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ENVIRONMENTAL RISKS OF AN INSECTICIDE (DIMILIN® 25 WP) ON THE SHRIMP *PALAEMON ADSPERSUS:* BIOCHEMICAL COMPOSITION OF CUTICLE AND OXIDATIVE STRESS

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ABSTRACT

The present study aimed to assess, under laboratory conditions, the side effects of a trade formulation of diflubenzuron (Dimilin® 25 WP), a chitin synthesis inhibitor (CSI), on the biochemical composition of the cuticle and on the level of the biomarker of environmental stress glutathione (GSH), in a non-target species *Palaemon adspersus* (Rathke, 1837) (Crustacea, Decapoda). The insecticide was added to the shrimps' rearing water at two concentrations: 16 ng of active ingredient/L and 1 µg/L. The shrimps were exposed to Dimilin from stage A (newly molted shrimp) to Stage C (Intermolt), where mineralization and deposition of the cuticular layers are completed. The results show a significant decrease ($p < 0.05$) in the amounts of chitin in the treated batches as compared to controls for both assays. Moreover, there was a significant difference ($p< 0.05$) in the cuticular proteins between treated and control individuals. However, the compound had no significant effect on the amounts of calcium salts. Regarding GSH level, there was a significant inhibition ($p< 0.05$) in treated individuals as compared to controls. Our results show an induction of the detoxification system and confirm the mechanism of action of Dimilin on chitin biosynthesis. and indicate that this product can pose side-effects on non-target Arthropod organism like shrimps.

KEYWORDS:
Crustacea, *Palaemon adspersus*, Insecticides, Diflubenzuron, Cuticle, Chitin, Calcium salts, Glutathione.

INTRODUCTION

Human actions are one of the main factors affecting the quality of the environment and water [1]. Pollution of the aquatic ecosystem is due to human development and its side effects such as radioactive discharges, organic matter, heavy metals [2, 3], industrial and domestic discharges, and the intensive use of pesticides by farmers [4, 5]. Some pesticides are applied directly to aquatic systems to reduce numbers of mosquito larvae (larvicides) and thereby reduce transmission of pathogens that mosquitoes vector to humans and wildlife [6]. Environmental imperatives [7] have pushed research towards the use of natural pesticides or biopesticides [8, 9, 10] and prompted the phytosanitary industry to develop more selective molecules with high metabolic and environmental stability [11, 12], acting on biochemical processes specific to the target organisms [13]. These new products are the insect growth disruptors (IGDs). These include the inhibitors of chitin synthesis which interferes in the formation of the cuticle [14, 15].

Dimilin (25% WP) is a trade formulation of diflubenzuron, a benzoylurea derivative and inhibitor of the synthesis of chitin [16, 17, 18, 19], one of the major compounds of the Arthropod cuticle. It is an approved insecticide in Algeria and is used on a large scale to fight forest defoliating insects. In addition, DFB was found a potent insecticide for controlling mosquito pullulations [20]. It was also Its leaching by rains contaminates the aquatic ecosystem including the sediments in which all stages of the life cycle of a crustacean take place. This prompted to study the degradation of the insecticide (DFB) in seawater [21].

In this study, we used the shrimp *Palaemon adspersus* (Rathke, 1837), a very abundant shrimp in the Northeastern region of Algeria and is of a relatively important interest to the local fishing industry. This shrimp was used as a bioindicator species in order to assess the quality of the water from the Mellah lagoon (Northeast, Algeria) [22] and as well as a model organism to test the effects of Novaluron, another chtin synthesis inhibitor (CSI), on chitin and cuticular proteins [23]. The Crustaceans from the gulf of Annaba have been the subject of several studies on the physiological [22-25] and toxicological aspects [23, 26, 27, 28, 29]. Within this context, the current study is a continuation of the previous research conducted by [23] who determined the amount of chitin and cuticular proteins of the same species when exposed to another molecule (Novaluron). This research adds to the latter in that it shows

the amount of calcium salts content of the cuticle and also measures the level of glutathione (GSH), a biomarker that plays a central role in intracellular antioxidant defense processes.

MATERIALS AND METHODS

Shrimp collection. *P. adspersus* (Decapoda, Palaemonidae) is a common shrimp in Norwegian coast and South of the Baltic Sea to the Mediterranean. This species is transparent, fairly uniform with yellow strips at the level of articulations and presents dark chromatophores on the ventral half of the rostrum. The shrimps (length: 8-10 cm; weight: 12-16 g) were collected from the lagoon of El Mellah (Northeast, Algeria) and transported to the laboratory. They were reared in aquariums (100 × 60 cm) filled with sea water under laboratory conditions (salinity 37 psu; temperature 24-27°C; photoperiod 14h light). They were daily fed with fresh mussels administered in the afternoon.

Shrimp datation. In crustaceans, the molt cycle, corresponding to the modifications that occur between two successive molts, is subdivided into five stages: A (early post-molt), B (late post-molt), C (intermolt) and D (pre-molt) and molting (E). Molt stages were determined according to [30] by microscopic examination of the uropod setae. Under these conditions, *P. adspersus* has a molt cycle of 20 ± 2 days, and the relative duration of each stage is 9% for A+B, 36% for C, and 59% for D.

Insecticide and treatment. Dimilin® (Wettable powder, 25% active ingredient, a.i.), a trade formulation of diflubenzuron, is an insecticide belonging to the benzoylphenylurea derivatives. It was kindly provided by Pr. G. Smagghe (Ghent University, Belgium). Dimilin was added to the rearing seawater at a final concentration of 1 µg active ingredient/L according to concentrations tested on several shrimp species [27, 28] and 16 ng/L corresponding to the LC_{50} determined against fourth instar larvae of the most abundant mosquito species *Culex pipiens* [20]. Newly molted adult shrimps (0-8 h old) were continuously exposed to treatment up to intermolt. Control shrimps were reared in seawater alone.

Biochemical procedure. Shrimps were sampled at the beginning of the inter-molt and killed by freezing. The entire carapace was removed and the cuticle was separated from adhering tissue, washed and dried at 60°C to constant weight and decalcified by means of 10% trichloroacid acetic (TCA) according to [29]. The decalcified cuticle was extracted in 2 N NaOH at 110°C for 3-4 h. The colorless residues were washed in distilled water and dried at 60°C to constant weight. The weight loss was supposed to be due to removal of proteins, and the residue to be chitin.

Glutathione assay. The glutathione was determined according to the method of [32] previously described [33]. The flesh of the shrimp was homogenized in 1 ml EDTA solution (0.02M; pH 6) and subjected to a deproteinisation with sulfosalysilic acid (SSA) at 0.025%. Then the homogenates were maintained for 15 min on ice and centrifuged (3000g, 15 min). The mixture was vortexed, left for 15 minutes in an ice bath and centrifuged (1000 rpm/min for 5 min). The supernatant (0.5 mL) was taken and then 1 mL of Tris-EDTA buffer (0.02 M, pH 9.6) and 0.025 mL of DTNB (0.01 M) were added. The mixture was then left for 5 min at room temperature. The optical density was measured at a wavelength of 412 nm after 5 min. GSH levels are expressed in µM /mg proteins.

Protein assay. The protein assay was prepared according to the method of [34] described previously [27]. This uses the Coomassie brilliant blue (BBC) G 250 as a reagent and a solution of bovine serum albumin (BSA) as a standard. The absorbance was read by the spectrophotometer at a wavelength of 595 nm.

FIGURE 1
***Palaemon. adspersus* (Crustacea, Decapoda).**

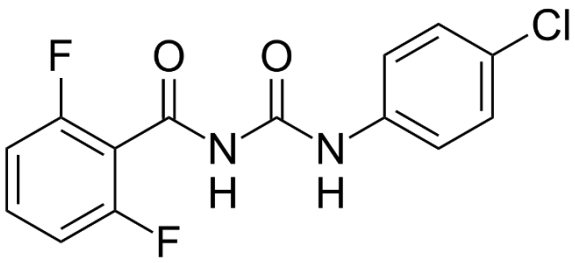

FIGURE 2
Molecular structure of diflubenzuron.

TABLE 1
Effect of Dimilin (1µg/L and 16ng/L) on cuticle chitin levels (%) of *P. adspersus* at intermolt stage in control and treated series (mean ± SD, n = 4-5).

Treatment	Control	DFB 16 ng/L	DFB 1 µg/L	p
Level (%)	58.46 ± 13.41 a	45.76 ± 8.47 b	30.68 ± 10.55 b	0.003

Values followed by a different letter are significantly different ($p < 0.05$).

TABLE 2
Effect of Dimilin (1µg/L and 16ng/L) on cuticle protein levels (%) of *P. adspersus* at intermolt stage in control and treated series (mean ± SD, n = 4-5).

Treatment	Control	DFB 16 ng/L	DFB 1 µg/L	p
Level (%)	41.54 ± 13.41 a	69.31 ± 10.55 b	69.17 ± 7.26 b	0.001

Values followed by a different letter are significantly different ($p < 0.05$).

TABLE 3
Effect of Dimilin (1µg/L and 16ng/L) on cuticle calcium salt levels (%) of *P. adspersus* at intermolt stage in control and treated series (mean ± SD, n = 4-5).

Treatment	Control	DFB 16 ng/L	DFB 1 µg/L	p
Level (%)	42.26 ± 6.21 a	49.40 ± 13.04 a	30.83 ± 7.26 a	0.065

Values followed by a different letter are significantly different ($p < 0.05$).

TABLE 4
Effect of Dimilin (1µg/L and 16ng/L) on GSH amounts (µM/mg protein) in the flesh of *P. adspersus* atintermolt stage in control and treated series (mean ± SD, n = 4-5).

Treatment	Control	DFB 16 ng/L	DFB 1 µg/L	P
Amounts	0.14 ± 0.02 a	0.13 ± 0.01a	0.09 ± 0.04 a	0.026

Values followed by a different letter are significantly different ($p < 0.05$).

Statistical analysis. Data are expressed as mean ± standard deviation (m ± SD) and were subjected to one-way analysis of variance (ANOVA) followed by Tukey test. All statistical analyses were performed using Minitab software (version 16, PA, State College, USA). With $p < 0.05$ considered as a statistically significant difference.

RESULTS

Effect on chitin levels. The amount of cuticular chitin noted in the control series was 58.46 ± 13.41%. The treatment with Dimilin led to a significant decrease ($p = 0.003$) in the chitin level (Table 1). The lowest level was observed in individuals exposed to a dose of 1 µg/L (30.68 ± 10.55%) compared with the dose of 16 ng/L (45.76 ± 8, 47%).

Effect on protein levels. Under normal conditions, the protein level at the intermolt is (41.54 ± 13.41%). In the treated series, there was a significant increase ($p \leq 0.001$) in the level of proteins for both doses: 69.31 ± 10.55% for the dose of 16 ng/L, and 69.17 ± 7.26% for the dose of 1 µg/L (Table 2).

Effect on calcium salt levels. The result for the calcium salt level in controls was 42.26 ± 6.21%. As for the treated series, it was noted that the level of calcium salts did not vary significantly ($p = 0.065$) compared to the levels in the control series (Table 3).

Effect on glutathione amounts. For the treated series, the GSH amounts recorded in stage C decreased significantly (0.13 ± 0.01 µM/mg proteins for the dose of 16 ng/L and 0.09 ± 0.04 for the dose of 1 µg/L) compared to the control series (0.14 ± 0.02 µM/mg proteins) (Table 4). The comparison of the mean values in the control and treated series shows that there was a significant difference in the GSH level ($p < 0.05$).

DISCUSSION

Crustaceans have an exoskeleton or cuticle which acts as a selective barrier between the internal and external environments [35, 36]. This non-extensible exoskeleton is periodically disposed of and replaced by a new cuticle during molting. The cuticle is a complex macromolecular structure. It is mainly composed of chitin fibers to which proteins bind.

These fibres are in turn associated to a protein matrix [37] and impregnated with limestone [38].

Regarding the effect of Dimilin on the amount of chitin at the intermolt, the compound was found to significantly reduce the level of chitin in the treated series as compared to controls. Our findings are consistent with those commonly reported and which assert that benzoylphenylurea derivatives interfere with the molting process by disrupting cuticular secretion via chitin synthesis [27, 39, 40, 41]. According to [42], diflubenzuron interferes with enzymes that contribute to the synthesis of chitin in crustacean species such as the benthic copepod *Tisbe battagliai*. Our results confirm previous studies using the same product on another shrimp species, *Penaeus kerathurus* [27]. It was also reported that Novaluron, another inhibitor of chitin synthesis, caused a decrease in the level of cuticular chitin of *P. adspersus* [23].

The proteins play a fundamental role within the organism of all living biological species [43]. Our results show a significant increase in their levels in the treated series compared to the control series. This is reflected in the composition of the cuticle, which consists of chitin which binds to proteins. Thus, there is a strong correlation between the two components [44]. Similar results have been reported by [26] and concluded that treatment with diflubenzuron causes an increase in the level of cuticular proteins in *P. kerathurus*. The same effect was observed in the treatment of *P. adspersus* with Novaluron [23].

Finally, regarding the effect of this xenobiotic on the calcium salt content, it is noted that Dimilin has no effect on the calcium salt levels. The same results were obtained in *P. kerathurus* following treatment with Dimilin [26]. Similarly, the work of [22] states that calcium salts in *P. adspersus* are not affected.

During the environmental stress caused by the presence of pollutants, biochemical responses are immediate in exposed organisms [45]. Little work has been done on the detoxification system in crustaceans. GSH is considered as one of the most important antioxidant agents involved in the protection of cell membranes against free radicals damage [46, 47, 48]. So in the treated series, our experiments show that Dimilin causes a decrease in GSH amounts measured at the intermolt (Stage C). In the work of [49], it was noted that the exposition of marbled crayfish (*Procambarus fallax f. virginalis*) to Cyperkill 25 EC (Pyrethroid insecticide) in concentration of 0.05 µg/L caused significant increase in GSH amounts ($p < 0.05$). Other research [50] shows that in shrimp *Palaemonetes pugio* and after 96-h exposure to phenothrin at five concentrations, the sublethal biomarkers glutathione were examined and there were no statistically significant differences in these levels in adults or larvae compared to controls.

CONCLUSION

Our experiments assessed the impacts of an inhibitor of chitin synthesis, Dimilin, on a non-target aquatic Arthropod *P. adspersus*. The obtained results confirm its mechanism of action on chitin biosynthesis. This compound can show secondary effects even at low doses on this edible shrimp species.

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