $p < 0.01$; 21 d: F = 122.7, $p < 0.01$. For ANOVA parameters between DE in the same Dose and Day, in all cases df = 4,40. At 200 ppm: for 7 d: F = 8.2, $p < 0.01$; for 14 d: F = 19.0, $p < 0.01$; 21 d: F = 60.7, $p < 0.01$. At 500 ppm: for 7 d: F = 28.7, $p < 0.01$; for 14 d: F = 44.2, $p < 0.01$; 21 d: F = 111.4, $p < 0.01$. At 1000 ppm: for 7 d: F = 51.2, $p < 0.01$; for 14 d: F = 120.7, $p < 0.01$; 21 d: F = 280.1, $p < 0.01$.

3.3. *Rhyzopertha dominica*

Adults of *R. dominica* were the least susceptible of the tested species to the DE formulations. Mortality rates of this species was not significantly affected by the doses of DE formulation at any of the exposure periods, with the exception of DE6 (Table 5). DE6 seemed to have some insecticidal activity against this pest, but this occurred only at the highest dose and at the longest exposure interval, reaching 62%.

Table 5. Mean (% ± SE) mortality of *R. dominica* adults after exposure for 7, 14 and 21 d on wheat treated with DE at three doses (means within each row followed by the same uppercase letter are not significantly different; means within each column for the same exposure followed by the same lowercase letter are not significantly different; HSD test at 0.05) *.

Dose		200 ppm	500 ppm	1000 ppm
7 d	DE1	3.3 ± 1.1 ABa	4.3 ± 1.4 ABa	6.6 ± 2.7 Ba
	DE2	2.2 ± 0.8 ABa	7.7 ± 1.4 Aa	7.7 ± 2.2 Ba
	DE3	5.0 ± 1.6 Aa	2.2 ± 0.8 Ba	6.6 ± 2.0 Ba
	DE5	3.3 ± 1.8 ABa	4.4 ± 1.3 ABa	6.6 ± 0.8 Ba
	DE6	0.0 ± 0.0 Bb	5.5 ± 2.1 ABb	33.3 ± 5.8 Aa
14 d	DE1	5.0 ± 2.2 ABa	7.5 ± 2.5 BCa	12.2 ± 2.9 Ba
	DE2	3.8 ± 1.1 ABb	13.3 ± 2.2 ABa	15.0 ± 3.1 Ba
	DE3	6.6 ± 2.2 ABa	6.6 ± 1.6 Ca	9.4 ± 2.5 Ba
	DE5	8.3 ± 2.2 Aa	9.4 ± 1.7 BCa	12.7 ± 2.2 Ba
	DE6	2.7 ± 1.2 Bc	16.6 ± 3.2 Ab	49.4 ± 7.4 Aa
21 d	DE1	8.3 ± 2.3 Bb	16.8 ± 4.7 Bab	21.1 ± 3.8 Ba
	DE2	8.3 ± 1.1 Bb	17.2 ± 3.0 Ba	20.0 ± 2.5 Ba
	DE3	11.1 ± 1.8 ABb	11.1 ± 2.0 Bb	17.2 ± 1.8 Ba
	DE5	15.0 ± 2.6 Aa	15.0 ± 2.6 Ba	18.8 ± 2.8 Ba
	DE6	9.4 ± 2.6 ABc	30.5 ± 4.4 Ab	62.2 ± 7.2 Aa

* For ANOVA parameters between doses in the same DE formulation, in all cases df = 14,120. For 7 d: F = 12.4, $p < 0.01$; for 14 d: F = 14.3, $p < 0.01$; 21 d: F = 15.8, $p < 0.01$. For ANOVA parameters between DE in the same Dose and Day, in all cases df = 4,40. At 200 ppm: for 7 d: F = 2.0, $p = 0.11$; for 14 d: F = 1.4, $p = 0.24$; 21 d: F =1.5, $p = 0.19$. At 500 ppm: for 7 d: F = 1.8, $p = 0.14$; for 14 d: F = 3.2, $p = 0.02$; 21 d: F =4.6, $p < 0.01$. At 1000 ppm: for 7 d: F = 13.3, $p < 0.01$; for 14 d: F = 16.3, $p < 0.01$; 21 d: F = 21.5, $p < 0.01$.

3.4. *Oryzaephilus surinamensis*

This species showed the greatest variability in mortality rates among DE formulations (Table 6). Exposure for 7 d was enough to control all adults (100%) in wheat treated with 1000 ppm of DE5 or DE6. On the contrary, mortality of the same intervals ranged from 1.1 to 2.7% in the case of DE1, DE2 or DE3. With longer exposure periods, the mortality continued to rise gradually, for all DE and doses. After 21 d of exposure on wheat treated with DE6 at 500 ppm, adult mortality was complete (100%).

Table 6. Mean (% ± SE) mortality of *O. surinamensis* adults after exposure for 7, 14 and 21 d on wheat treated with DE at three doses (means within each row followed by the same uppercase letter are not significantly different; means within each column for the same exposure followed by the same lowercase letter are not significantly different; HSD test at 0.05) *.

Dose		200 ppm	500 ppm	1000 ppm
7 d	DE1	2.7 ± 1.2 Ca	2.7 ± 1.6 Ca	2.2 ± 0.8 Ba
	DE2	0.5 ± 0.5 Ca	0.5 ± 0.5 Ca	1.1 ± 0.7 Ba
	DE3	1.1 ± 1.1 Ca	0.5 ± 0.5 Ca	2.7 ± 0.8 Ba
	DE5	16.6 ± 3.3 Bc	51.1 ± 11.2 Bb	100.0 ± 0.0 Aa
	DE6	45.5 ± 9.3 Ab	96.1 ± 2.1 Aa	100.0 ± 0.0 Aa
14 d	DE1	5.5 ± 1.5 Cb	15.5 ± 4.1 Ca	11.1 ± 3.0 Cab
	DE2	2.7 ± 0.8 Ca	5.0 ± 1.6 Da	5.5 ± 1.5 Ca
	DE3	6.1 ± 2.0 Cb	2.7 ± 1.2 Db	21.1 ± 4.6 Ba
	DE5	70.5 ± 7.7 Bb	90.0 ± 3.6 Ba	100.0 ± 0.0 Aa
	DE6	90.5 ± 5.2 Ab	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa
21 d	DE1	11.6 ± 1.6 Bc	40.5 ± 6.6 Bb	67.7 ± 4.7 Ba
	DE2	6.6 ± 1.4 Bb	40.5 ± 6.9 Ba	37.2 ± 7.9 Ca
	DE3	7.7 ± 2.3 Bb	11.6 ± 4.0 Cb	61.1 ± 6.5 Ba
	DE5	85.5 ± 4.4 Ab	97.2 ± 2.0 Aa	100.0 ± 0.0 Aa
	DE6	91.6 ± 4.7 Ab	100.0 ± 0.0 Aa	100.0 ±0.0 Aa

* For ANOVA parameters between doses in the same DE formulation, in all cases df = 14,120. For 7 d: F = 100.5, $p < 0.01$; for 14 d: F = 170.4, $p < 0.01$; 21 d: F = 70.0, $p < 0.01$. For ANOVA parameters between DE in the same Dose and Day, in all cases df = 4,40. At 200 ppm: for 7 d: F = 18.1, $p < 0.01$; for 14 d: F = 93.0, $p < 0.01$; 21 d: F = 180.9, $p < 0.01$. At 500 ppm: for 7 d: F = 68.1, $p < 0.01$; for 14 d: F = 336.8, $p < 0.01$; 21 d: F = 66.8, $p < 0.01$. At 1000 ppm: for 7 d: F = 6910.5, $p < 0.01$; for 14 d: F = 341.3, $p < 0.01$; 21 d: F = 28.12, $p < 0.01$.

3.5. *Cryptolestes ferrugineus*

Although significant differences were found among the different DE and dose combinations, mortality was always high, classifying this species as the most susceptible of those tested (Table 7). Mortality ranged from 39 to 96% at 7d of exposure in the lowest dose and 100% at the highest dose for all DE formulations, with the exception of DE1. Comparing the DE formulations among all exposure intervals and at 200 and 500 ppm, DE3 always gave the lowest mortality rates. Conversely, DE5 was the formulation that controlled 100% of the adults in all doses after 14 d of exposure.

Table 7. Mean (% ± SE) mortality of *C. ferrugineus* adults after exposure for 7, 14 and 21 d on wheat treated with DE at three doses (means within each row followed by the same uppercase letter are not significantly different; means within each column for the same exposure followed by the same lowercase letter are not significantly different; HSD test at 0.05) *.

Dose		200 ppm	500 ppm	1000 ppm
7d	DE1	72.7 ± 3.8 Bb	98.3 ± 1.1 Aa	98.8 ± 1.1 Aa
	DE2	84.4 ± 7.5 ABa	93.3 ± 5.4 Aa	100.0 ± 0.0 Aa
	DE3	39.4 ± 5.6 Cb	43.8 ± 4.5 Bb	100.0 ± 0.0 Aa
	DE5	85.0 ± 2.0 ABb	98.8 ± 1.1 Aa	100.0 ± 0.0 Aa
	DE6	96.6 ± 1.4 Ab	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa
14d	DE1	83.3 ± 3.2 Bb	100.0 ± 0.0 Aa	98.8 ± 1.1 Aa
	DE2	84.4 ± 7.5 Bb	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa
	DE3	51.6 ± 8.1 Cb	66.1 ± 4.7 Bb	100.0 ± 0.0 Aa
	DE5	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa
	DE6	97.7 ± 1.2 ABb	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa
21d	DE1	95.0 ± 2.2 Ab	100.0 ± 0.0 Aa	98.8 ± 1.1 Aab
	DE2	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa
	DE3	66.1 ± 7.8 Bb	79.4 ± 3.4 Bb	100.0 ± 0.0 Aa
	DE5	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa
	DE6	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa

* For ANOVA parameters between doses in the same DE formulation, in all cases df = 14,120. For 7 d: F = 37.3, $p < 0.01$; for 14 d: F = 20.7, $p < 0.01$; 21 d: F = 18.1, $p < 0.01$. For ANOVA parameters between DE in the same Dose and Day, in all cases df = 4,40. At 200 ppm: for 7 d: F = 21.9, $p < 0.01$; for 14 d: F = 13.7, $p < 0.01$; 21 d: F = 16.3, $p < 0.01$. At 500 ppm: for 7 d: F = 54.9, $p < 0.01$; for 14 d: F = 50.4, $p < 0.01$; 21 d: F = 34.8, $p < 0.01$. At 1000 ppm: for 7 d: F = 1.0, $p = 0.41$; for 14 d: F = 1.0, $p = 0.41$; 21 d: F = 1.0, $p = 0.41$.

3.6. Progeny Production

For all tested species, all main effects and associated interactions were significant (Table 8). For the five DE formulations and the three dose rates, progeny production of the species tested is summarized in Table 9. Progeny production was practically found in all treatments for all species, with the exception of *O. surinamensis* and *C. ferrugineus*. Regarding *T. confusum*, progeny production was low in all doses, but in vials treated with DE5 and DE6, progeny production was generally lower than that of the other treatments (<2 adults per vial). No significant differences were observed in progeny production of *S. oryzae* at all intervals, with the exception of DE1, DE2 and DE3, but adult emergence decreased with the increase in dose. Given that a considerable proportion of *R. dominica* adults survived, no significant differences were observed between progeny of untreated wheat, and wheat treated with DE1 and DE2. For all the other formulations, progeny production was at lower numbers than those of the control, but usually over 100 adults per vial. No adult emergence of *O. surinamensis* was recorded at the highest dose (1000 ppm) of DE5 and DE6. Regarding *C. ferrugineus*, progeny production was recorded mostly where parental mortality did not reach 100% after 21 d of exposure. For all the other intervals, offspring emergence was negligible.

Table 8. ANOVA parameters for main effects and interactions for progeny production for each species.

Effect (Source)		F	*p*
Tribolium confusum	DE	26.9	<0.01
	Dose	81.8	<0.01
	DE * Dose	5.7	<0.01
Sitophilus oryzae	DE	19.0	<0.01
	Dose	15.6	<0.01
	DE * Dose	3.6	<0.01
Rhyzopertha dominica	DE	14.9	<0.01
	Dose	9.0	<0.01
	DE * Dose	2.1	0.01
Oryzaephilus surinamensis	DE	54.8	<0.01
	Dose	51.1	<0.01
	DE * Dose	7.3	<0.01
Cryptolestes ferrugineus	DE	39.5	<0.01
	Dose	239.4	<0.01
	DE * Dose	15.1	<0.01

For each species: DE df = 4, Dose df = 3, DE * Dose df = 12; total df = 179.

Table 9. Mean (adults per vial ± SE) progeny production of adults per species after 65 d on wheat treated with DE at three doses (within each row, means followed by the same letter are not significantly different; HSD test at 0.05) *.

Dose		0 ppm (Control)	200 ppm	500 ppm	1000 ppm
Tribolium confusum	DE1	22.5 ± 1.5 a	22.0 ± 2.9 a	10.2 ± 1.9 b	10.2 ± 1.9 b
	DE2	22.5 ± 1.5 a	11.5 ± 2.7 bc	9.6 ± 0.9 c	15.4 ± 2.1 b
	DE3	22.5 ± 1.5 a	10.2 ± 3.6 b	8.3 ± 2.6 b	15.0 ± 1.7 b
	DE5	22.5 ± 1.5 a	1.4 ± 0.4 b	1.7 ± 0.6 b	1.7 ± 0.5 b
	DE6	22.5 ± 1.5 a	0.7 ± 0.2 b	1.0 ± 0.5 b	0.0 ± 0.0 b
Sitophilus oryzae	DE1	163.8 ± 22.8 a	41.8 ± 8.6 c	123.8 ± 13.0 ab	87.1 ± 19.6 bc
	DE2	163.8 ± 22.8 a	5.8 ± 2.1 b	1.3 ± 0.4 b	27.8 ± 11.5 b
	DE3	163.8 ± 22.8 a	91.1 ± 33.8 b	43.3 ± 8.1 b	35.5 ± 9.4 b
	DE5	163.8 ± 22.8 a	144.2 ± 31.7 a	201.7 ± 22.2 a	183.8 ± 28.4 a
	DE6	163.8 ± 22.8 a	90.7 ± 14.7 a	111.5 ± 42.6 a	168.8 ± 19.8 a
Rhyzopertha dominica	DE1	231.1 ± 27.7 a	272.5 ± 24.7 a	253.2 ± 20.2 a	245.1 ± 13.6 a
	DE2	231.1 ± 27.7 a	202.2 ± 35.1 a	231.1 ± 23.0 a	181.2 ± 25.7 a
	DE3	231.1 ± 27.7 a	232.4 ± 20.8 a	203.7 ± 11.7 ab	165.3 ± 8.3 b
	DE5	231.1 ± 27.7 a	173.7 ± 26.8 ab	147.6 ± 17.9 b	151.7 ± 27.5 b
	DE6	231.1 ± 27.7 a	136.6 ± 21.0 b	94.2 ± 16.8 bc	51.3 ± 12.4 c

Table 9. *Cont.*

Dose		0 ppm (Control)	200 ppm	500 ppm	1000 ppm
Oryzaephilus surinamensis	DE1	102.0 ± 12.9 a	76.3 ± 7.9 b	55.1 ± 3.0 b	25.5 ± 4.0 c
	DE2	102.0 ± 12.9 a	110.2 ± 9.0 a	82.2 ± 6.9 a	91.0 ± 11.0 a
	DE3	102.0 ± 12.9 ab	110.6 ± 12.7 a	83.7 ± 15.3 ab	66.0 ± 8.8 b
	DE5	102.0 ± 12.9 a	3.8 ± 1.7 b	1.5 ± 0.6 b	0.0 ± 0.0 b
	DE6	102.0 ± 12.9 a	0.5 ± 0.2 b	0.0 ± 0.0 b	0.0 ± 0.0 b
Cryptolestes ferrugineus	DE1	92.3 ± 7.4 a	43.2 ± 8.2 b	0.0 ± 0.0 c	0.0 ± 0.0 c
	DE2	92.3 ± 7.4 a	0.2 ± 0.2 b	0.0 ± 0.0 b	0.0 ± 0.0 b
	DE3	92.3 ± 7.4 a	84.4 ± 13.1 a	90.0 ± 10.5 a	0.0 ± 0.0 b
	DE5	92.3 ± 7.4 a	14.4 ± 6.3 b	2.2 ± 0.8 bc	0.0 ± 0.0 c
	DE6	92.3 ± 7.4 a	3.2 ± 1.4 b	0.3 ± 0.2 b	0.4 ± 0.4 b

* For ANOVA parameters among doses in the same DE formulation in all cases df = 3,32. For DE1: *T. confusum*: $F = 10.1$, $p < 0.01$; *S. oryzae*: $F = 9.3$, $p < 0.01$; *R. dominica*: $F = 0.6$, $p = 0.61$; *O. surinamensis*: $F = 16.3$, $p < 0.01$; *C. ferrugineus*: $F = 62.8$, $p < 0.01$; for DE2: *T. confusum*: $F = 8.3$, $p < 0.01$; *S. oryzae*: $F = 35.8$, $p < 0.01$; *R. dominica*: $F = 1.1$, $p = 0.35$; *O. surinamensis*: $F = 1.4$, $p = 0.2$; *C. ferrugineus*: $F = 154.2$, $p < 0.01$; for DE3: *T. confusum*: $F = 6.1$, $p < 0.01$; *S. oryzae*: $F = 7.6$, $p < 0.01$; *R. dominica*: $F = 2.7$, $p = 0.05$; *O. surinamensis*: $F = 2.4$, $p = 0.08$; *C. ferrugineus*: $F = 23.4$, $p < 0.01$; for DE5: *T. confusum*: $F = 130.0$, $p < 0.01$; *S. oryzae*: $F = 0.8$, $p = 0.4$; *R. dominica*: $F = 2.2$, $p = 0.09$; *O. surinamensis*: $F = 58.6$, $p < 0.01$; *C. ferrugineus*: $F = 80.4$, $p < 0.01$; for DE6: *T. confusum*: $F = 170.6$, $p < 0.01$; *S. oryzae*: $F = 2.0$, $p = 0.1$; *R. dominica*: $F = 14.3$, $p < 0.01$; *O. surinamensis*: $F = 61.6$, $p < 0.01$; *C. ferrugineus*: $F = 144.4$, $p < 0.01$. We used one set of controls for all DE doses.

4. Discussion

The effect of the granulometry and the percentage of diatoms a given DE powder has and whether their modification can alter its insecticidal efficacy, was verified in this study. Formulations of DE originated from the same location, but with different particle size and diatom content, showed a variation in their insecticidal efficacy against the tested species. Chiu [34] and McLaughlin [30] were the first who suggested that the size of DE particles may play an important role in the insecticidal value of a given DE. Later, other authors added more data that confirmed this observation [2,24,27]. Through their experiments, the authors claimed that DE particles smaller than 45 µm were significantly more effective against the targeted species. On the contrary, Korunić and Ormesher [31] through their experiments, stated that a mean particle size below 15 µm and diatom shape were not correlated with insecticidal activity. It was found that DE formulations originated also from Elassona, Greece, had large particles (45–150 µm) that were in some cases more effective than smaller ones in other tested DE [27]. This indicates that the insecticidal value of a DE cannot be predicted only from its particle size but from additional factors, such as larger available active surface and oil adsorption, the diameter of inner pores of particles, moisture content, SiO_2 content and tapped density [24]. However, in any case, large particles (>150 µm) usually correspond to rocks, sand and very large diatoms, which should be removed during the formulation process, as they can reduce DE performance [24]. Hence, a first step in mined DE processing and purification is the removal of the larger particles as completely as possible. The next step should be the separation in such a way to reduce the fracture of the diatoms as little as possible and then to carefully separate the intact from the fractured diatoms—all in a cost-effective way [35]. In contrast with the large number of published studies of the insecticidal effect of DE against different pests, there are serious bibliography gaps for methods in diatom separation from other materials; therefore, more research must be carried out towards this direction. In the current study, the aforementioned separation was achieved by drying the mined material, grinding it into fine powder and using air to separate the diatoms mainly from clay and quartz. DE5 and DE6 formulations had a smaller mean particle size to DE1 and DE2, which can partially explain their higher insecticidal efficacy. DE1 was the formulation which contained mostly physically intact diatoms but had no effect on most of the tested species; DE2 and DE5 had semifractured diatoms, but only the later formulation was efficient; DE3 and DE6 contained only fractured diatoms with the later formulation to cause high mortality rates in the target species. Based on these data, we assume that the presence of fractured or no-fractured diatoms in the DE formulation has no, at least direct, effect on its insecticidal value.

A few studies have addressed why there are differences in efficacy among insect species. Ebeling [4] stated that this diversity is due to morphological (body size, quantitative and qualitative differences in epidermal lipids, presence of hair in the body) and physiological (different tolerance or resistance to dehydration, mobility, eating habits) characteristics of each species. Carlson and Ball [36], in an experiment carried out to demonstrate the susceptibility of eight stored product insect species to a commercially available DE formulation, concluded that the lined flat bark beetle, *Cryptolestes pusillus* (Schönherr) (Coleoptera: Laemophloeidae) was by far the most susceptible, in comparison with *S. oryzae, Sitophilus granarius* (L.) (Coleoptera: Curculionidae), *R. dominica, O. surinamensis,* the red flour beetle, *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) and *T. confusum*, which are given here from the most to the least susceptible. In contrast, Korunić [28], indicated that *O. surinamensis* was more susceptible than *S. oryzae, S. granarius* and *R. dominica*. In the present study, when DE1, DE2 or DE3 were applied in soft wheat, *T. confusum* was found to be the most tolerant, followed by *S. oryzae* and *R. dominica*. However, when DE5 or DE6 were used in the same intervals, *R. dominica* was the most tolerant, followed by *T. confusum* and *S. oryzae*. For all the tested DE, *C. ferrugineus* appeared to be the most susceptible of all species. In our experiments, *S. oryzae* and *R. dominica* offspring were found in all intervals, regardless of the DE and dose. Athanassiou et al. [23] reported that neither the available mined nor the commercial DEs that were tested could eliminate the progeny production capacity of *S. oryzae* and *R. dominica* in wheat, maize, rice or barley. This is somehow expectable, as in both species, the immature development occurs in the inner part of the kernel; the female *S. oryzae* oviposits directly inside the kernel, and the larva develops and completes its development in the internal kernel part, while *R. dominica* deposits its egg in clusters on grain, the newly hatched larva chews into kernel and remains inside until adulthood. Consequently, once the larvae bore into the kernel, they were not affected by agents that were applied on the external kernel part [37,38]. Hence, any differences occurred in offspring emergence in our experiments could be attributed to these differences. Moreover, we found that progeny production suppression was not always comparable with parental mortality rates, and, in some of the cases tested, low parental mortality resulted in low progeny production. This may indicate that the DE tested here were more effective in immatures than in parental adults, as in the case of *R. dominica*. In this context, the reduced offspring emergence that was noted for secondary colonizers is due to the fact that their immatures develop at the external kernel part [39–41].

5. Conclusions

In the present study, formulations originating from the same DE but with different granulometry and percentage of diatoms were evaluated for their insecticidal activity. Particle size and the percentage of diatoms in the formulation were found to be the main aspects for an effective formulation. The existence of fractured or physically intact diatoms seems to not be a contributing factor in the insecticidal value of DE. On the contrary, the biological, morphological and physiological aspects of each target insect species may play an important role in parental mortality and the subsequent progeny production capacity and population growth. Knowledge of the target species that is to be controlled should be the first step in the selection of a DE formulation, and its application rate.

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