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Low-Dose Effects: Nonmonotonic Responses for the Toxicity of a *Bacillus thuringiensis* Biocide to *Daphnia magna*

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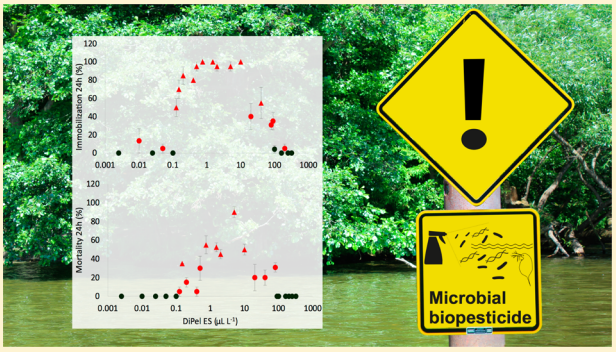
Supporting Information

ABSTRACT: Currently, there is a trend toward an increasing use of biopesticides assumed to be environmentally friendly, such as *Bacillus thuringiensis* (Bt). Studies of the Bt toxicity to nontarget organisms have reported low effects at high exposure levels, which is interpreted as indicating negligible risk to nontarget organisms. We investigated the response of the nontarget organism *Daphnia magna* to waterborne DiPel ES, a globally used Bt formulation. Neonates and adults were exposed for 48 h to a wide range of concentrations, and immobilization and mortality were monitored. Whole body biomarkers (body weight, protein, chitobiase, catalase, xenobiotic metabolism, and acetylcholinesterase) were measured in the adults. The immobilization and mortality of the neonates were affected in a nonmonotonic and inverted U-shaped pattern with EC_{50}s that were ~10^5-fold lower than those reported by the manufacturer. The immobilization of adults demonstrated a similar pattern, but significant mortality was not observed. The biomarker results revealed multiphasic dose−response curves, which suggested toxicity mechanisms that affected various physiological pathways. The main particle size in exposure media was in the size range of bacterial spores and crystal toxins. However, the chemical heterogeneity was nonmonotonic, with a change in the phase at the maximum of toxicity (~5 μL L^{-1}), which might explain the observed nonmonotonic effects. These results demonstrate the vulnerability of a nontarget organism to a biopesticide that is considered to be safe, while challenging the universal applicability of the central ecotoxicological assumption of monotonicity.



1. INTRODUCTION

Currently, there is a trend to replace conventional agrochemicals that have known adverse side effects on environmental health with biopesticides, which are considered to be environmentally friendly and safe for nontarget organisms.[1] In this context, products based on *Bacillus thuringiensis* (Bt) are globally among the leading biorational insecticides,[2] but their usage has raised some concerns regarding potentially adverse ecological effects.[3,4] Bt is a ubiquitous entomopathogenic Gram-positive, spore-forming bacterium that occurs naturally in soils, leaves, and dead insects. It synthesizes parasporal bodies with crystal endotoxins,[1] several cytolytic proteins, exotoxins, and side metabolites that act synergistically with the crystal endotoxins.[5] The commercial formulations of Bt are broadly used to control Lepidoptera, Diptera, and Coleoptera, which are vectors for human diseases as well as pests in agriculture and forestry.[6−8]

Several tests in which nontarget organisms were exposed to high levels of Bt formulations did not detect deleterious effects.[8−10] Exposure concentrations 2−5 orders of magnitude higher than those recommended for field application often resulted in negligible effects.[1] Additionally, the common mechanism of toxicity of Bt to target organism involves at least four major steps. First, the target insect ingests Bt and/or its toxins.[7] Second, enzymes activate the toxins by proteolytic processing under the alkaline conditions of the midgut.[11] Subsequently, the toxins bind to specific receptors on the gut cells.[5] Finally, the toxins insert through the cell membrane, which causes loss of ions and electrolytes that result in cytolysis and lead to organism death.[12] The requirement for this particular sequence of processes to induce toxicity in target insects has been credited as the reason for the high specificity of Bt insecticides.[5,8,10] Thus, Bt biocides and genetically modified crops that express Bt toxins are booming worldwide (Supporting Information, SI).[13−17] Bt microbial pesticides represent approximately 90% of biological control agents used in the

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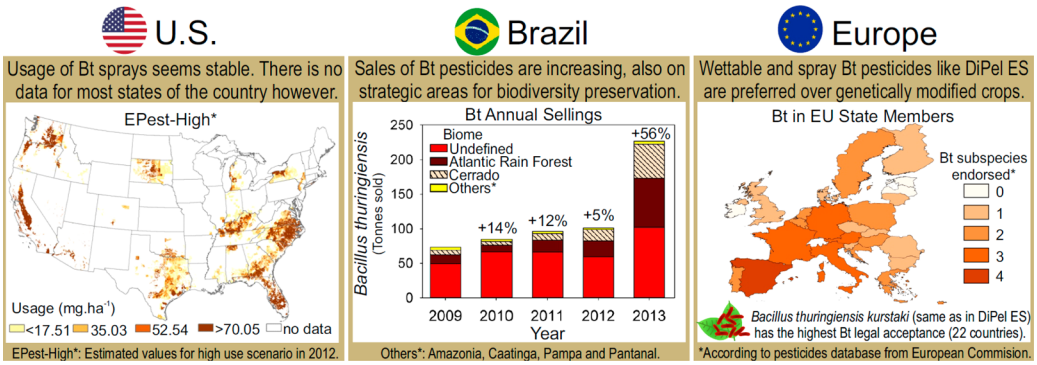

Figure 1. *Bacillus thuringiensis* (Bt) is the active compound of the most popular microbial pesticides. The U.S. data refer to all monitored Bt subspecies.[17] For the Brazilian data, the numbers from Brazilian authorities were extrapolated from the scale of states to biomes by the authors. The data for the European countries were compiled in 2015 from public databases of the European Commission.[18]

world, about 2% of the insecticides used globally, and 1% of total pesticides.[8,9]

Claimed to be natural and specific, Bt-based microbial pesticides have achieved notably broad acceptance (Figure 1). Dipel is a Bt formulation that is among the most-used biopesticides for the control of caterpillars worldwide.[10] One of its commercial forms available in Europe is DiPel ES, which is presumably a mixture of *Bacillus thuringiensis kurstaki* (Btk), its spores, crystalline endotoxins, fermentation chemicals and solids, Btk metabolites and exotoxins, and formulation substances (inert and proprietary compounds). Large amounts of Dipel have been sprayed over large areas of Europe, with potential exposure of aquatic ecosystems. For instance, DiPel ES was applied by aerial spraying to over 185 ha and by manual processes to 5500 additional individual oaks in the forest and urban areas of Frankfurt/Main (Germany) in the year of 2015. Similar management actions are performed in many other German, British and French cities (see Figure S1). However, to the best of our knowledge, no detailed studies are available that have addressed dose−response curves using environmentally relevant concentrations of DiPel ES to aquatic organisms. Thus, we investigated the lethal and sublethal responses (immobilization and biomarkers) in an aquatic model using the nontarget organism *Daphnia magna* to waterborne DiPel ES over a broad range of concentrations.

2. EXPERIMENTAL SECTION

2.1. Physico-Chemical Analyses. Physico-chemical measurements were performed in duplicate. The chemical behavior of DiPel ES in the medium that was used to expose the daphnia was analyzed in terms of chemical heterogeneity (polydispersity index) and the modes and importance of particle size distribution using light-scattering measurements (22 °C, scattering angle 173°) with the Zetasizer nano ZSP (Malvern, Worcestershire, U.K.). These measurements were not stable below 0.1 μL DiPel ES L^{-1}, therefore only higher concentrations were considered for the light scattering analysis. The carbon concentration was additionally measured with a C/N-Analyzer (TOC 5000, Shimadzu, Kyoto, Japan), which had a detection limit of 1 mg C/N L^{-1}. Basic water chemistry parameters (pH, dissolved oxygen) were also determined.

2.2. *Daphnia magna* Exposures. A *D. magna* culture originating from a female from Lake Großer Müggelsee (Berlin, Germany) has been maintained in the Ecophysiology Laboratory of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries for ∼7 years.[19] DiPel ES (Cheminova Deutschland GmbH & Co. KG; Valent BioSciences, Libertyville-U.S.), hereafter referred to as Dipel, contains the Btk ABTS 351 HD-1 and was obtained as a sample of the product that was recently sprayed over the state of Brandenburg (Germany). Neonates (<24 h old) of *D. magna* were exposed for 48 h to waterborne Dipel at 24 different Dipel concentrations (0.0025 to 320 μL L^{-1}) using 3−6 replicates, each of which consisted of 10 mL of ISO test water with ∼5 neonates (each in 20 mL glass flasks), as well as the controls according to OECD guidelines.[20] Adult females of *D. magna* (17−21 days-old) born in the same period as the neonates were exposed in the same test water to 9 Dipel concentrations (0.01 to 500 μL L^{-1}, 4−6 replicates, each consisting of 50 mL and ∼4 adults) plus controls. The adult exposure was repeated 5 months later to confirm the reproducibility of the dose−response pattern and to provide enough material for the biochemical analyses. The daphnids were evaluated after 24 and 48 h of exposure for immobilization and mortality. Animals unable to swim within 15 s after gentle agitation of the test vessel were considered to be immobilized.[20] Mortality was assumed if a complete absence of macroscopic movement was observed during the same 15 s period. Dead daphnia were an opaque white color that confirmed absence of life-sustaining functions. After exposure, the neonates were discarded, whereas each adult was quickly and gently dried in paper napkins, weighed, and preserved individually in bullet tubes at −70 °C for the subsequent biochemical analyses. Totals of 1145 daphnia (567 neonates and 578 adults) were exposed.

2.3. Biomarker Measurements. The adults from both exposures were used for biomarker analyses. The whole body of each animal was homogenized in 200 μL of cold phosphate buffer (0.1 M, pH 7.5) for 1.5 min at 18 cycles s^{-1} using TissueLyser (Qiagen-Retsch Stokcach, Germany). The biomarkers were measured in these homogenates. The number of animals used for each biomarker per treatment (N) varied according to our experience on the biomarker variance as well as to the amount of tissue required and available. The total protein in these homogenates was measured using a Bradford assay kit (N = 18−23, Sigma-Aldrich, Germany). Chitobiase activity, a biomarker for crustacean growth, was measured according to Avila et al.[21] with the modification that phosphate buffer (0.1 M, pH 7.0) was used as the reaction media (N = 10−12). Acetylcholinesterase activity was measured according to Ellman et al.[22] as a biomarker for neurotransmission (N = 4−9). Finally, catalase (N = 10−12), glutathione S-transferase (N = 4−6), and glutathione reductase (N = 5−6) activities were analyzed as biomarkers for antioxidant defense and xenobiotic metabolism.

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Catalase was measured according to Beutler,[23] while glutathione S-transferase and glutathione reductase were estimated according to Keen et al.,[24] and Carlberg and Mannervik,[25] respectively. All assays were adapted to use 96 well-microplates in which the absorbance or fluorescence was read using a Tecan plate reader (Infinite M200, Männedorf, Switzerland).

2.4. Statistical Analysis. The Trimmed Spearman-Karber method was used to estimate LC_{50} (mortality) and EC_{50} (immobilization)[26] using TSK software. This method is recommended by the Environmental Protection Agency (U.S. EPA)[26] and is among the most common methods used to estimate LC_{50} and EC_{50}. For the determination of the LC_{50} and EC_{50} values, a subset of the test concentration had to be used because the TSK method requires monotonicity and limits the number of exposure concentrations to a maximum of 10. Therefore, estimation of the LC_{50} and EC_{50} values for the neonates was based on concentrations up to 5 μL Dipel L^{-1}, whereas for adults concentrations up to 10 μL Dipel L^{-1} were chosen (see details in the SI). Selection of data was a requirement for the method and did not affect the p values presented here. Additionally, no selection of data was performed for any other statistical analyses.

Significant differences in the mortality and immobilization were detected using the Fisher test,[27] and differences in the biomarkers were detected using the Kruskal-Nemenyi test with Tukey post hoc test for the complete data set.[28] Linear correlations between the Dipel concentrations and biomarkers were also tested in the complete data set.[27] For all analyses, the significance level was 5% ($\alpha = 0.05$), and all data discussed here are available in the SI.

3. RESULTS AND DISCUSSION

Bt sprays such as Dipel are applied several times in a growing season to reach the entire larval pest population, which results in considerable amounts of total deposition.[10] Other Bt products, i.e., those based on Bti, are applied directly into water environments, which increases the risk of exposure to nontarget aquatic biota. Both Bt toxins and spores have the potential for indirect ecological side-effects[2,3] because they persist for weeks to years in lentic and lotic environments.[5,11] Nonetheless, little scientific attention has been given to the direct effects of Bt pesticides on nontarget organisms.[7]

Concomitantly, there is growing discussion regarding the relevance of nonmonotonic ecotoxicological responses.[29] Some studies have suggested that a central ecotoxicological principle, i.e., that toxicity increases monotonically with the exposure levels, might not be universally correct.[30] These authors have argued that nonmonotonicity has been generally neglected by ecotoxicology due to constraints of experimental design and lack of proper dose−response curves. Likewise, environmental agencies,[31] the European Commission and agencies,[32] and several American scientific societies[33] have expressed concern with respect to whether such currently accepted testing paradigms and government review practices are adequate.

Therefore, the present results are scientifically and socially relevant for two main reasons. First, they show the potentially high toxicity of a biopesticide that has been assumed to be safe to a relevant nontarget ecotoxicological model organism. Second, the present results report unprecedentedly unusual nonmonotonic dose−responses. In the next paragraphs, we present the results from Dipel chemical behavior in the exposure media. Next, we explore the inverse U-shaped dose−response for the organism toxicity and how it relates to Dipel behavior. Then, we address the multiphasic responses of the physiological biomarkers. Finally, we discuss the implications of these observations for environmental health regulation. Investigations of the direct or indirect effects of single components of Dipel mixture (e.g., Bt cells, Bt spores, and Bt toxins) were beyond the scope of the current study.

3.1. Particle Size and Water Chemistry. Dissolved oxygen and pH were relatively constant over the range of concentrations tested (8.71 ± 0.01 mg O_2 L^{-1} and 7.74 ± 0.01, respectively). Organic carbon could be detected but not quantified at 500 μL Dipel L^{-1}; therefore, it complied with the OECD criteria (total organic carbon <2 mg L^{-1}, total particulate solids <20 mg L^{-1})[20] in all experimental treatments.

Light scattering analyses revealed that the various Dipel concentrations generated diverse particle size distributions in the exposure media (Figure 2). The heterogeneity of the particle

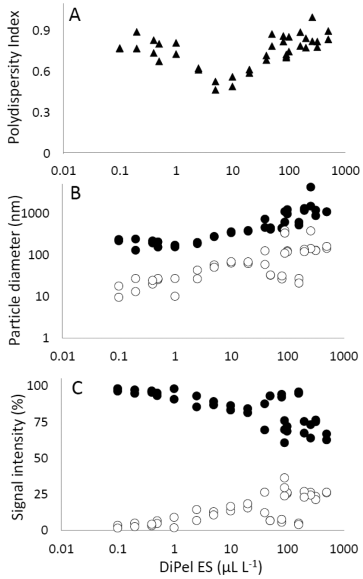

Figure 2. Dipel behavior at various concentrations (each point represents an individual measurement). A: The triangles represent the polydispersity index. B: Main mode (black-filled circles) and secondary mode (white-filled circles) of the particle sizes in the exposure media. C: Importance of main (black-filled circles) and secondary mode (white-filled circles) of particles.

sizes in the exposure media (as indicated by the polydispersity index) decreased with increasing Dipel concentrations up to ~5 μL L^{-1} (Figure 2A) and increased at concentrations above that level. Particles in the size range of bacteria spores and crystal endotoxins (~100−300 nm) predominated, whereas smaller and larger particles were observed at the lowest and highest concentrations (Figure 2B). This generated a bimodal distribution with two particle size modes in the exposure media (Figure 2C).

3.2. Inverted U-Shaped Dose−Response for Organism Level Responses. Dipel affected the immobilization and mortality of the neonates in an inverted U-shaped dose−response curve ($p < 0.001$, Figure 3). For the concentrations less than and equal to 5 μL L^{-1}, the toxicity levels were $EC_{50,24h}$ = 0.148 (0.129 − 0.171) μL Dipel L^{-1}, $EC_{50,48h}$ = 0.148 (0.130 − 0.168) μL Dipel L^{-1}, $LC_{50,24h}$ = 0.880 (0.595 − 1.302) μL Dipel L^{-1}, and $LC_{50,48h}$ = 0.286 (0.238 − 0.342) μL Dipel L^{-1}.

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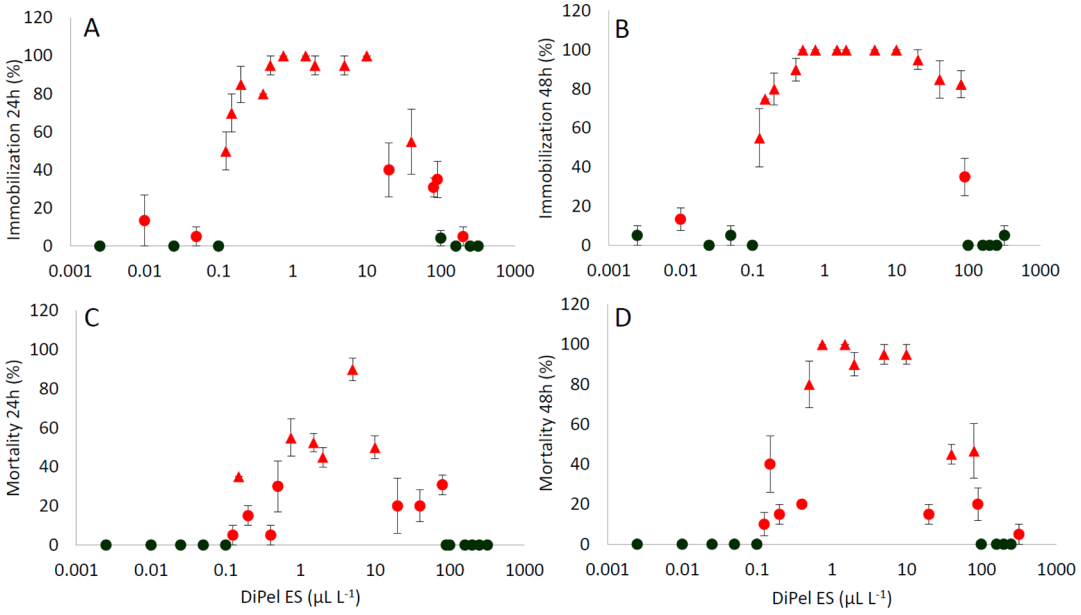

Figure 3. Organism-level toxicity of Dipel to *Daphnia magna* neonates (average ± SEM, 4 replicates per treatment, 14 replicates of the controls, $N = 5$). The circles indicate values that were not significantly different from the control. The triangles indicate values that were significantly different from the control. The red-filled symbols indicate treatments with averages higher than the control average ± SEM, and the black-filled symbols indicate treatments within the control ± SEM. A: Immobilization at 24 h of exposure (control = 3 ± 2%); B: immobilization at 48 h of exposure (control = 6 ± 4%); C: mortality at 24 h of exposure (control = 0 ± 1%); D: and mortality at 48 h of exposure (control = 0 ± 1%).

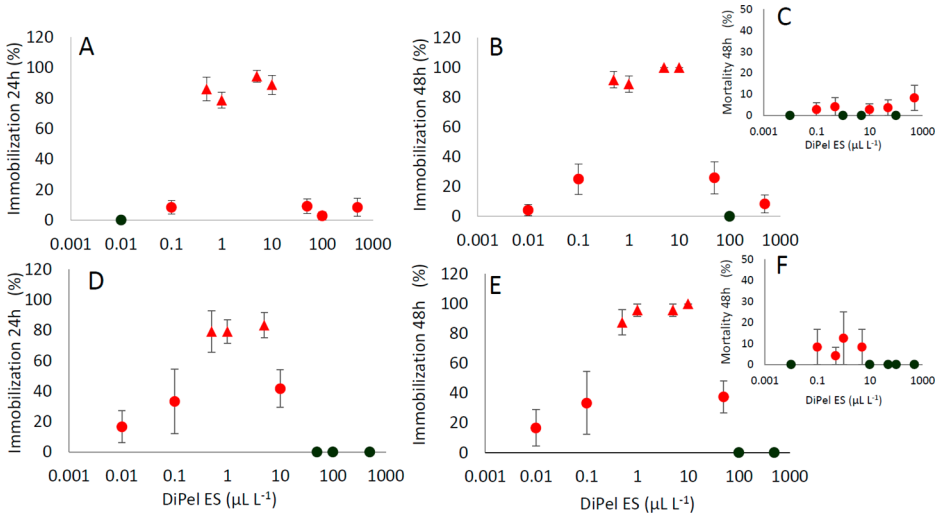

Figure 4. Organism-level toxicity of DiPel ES to *Daphnia magna* adults (average ± SEM, 7−12 replicates per treatment, 20 replicates of controls, $N = 3$). The circles indicate values that were not significantly different from the control. The triangles indicate values that were significantly different from the control. The red-filled symbols indicate treatments with averages higher than the control average ± SEM, and the black-filled symbols signify treatments within the control ± SEM. A: Immobilization at 24 h of adults exposed at the same time as neonates (control = 0 ± 0%); B: immobilization at 48 h of adults exposed at the same time as neonates (control = 0 ± 0%); C: mortality at 48 h of adults exposed at the same time as neonates (control = 0 ± 0%); D: immobilization at 24 h of adults exposed 5 months later than neonates (control = 0 ± 0%); E: immobilization at 48 h of adults exposed 5 months later than neonates (control = 0 ± 0%); and F: mortality at 48 h of adults exposed 5 months later than neonates (control = 0 ± 0%).

Immobilization of the adults was also affected by Dipel ($p < 0.001$). The EC_{50} values for the adults exposed simultaneously with the neonates were $EC_{50,24h}$ = 0.949 (0.735 − 1.225) μL Dipel L^{-1}, $EC_{50,48h}$ = 0.292 (0.194 − 0.441) μL Dipel L^{-1}. The adults exposed 5 months later demonstrated a similar response pattern ($EC_{50,24h}$ = 0.175 (0.081 − 0.378) μL Dipel L^{-1}, $EC_{50,48h}$ = 0.143 (0.076 − 0.271) μL Dipel L^{-1}) (Figure 4): the effects on mortality were nonsignificant in adults. The immobilization and mortality decreased at concentrations greater than 10 μL Dipel L^{-1} and generally disappeared at concentrations higher than 90 μL Dipel L^{-1} for adults and neonates. The acute EC_{50} values presented here are ∼10^5-fold lower than the chronic concentrations indicated by the Dipel manufacturer ($EC_{50,32days}$ = 14 mg L^{-1}). Indeed, given these results, Btk in Dipel formulation seems to be more toxic to *D. magna* than *Bacillus thuringiensis israelensis* (Bti) to the target organism *Aedes vexans*.[6]

Such inverse U-shaped toxicity at organismal level is rather unusual. It has been observed mostly for the chronic toxicity caused by carcinogenics and endocrine disruptors.[29,34] On the basis that the neonates were immobilized within a few minutes

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after exposure, a more acutely effective toxicity mechanism than genotoxicity might occur.

Bt toxins cause cytotoxicity, ionic disruption, and osmolyte loss in vertebrate and invertebrate cell cultures.[5,11,35] However, such effects remain to be demonstrated in vivo in nontarget organisms. Additionally, the pH in the digestive tract of *D. magna* ranges from 6 to 7.2, at which activation of the endotoxin crystals is unlikely.[5,11] Thus, it is possible that other Bt or Dipel-related stressors are responsible for the toxicity to *D. magna*.

In this context, the chemical heterogeneity varied as a function of the Dipel concentration in an U-shaped fashion. The particle size present throughout the exposure concentrations was 100–300 nm in diameter, in the range of the sizes of both the Bt parasporal inclusions (crystal endotoxins) and Bt spores. At the highest concentrations, additional larger particle sizes were observed. This is attributable to the higher instability in the solubility of Dipel colloids, where large aggregates could potentially encapsulate toxic compounds, which would reduce the bioavailability. Therefore, interactions between the chemical behavior at the various concentrations of Dipel and the physiology of daphnids might explain the observed non-monotonic effects.

These results challenge the idea that low toxicity at high exposure implies lower or no toxicity at lower concentrations. The current standard ecotoxicological techniques could not determine LC_{50} and EC_{50} values based on the full data set due to the clearly biphasic and inverted U-shaped response. Hence, only the low concentrations were used to determine these parameters because otherwise two EC_{50}s could be derived, i.e., when toxicity is increasing or decreasing. Similarly, multiple no-observed effect concentrations (NOEC) exist. Finally, in addition to the lowest observed effect concentration (LOEC), it is necessary to conceptualize a maximum observed effect concentration (MOEC), which in the present study was ~80 μL L^{-1}. Concentrations above MOEC yielded no detectable effects.

It is worth mentioning that without the monotonicity assumption, NOECs, LOECs, and MOECs are properties of the experimental design and not of the toxicant. In our experiments, the exposure limit was 320 μL L^{-1} for neonates and 500 μL L^{-1} for adults. Above this range, turbidity prevented observation of the organisms and classification of swimming ability. Presumably, concentrations much higher than the observed MOEC would cause further effects.

3.3. Multiphasic Dose–Responses for Physiological Responses. Effects of Dipel were observed for most biomarkers, and the differences are stronger when compared among treatments than with the controls. Dipel exposure affected the body weight and chitobiase activity of *D. magna* ($p < 0.01$, Figure 5). There was a trend for an increase in the body weight with exposures higher than 1 μL Dipel L^{-1} ($r^2 = 0.17$, $p < 0.001$). There were no significant changes in the total protein. Despite the significant effects observed for the chitobiase activity, none of the tested concentrations was different from the control, i.e., the differences were only significant among the treated groups. Feeding of the exposed organisms on Bt might explain the effects on body weight, i.e., the digestive tracts of daphnids exposed to high concentrations were filled. Indeed, *D. magna* feeds on particles from 1 to 50 μm, which include the sizes of bacteria and Bt spores. In turn, the balance between the energy obtained from food and the metabolic costs of Dipel detoxification could determine the effects on the growth biomarker chitobiase.

Catalase, glutathione S-transferase, and glutathione reductase also demonstrated nonmonotonic and multiphasic responses.

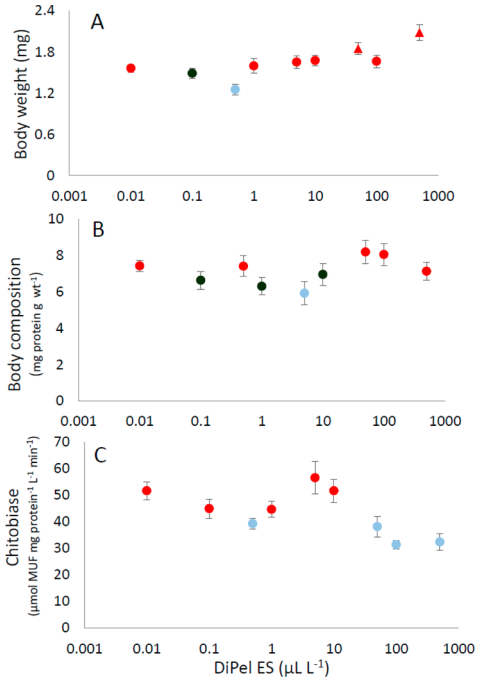

Figure 5. General health biomarkers on *Daphnia magna* adults after a 48 h Dipel exposure (average ± SEM). The circles indicate values that were not significantly different from control. The triangles indicate values that were significantly different from the control. The red-filled symbols indicate treatments with averages higher than the control average ± SEM, the blue-filled symbols indicate treatments with averages lower than the control ± SEM, and the black-filled symbols indicate treatments within the control ± SEM. A: Body weight ($N = 21 \pm 1$, control = 1.45 ± 0.08 mg); B: body composition ($N = 21 \pm 1$, control = 6.6 ± 0.5 mg protein g wt^{-1}); and C: chitobiase activity ($N = 12 \pm 0$, control = 41.82 ± 1.78 μmol MUF mg $protein^{-1}$ L^{-1} min^{-1}).

Catalase ($p < 0.01$), glutathione S-transferase ($p < 0.05$), and glutathione reductase ($p < 0.001$) were significantly affected by waterborne Dipel, but the values were not different from controls (Figure 6). Oxidative stress, glutathione metabolism, and glutathione S-transferase have sometimes been reported to be directly or indirectly involved in Bt detoxification and resistance.[35,36] Such biochemical biomarkers are known to present nonmonotonic responses.[37] Organisms facing stress activate a cascade of detoxification mechanisms. If the stressors accumulate to a certain level, then the detoxification strategies shift, i.e., some scavengers decrease while others increase.[38] Indeed, a consistent change in the biomarker responses at intermediate concentrations (1–10 μL Dipel L^{-1}: concentrations at which the organism toxicity was highest) was observed for all of the detoxification biomarkers measured (Figure 6).

Acetylcholinesterase was not significantly affected by Dipel (Figure 6). This lack of effect suggests that the immobilization was not related to the disruption of the respective neurotransmission processes. Notwithstanding this result, the generalized and diverse Dipel effects on most of the biomarkers suggest the existence of tissue-nonspecific targets and mechanisms of toxicity that are able to affect the whole organism at once.

3.4. Environmental Health Implications of Nonmonotonic Responses. The core assumption of environmental health and current regulatory toxicology is that toxicity increases monotonically with contaminant exposure. In the U.S., for instance, environmental risk assessment for chemicals is

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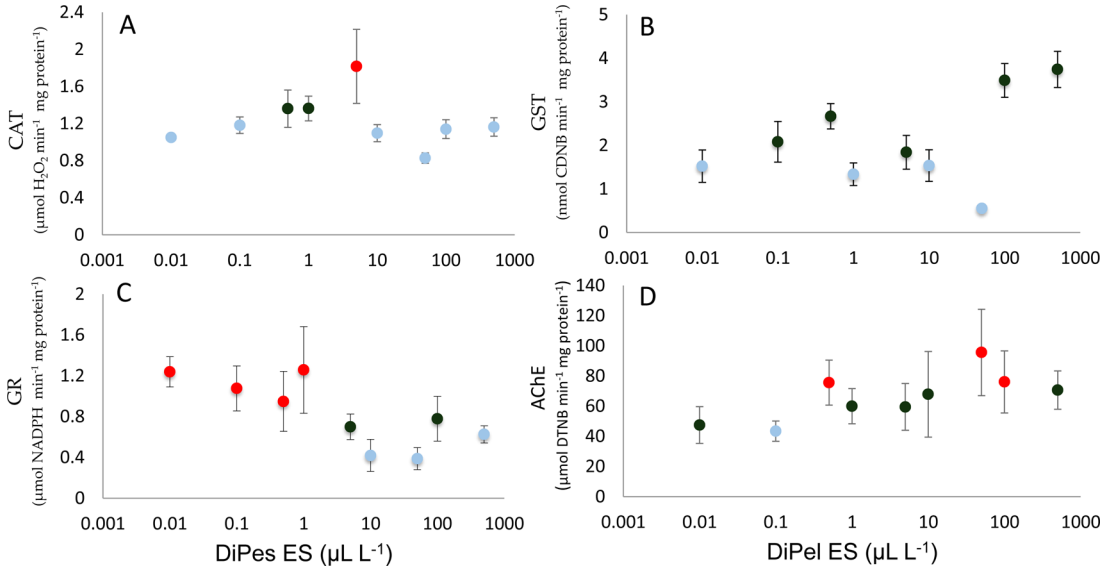

Figure 6. Biochemical biomarkers in *Daphnia magna* adults after 48 h of exposure to DiPel ES (average ± SEM). The circles indicate that the values were not significantly different from the control. The red-filled symbols indicate the results of treatments with averages higher than the control average ± SEM, the blue-filled symbols indicate treatments with averages lower than the control ± SEM, and the black-filled symbols indicate treatments within control ± SEM. A: Catalase activity ($N = 12 \pm 0$, control = 1.31 ± 0.11 μmol H_2O_2 min^{-1} mg $protein^{-1}$); B: glutathione S-transferase activity ($N = 6 \pm 0$, control = 3.19 ± 1.41 nmol CDNB min^{-1} mg $protein^{-1}$); C: glutathione reductase activity ($N = 6 \pm 0$, control = 0.77 ± 0.13 μmol NADPH min^{-1} mg $protein^{-1}$); and D: acetylcholinesterase activity ($N = 7 \pm 1$, control = 60.32 ± 13.98 μmol DTNB min^{-1} mg $protein^{-1}$).

performed in three TIERs.[1] First, organisms are exposed to high concentrations (orders of magnitude higher than expected in the environment). If relevant effects are observed, then the second TIER is to provide dose−response curves for estimation of NOECs. If concerns about toxicity at environmental levels remain, then chronic exposure of several organisms is performed (third TIER). Because most of the Bt biocides present low toxicity at high concentrations,[8] none of approximately 180 Bt biocide products registered in the U.S. have been required by U.S. EPA to undergo to TIER 2.[1] In other words, no moderate or significant hazards or risks have been detected with any Bt subspecies against any of the nontarget organisms studied,[39] and all Bt insecticides are exempted from a food tolerance requirement.[1]

Several countries have applied a similar strategy, and some integrate the U.S. EPA decisions into their considerations,[15] amplifying a sense of safety that does not hold under nonmonotonicity. Moreover, numerous studies that have reported no adverse effects were part of the registration process but are proprietary, and the data are therefore not publicly available.[8]

Nevertheless, the concepts of nontoxicity to nontarget organisms and high specificity of a Bt type have been extended to all subspecies and crops that express Bt toxins. Consequently, the low requirements have contributed to fewer tests and low registration costs of Bt pesticides. These factors have reduced the costs by approximately 40-fold,[6] and reinforce the concept that this biopesticide lacks effects. This might be misleading since nearly 25% of the studies about Bti and nontarget organisms have described impacts at environmentally relevant (field-operational) exposure levels.[11]

The results reported here also raise questions about how to regulate and biomonitor compounds for which multiple EC_{50} values for the same time and end point could be estimated. In an environmental health assessment context, pollution sources that result in concentrations higher than MOEC would be perceived as having effects only far enough away in time and/or space to dilute the Dipel to the levels at which toxicity occurs. Effects of Dipel cannot be appropriately detected or properly attributed using current biomonitoring tools that work under the monotonicity assumption.

Extrapolation of our laboratory results to the field cannot be linear because the "active compound" of Dipel is a living organism and populations might behave also nonmonotonically. Similarly, Bt produces incompletely described endotoxins,[40] exotoxins, cytolytic toxins, and a number of metabolites (phospholipases, chitinases, antibiotics, antifungals, among others) that present synergistic toxic effects.[5] Bt commercial strains have been strongly genetically manipulated to potentiate their virulence through the expression of toxicity mechanisms that are not completely understood.[10] Thus, each component of Dipel might have a different environmental fate, which could have positive or negative impacts on the toxicity.[11] To the best of our knowledge, studies that have compared the effects of two concentrations of Bti commercial formulations have found little or no impact on *Daphnia* populations in the presence of alternative food.[41−44] In fact, Duchet et al.[42] have proposed that nonmonotonic (hormetic) effects of Bti on daphnids should be investigated. Given the lack of appropriate tools to evaluate the toxicity of biopesticides,[45] more studies are required before scientifically sound predictions of the environmental toxicity can be deduced.

The results described above do not deny the usefulness of Dipel or Bt biocides. Such products have successfully contributed to control vectors of human diseases and to promote well-being and food security.[1,6,8,11,14] However, the present data demonstrate that the Bt formulation, Dipel, has the potential to cause direct lethal and sublethal toxicity to nontarget aquatic species at environmentally relevant levels. Mortality and immobilization were observed at concentrations approximately 5 orders of magnitude lower than those described by the manufacturer. Biphasic dose−responses were observed for lethal toxicity and

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immobilization, while multiphasic dose−responses were observed for the biochemical biomarkers. These observations suggest that the central ecotoxicological principle of monotonic toxicity might not be applicable to this contaminant. More scientific awareness and further studies are required to clarify the toxicity mechanisms of Dipel in *D. magna* and to determine whether the nonmonotonic effects occur in other nontarget species. Given the magnitude of the Dipel and Bt usage worldwide, the potential ecotoxicological effects described here represent, at least, a yellow warning sign to this globally applied green pesticide.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b03056.

All the data discussed in the current article (ZIP)

A figure showing the global context of Bt as a biopesticide (PDF)

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Notes

The authors declare no competing financial interest.

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