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## Impact of insect growth disruptor, novaluron, on biochemical composition of cuticle from the shrimp *Palaemon adspersus*

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#### Abstract

Le novaluron (20% Wettable Powder), is a recently developed benzoylurea insecticide which is considered as chitin synthesis inhibitor with excellent activity against mosquito larvae. The current study aimed to evaluate under laboratory conditions the potential side-effects of novaluron on non-target shrimp, *Palaemon adspersus* Rathke, 1837 (Decapoda, Palaemonidae). The compound was tested at two concentrations (0.91 µg/L and 4.30 µg/L) corresponding respectively to LC<sub>50</sub> and LC<sub>90</sub> determined against fourth-instar larvae of *Culiseta longiareolata* (Diptera, Culicidae). Novaluron was added to the rearing seawater of newly-ecdysed adult shrimps of *P. adspersus* and exposed for 15 days, i.e. until stage D during the molting cycle. The weight of cuticles and their biochemical composition (chitin, proteins) were measured in peripheral integument by a gravimetric method. Under normal conditions, the weight of cuticle increase significantly during the period from the stage A until the stage D. Novaluron-treatment was found to reduce the weight of cuticle at the stage B, C and D. The chitin percentage, increase from stage A to stage C followed by a decrease at stage D. Exposition of shrimps to novaluron resulted in a significant decrease of values at all molting stages with a dose-dependent manner. Lastly, the percentage of cuticular protein showed a decrease from stage B to stage D in controls. Novaluron can cause a significant increase in cuticular proteins. Therefore, the overall data confirm the primary mode of action of novaluron to inhibit chitin biosynthesis.

**Keywords:** Cuticle, chitin, protein, Novaluron, *Palaemon adspersus*.

#### Introduction

The use of pesticides in agriculture is the most widespread method for pest control [1]. However, the intensive utilisation of neurotoxin insecticides becomes environmentally hostile and ecologically hazardous [2]. So, in this context, search for new insect-selective insecticides with minimal ecotoxicological risks is relevant. Insect growth disruptors (IGDs) seem promising because of their specific mode of action on insect and their lower toxicity against non-target organisms than conventional insecticides [3, 4]. On the basis of the mode of action, these compounds can be grouped on the substances that interfere with the action of insect hormones (i.e. juvenile hormones, ecdysteroids) and chitin synthesis inhibitors (i.e. of cuticle formation). The prototype of a benzoylurea insecticide is diflubenzuron, which was developed as a commercial insecticide in the early 1970s. In the following years, many derivatives with insecticidal activities enhanced have been successfully established such as triflumuron [5], chlorfluazuron [6], teflubenzuron [7], hexaflumuron [8], flufenoxuron [9], lufenuron [10] and more recently, novaluron [11]. Novaluron is a new derivative of the benzoylurea class which is considered as a chitin synthesis inhibitors [12], with excellent activity against several important insect pests with a high toxicity level and effectiveness against several mosquito larvae as, *Culiseta longiareolata*, [13] *Aedes aegypti* [14] and *Culex pipiens* [15]. The US Environmental Protection Agency (EPA) and Canadian Pest Management Regulatory Agency (PMRA) designed novaluron a "reduced-risk/organophosphorus alternative" as it exhibit low acute mammalian toxicity and no significant subchronic effects in mammals [16-19]. According to these agencies, novaluron was considered a low risk to the environment and non target organisms. Use of novaluron can contaminate rivers which diverse their pollutants into the lakes of El- kala and the Annaba gulf (Northeast Algeria). Thus, in the present study, we examine the impact of novaluron on a non-target organism, shrimp *Palaemon adspersus* Rathke, 1837 (Decapoda, Palaemonidae) abundant in the lagoon El-Mellah (Northeast Algeria) and a relatively important species for the

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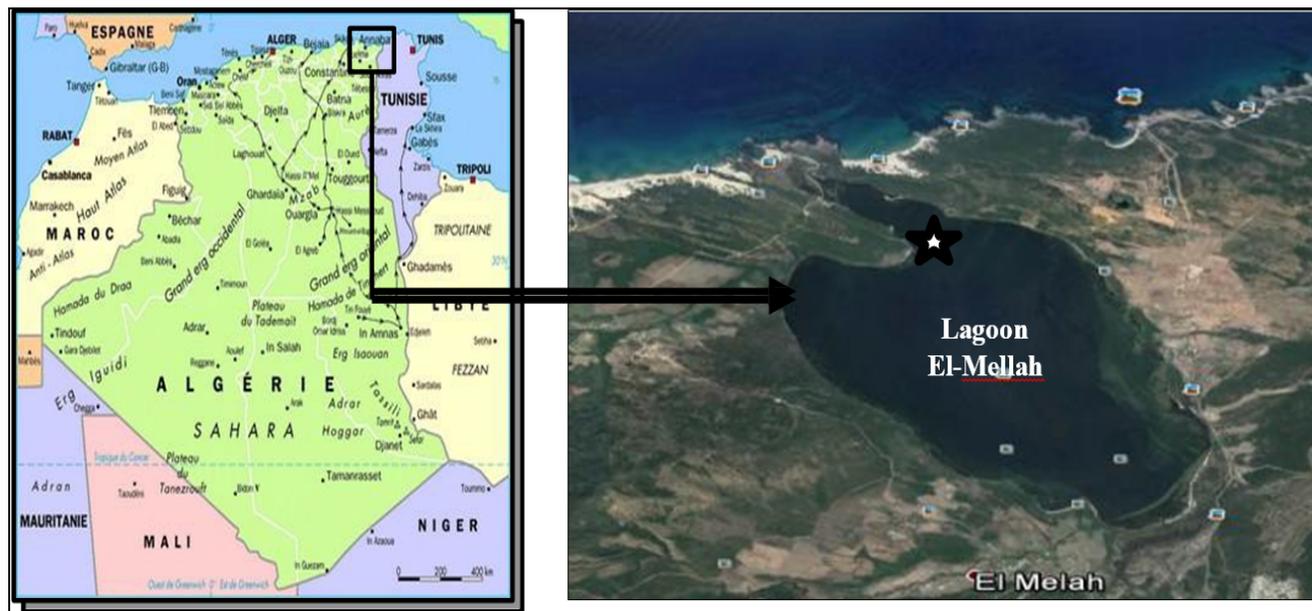
local fishery industry. Novaluron was added to the rearing seawater of newly-ecdysed adult shrimps during a molt cycle. The effects of this compound were examined on the weight and biochemical composition of cuticle (chitin, proteins).

## Materials and Methods

### Collection and rearing of shrimps

*P. adspersus* Rathke, 1837 (Decapoda, Palaemonidae) were collected from the lagoon El-Mellah (Northeast Algeria), in the constriction zone of the channel that leads to the Mediterranean Sea (Fig. 1). This site is far from any source of

pollution and expected as a relatively clean site away from pollution sources [20, 21]. Shrimps were transported to the laboratory alive and reared in laboratory conditions by maintaining them in glass aquaria (100 x 60 x 80 cm) filled with sea water (salinity 37 psu; temperature 22-25 °C; photoperiod 14 h of light). Filtration is performed by water filter having a flow rate of 180 l/h (Rena 225). The animals were daily fed with fresh mussels distributed in the afternoon. Prior to exposure, shrimps were acclimated to laboratory conditions for a week. Shrimps with of similar size (length: 25 mm and weight: 850 mg) were used in the experiment.



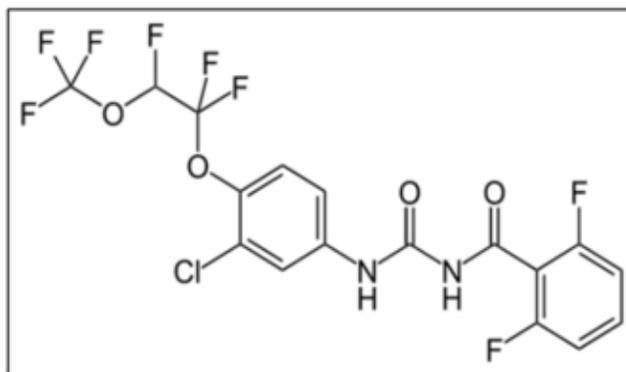
**Fig 1:** Geographical location of the sampling sites of lagoon El Mellah, El-Kala (Northeast Algeria). \*: constriction zone of the channel with Mediterranean, (www.Google Earth.com).

### Shrimp datation

*P. adspersus* is a Decapod Palemonides a common shrimp in Norwegian coast and South of the Baltic Sea to the Mediterranean. This species is clear with no lines or bands, excepting presence of dark chromatophores on the half ventral rostrum. Shrimps were fished by the seine net (mesh: 4 mm; length: 1.80 m). The moult cycle is divided into five stages: A (early postmolt), B (late postmolt), C (intermolt) and D (pre-molt) and moulting (E). The datation was made according to the method of [22], based on morphogenesis at the uropod. This method is simple, fast and efficient. Under these conditions, *P. adspersus* has a molt cycle of 20 days with 20% for A+B, 25% for C, and 65% for D.

### Insecticide and treatment

Novaluron (wetttable powder 20% active ingredient), was kindly provided by Pr. G. Smagghe (Ghent University, Belgium) (Fig. 2). The compound was added to the rearing sea water at two final concentrations (0.91 µg/L and 4.30 mg/L) corresponding respectively to the LC<sub>50</sub> and LC<sub>90</sub> obtained with respect to the fourth-stage larvae *Culiseta longiareolata* (Diptera, Culicidae) [13]. Newly-ecdysed adult shrimps (0-8 h old) were exposed continuously to treatment. Control shrimps were reared in sea water only. Samples (cephalothorax) were collected from each shrimp at different stages of molt cycle (A, B, C, and D) in control and treated series.



**Fig 2:** Molecular structure of novaluron

### Biochemical procedure

The biochemical composition of cuticle was determined in peripheral integument at different stages during the molting cycle. The percentage of chitin and cuticular proteins were performed following the procedure of [23]. Samples were washed and dried at 60 °C to constant weight and decalcified by means of 10% TCA. The decalcified cuticle was extracted in 2 N NaOH at 110 °C for 3-4 h. The colourless 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.

### Statistical analysis

Results were represented as mean  $\pm$  standard deviation (SD). The statistical analyses were performed using the Prism software version 6.01 for Windows (GraphPad Software Inc., www.graphpad.com). The homogeneity of variances was checked by Bartlett's test. Data were subjected to two-way analysis of variance (ANOVA) followed by a post-hoc HSD Tukey test.

### Results

#### Effect on the cuticular weight

In control series, the cuticular weight of *P. adspersus* increased progressively during the period from the stage A (5.1 $\pm$ 0.69 mg) until the stage D (7.29 $\pm$ 0.26 mg) at the end of the cycle (Table 1). Treatment with novaluron at the two tested concentrations (LC<sub>50</sub> and LC<sub>90</sub>), reduced very significantly ( $p \leq 0.01$ ) weight of cuticle at all stages as compared to controls. ANOVA indicated significant effects of concentration (F 2, 36 = 16.55;  $P < 0.0001$ ) and stage (F 3, 36 = 28.77;  $P < 0.0001$ ) and interaction stage/treatment (F6, 36 = 1.67;  $P = 1569$ ).

**Table 1:** Effect of novaluron (LC<sub>50</sub> = 0.91  $\mu$ g/l and LC<sub>90</sub> = 4.30  $\mu$ g/l) on the cuticular weight (mg) of *P. adspersus* during the molt cycle in control and treated series (mean  $\pm$  SD, n = 4-5).

Stages	Control	Novaluron (LC <sub>50</sub> )	Novaluron (LC <sub>90</sub> )
A	5.1 $\pm$ 0.69 a A	5.15 $\pm$ 0.35 a A	4.81 $\pm$ 0.31 a A
B	6.17 $\pm$ 0.39 a B	5.54 $\pm$ 0.56 a A	5.19 $\pm$ 0.26 b A
C	7.11 $\pm$ 0.57 a C	6.66 $\pm$ 0.56 ab B	5.8 $\pm$ 0.38 b AB
D	7.29 $\pm$ 0.26 a C	6.58 $\pm$ 0.06 ab B	6.07 $\pm$ 0.41 b B

Different capital letters indicate a significant difference between stages of the same series; different small letters indicate a significant difference between control and treated series of the same stage ( $p > 0.05$ ).

#### Effect on the percentage of chitin

Under normal conditions, the percentage of chitin showed a progressive increase from stage A (44.09 $\pm$ 2.12%) until stage C to reach a maximum of 58.33  $\pm$  1.19% and decreased at stage D (45.13 $\pm$ 3.18%) compared to the stage C. Novaluron treatment (LC<sub>50</sub> and LC<sub>90</sub>) resulted in a significant ( $p \leq 0.01$ ) decrease in the percentage of chitin with a dose-response relationship comparatively to controls. The percentage recorded at stage C was 52.97 $\pm$ 2.94% with novaluron at the LC<sub>50</sub> and 43.09 $\pm$ 1.80% with the LC<sub>90</sub> as compared to the controls (Table 2). ANOVA showed significant effects of concentration (F 2, 41 = 43.41;  $P < 0.0001$ ), stage (F 3, 41 = 53.11;  $P < 0.0001$ ) and interaction concentration/stage (F6, 41 = 5.63;  $P = 0,0002$ ).

**Table 2:** Effect of novaluron (LC<sub>50</sub> = 0.91  $\mu$ g/l and LC<sub>90</sub> = 4.30  $\mu$ g/l) on the percentage (%) of chitin in the cuticle of *P. adspersus* during the molt cycle in control and treated series (mean  $\pm$  SD, n = 4-5).

Stages	Control	Novaluron (CL <sub>50</sub> )	Novaluron (CL <sub>90</sub> )
A	44.09 $\pm$ 2.12 a A	42.04 $\pm$ 1.30 a A	35.34 $\pm$ 5.46 b A
B	45.07 $\pm$ 0.93 a A	35.61 $\pm$ 3.30 b B	37.61 $\pm$ 6.10 b A
C	58.33 $\pm$ 1.19 a B	52.97 $\pm$ 2.94 b C	43.09 $\pm$ 1.80 c B
D	49.13 $\pm$ 3.18 a A	45.13 $\pm$ 3.18 a B	33.64 $\pm$ 3.77 b A

Different capital letters indicate a significant difference between stages of the same series; different small letters indicate a significant difference between control and treated series of the same stage ( $p > 0.05$ ).

### Effect on cuticular proteins

In control series, the highest percentage of cuticular proteins were observed at stage A (60,54 $\pm$ 4,26%). Then they decrease at the three stages of the molt cycle, B (54,92 $\pm$ 0,93%), C (41.25 $\pm$ 1.28%) and D (49,02 $\pm$ 3,67%). In treated series by novaluron at the two tested concentrations (LC<sub>50</sub> and LC<sub>90</sub>), presented a significant increase ( $p < 0.01$ ) at stages B, C and D as compared to control series. The values recorded at the end of the cycle were in order of 52,87 $\pm$ 1,69% and 66,35 $\pm$ 3,77% with LC<sub>50</sub> and LC<sub>90</sub>, respectively (Table 3). In addition, ANOVA revealed a significant effects of concentration (F 2, 38 = 635.7;  $P < 0.0001$ ), stage (F 3, 38 = 545.6;  $P < 0.0001$ ) and interaction concentration /stage (F6, 38 = 14.67,  $P = 0.1237$ ).

**Table 3:** Effect of novaluron (LC<sub>50</sub> = 0.91  $\mu$ g/l and LC<sub>90</sub> = 4.30  $\mu$ g/l) on the percentage (%) of cuticular protein in the cuticle of *P. adspersus* during the molt cycle in control and treated series (mean  $\pm$  SD, n = 4-5).

Stages	Control	Novaluron (LC <sub>50</sub> )	Novaluron (LC <sub>90</sub> )
A	60,54 $\pm$ 4,26 a A	63,37 $\pm$ 2,79 a A	73,12 $\pm$ 0,78 b A
B	54,92 $\pm$ 0,93 a AC	57,41 $\pm$ 3,84 a B	62,38 $\pm$ 6,10 b B
C	41,25 $\pm$ 1,28 a B	48,93 $\pm$ 1,13 b C	56,28 $\pm$ 1,09 c C
D	49,02 $\pm$ 3,67 a C	52,87 $\pm$ 1,69 a CB	66,35 $\pm$ 3,77 b B

Different capital letters indicate a significant difference between stages of the same series; different small letters indicate a significant difference between control and treated series of the same stage ( $p > 0.05$ ).

### Discussion

Chitin a polymer of *N*-acetyl-D-glucosamine, contribute considerably to barrier and shaping function of exoskeletons or cuticle which is an extracellular matrix covering the animal [24]. It constitutes up to 40% of the exuvial dry mass depending on the species and varies considerably with the different cuticle types even in a single organism [25]. In this current study, weight and biochemical composition of cuticle in *P. adspersus* adults were determined by the gravimetric method during a molting cycle. In control individuals weight increased progressively during the period from the stage A until the stage D, at the end of the cycle. While, the chitin percentage showed a progressive increase from stage A until stage C to reach a maximum and decreased at stage D. These variations are correlated with the apolysis, the secretion of the new cuticle and the digestion of the old cuticle [26]. So according to [27] the chitin content varied between 66 and 72% during molting cycle in shrimp *P. kerathurus*. The same authors reported an incorporation of two precursors, D-[3-3H(N)]-glucose and N-acetyl- D-[1-3H]-glucosamine (NAGA) in the postmolt (stage A and B) leading to the secretion of endocuticle, followed by a decrease at the intermolt (stage C) (where the secretion of cuticle is complete) and the least content of incorporation of the two precursors is noted in premolt (stage D) where exocuticle secretion is completed. Novaluron-treatment increased significantly the percentage of chitin with a dose-response effect probably by inhibit of the incorporation of sugars into the growing chitin chain. In addition, the modification of the produced cuticle could be explained by the decrease in the chitin and thereby the weight of cuticle. This is in accordance with a previous report made with diflubenzuron, another chitin synthesis inhibitor on *P. kerathurus* [28, 29] and several benzoylurea derivatives which interfere with the molting process by disrupting secretion

cuticular, *via* the synthesis of chitin [3, 30]. The effectiveness of novaluron was demonstrated by several studies in respect of several species [31, 32]. Indeed, this insecticide is very effective against mosquito larvae such as *C. longiareolata* [13], *A. aegypti* [14] and *C. pipiens* [15].

Cuticular proteins are suggested to be involved in the calcification process [33] and in chitin binding in crustacean exoskeleton. The evolution of cuticular proteins in control series of *P. adspersus*, showed a significant decrease at all stages of the molt cycle. [23] Found that the *Astacus fluviatilis* shell consists of 30% of cuticular proteins and 35.3% in *Penaeus duorum*. The cuticular protein content is much more important after a recent exuviation than the intermolt stage [27]. The results for the Novaluron-effect on cuticular proteins reveal a significant increase at stages B, until stage D as compared to control series. Also [29] reported that diflubenzuron, showed an increase in the amount of cuticular proteins on *P. kerathurus*. Variations in the cuticular weight, chitin and proteins during the molting cycle of *P. adspersus* were modified by novaluron. Thus, the biochemical composition of their cuticle, the crustaceans can be the potential targets of these benzoylurea derivatives.

### Conclusion

In current study, *P. adspersus* present a significant variation in the biochemical composition of cuticle during the molt cycle. Novaluron-treatment resulted in biochemical modification of the produced cuticle as evidenced by a decrease in the chitin and thereby the weight of cuticle. Therefore, novaluron caused side-effects on the non target organism the shrimp *P. adspersus*. These effects could be explained by different modes of action, such as a blockage of transport and incorporation of the precursor biosynthetic chitin, N-acetyl-D-glucosamine (GlcNAc), or by direct inhibition of chitin synthesis. However, these mechanisms of action remain to be clarified.

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