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Original Research Paper

Acute toxicity tests of two herbicides diuron and atrazine on the beetle *Crenitis sp* in Volta Basin, Burkina Faso

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Acute toxicity tests were performed on *Crenitis spp* (Coleoptera; Hydrophilidae) using two herbicides, atrazine and diuron in the laboratory. The experiment was to investigate the effect of high doses of these pollutants in individually and together for 12 h each on *Crenitis spp* a species that abounds during the dry period in the shallow hydro-agricultural waters reservoirs of the Volta Basin. Individual macroinvertebrates were collected from puddle areas of the shoreline of Bama Reservoir in the Volta basin. The dry period is the period of rest for agricultural activities at the reservoir. Tests have shown that the toxic effects of the two herbicides on the species of beetles can be enhanced when the both products act synergistically. For diuron, the effective concentration that immobilizes 50% (EC_{50}) of the insects is 44.96 g/l only, but drops to 11.72 g/l in the mixture; while in the same order, atrazine shows 11.75 g/l only and then drops to 7.33 g/l in synergy. It is concluded from this study that works on ecotoxicology should consider the additive or synergistic effects of herbicides to define the bio-ecological traits of macroinvertebrate species living in frequently polluted hydro-agricultural systems.

Key words: Herbicides, atrazine, diuron, acute toxicity, *Crenetis sp,* Bama reservoir, Volta Basin, Burkina Faso.

INTRODUCTION

In a recent study on the state of the benthic fauna of hydro-systems under the impact of agricultural pollutants Sanogo et al. (2014) reported that pollution-sensitive and –resistant macroinvertebrates can be considered as potential bioindicators of water quality in the Volta Basin. Indeed, these ecosystems are facing diverse assaults caused by chemical agents due to intensification of agricultural activities (Leight et al., 2010; Sass et al., 2010; Venot and Cecchi, 2011). However single and synergist effects of these agents on aquatic organisms and their resilience time remain questions to highlight.

FAO (2010) indicates that the use of diuron-based herbicides are authorized by the Sahelian Pesticides Committee (CSP); however famers often use other types of prohibited herbicides in areas of intensified agricultural activities. This is the case of herbicides containing atrazine for which the direct toxicity to humans is well established (Toé et al., 2013). In addition, Kurt (2005) believes that environmental risks caused by these pesticides are accentuated by the additive effects of certain pollutants. Such additive effects can be demonstrated on pollution-resistant beetles according to the findings of Barbour et al. (1999) that confirmed that aquatic benthic macroinvertebrates are the most suitable for the bio-indication studies of water quality. Gnohossou (2006) and Foto et al. (2011) reported that investigations on the impact of pesticides should be carried on *in situ* organisms because levels of differential sensitivities can be observed between different continents (for example Africa compared to Europe or America). Soleri (2013) has shown the presence of atrazine and diuron in the hydro-agricultural dams in the Volta Basin using passive sensors for chemical agents screening. The same author emphasized that atrazine and diuron were among the most commonly used

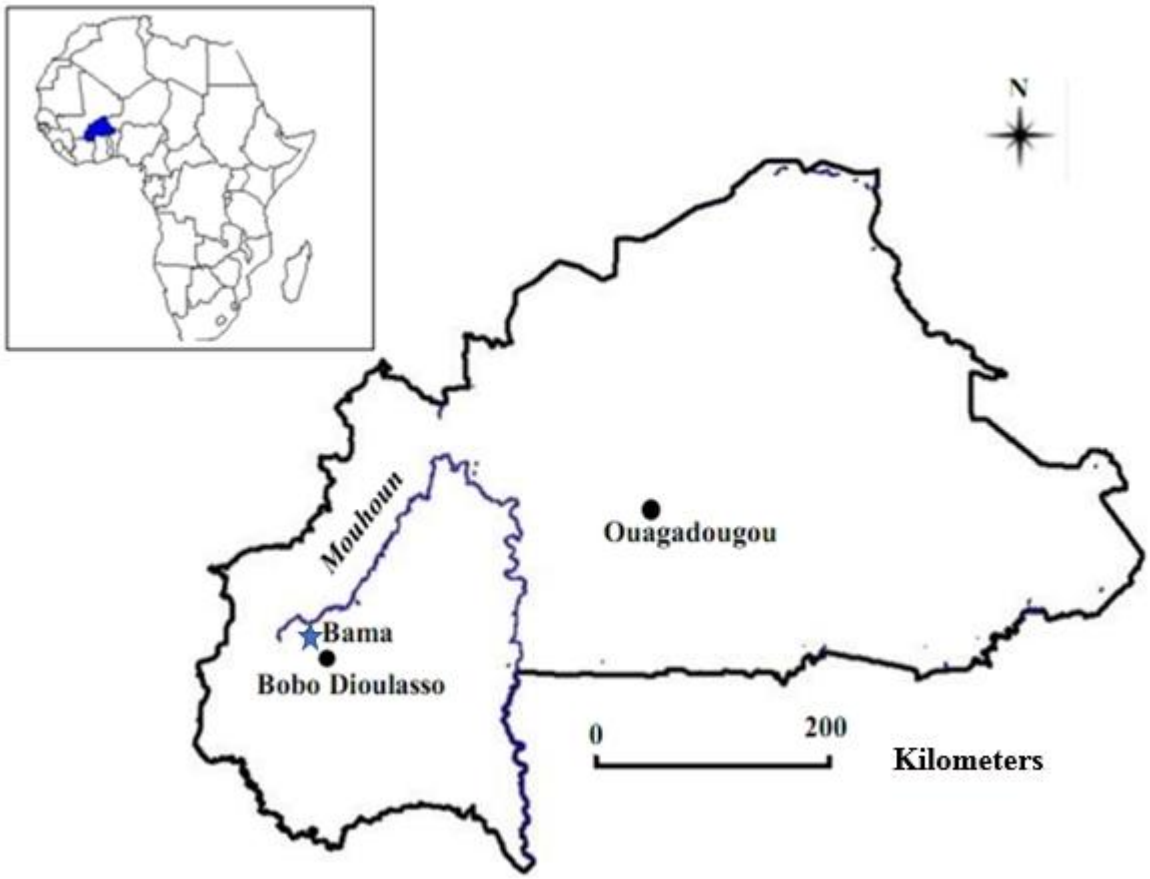

Figure 1. Sitting of the Bama area where the Bama Reservoir is located.

chemical agents in agricultural areas. Sanogo et al. (2014) found the proliferation of certain species of macroinvertebrates at these sites including *Crenetis spp* which is a pollution-resistant beetle of the Hydrophilidae family as well as *Hydrocanthus ferruginicollis* (Noteridae). They further stated that pollution-resistance could be used as a bioindicator of water quality in the Volta Basin.

This bioassay study was conducted on *Crenitis spp* in order to provide useful data on the single and combined effects (additive or synergist) of the two pollutants on one hand and their resilience times in pure water environments on the other hand. The *Crenitis* genus has been described by Bilton (2013) as a well-known panzootic genus in Africa, Europe and America.

METHODOLOGY

Study environment

Macroinvertebrates were collected from the Bama Reservoir (11° 23'N; 4° 24' W) located 30 km from the city of Bobo-Dioulasso (Figure 1). It is located in the heart of irrigation facilities covering a total area of nearly 3000 ha where an irrigated rice scheme was established in 1972 in 1,260 ha. Countless plots are also cultivated (bananas, corn, papaya, cabbage and vegetables) in the dry season. Covering an area of about 50 ha, Bama reservoir is the result of technical problems and lack of maintenance of hydraulic infrastructures. It is centrally located thus many plots drain their used outflow waters into it. Genetic mutations have favored the adaptation of mosquitoes to insecticides in the area and have been reported by Dabiré et al., (2008) attesting to the high contamination levels.

Materials and collection of sample

A total of 3,690 *Crenitis spp* were collected at the Bama Reservoir; precisely from the puddles still present in the perimeters of crops during the dormant period of agricultural activities in order to minimize the effects of herbicides. It is well known that in testing chemical effect it is required that the organisms used in the test be previously raised in a laboratory (ISO 6341, 1996). However, this experiment was inspired by that of Fossati et al. (1992) who used crustacean macroinvertebrates captured directly from natural waters. A kick net of 30 cm diameter and 1 mm^2 mesh size aided in this cropping of

Table 1. Mineral chemical composition of the water used to dilute the two herbicides.

Chemical composition	Concentration (mg/l)
Calcium Ca^{++}	2
Magnésium Mg^{++}	0.70
Potassium K^{+}	0.24
Sodium Na^{+}	1
Bicarbonate HCO_3^{-}	12
Sulfate SO_4^{--}	1
Silice SiO_2^{--}	15.4

Table 2. Different concentrations of atrazine (Atrazila 500g/l) that were used in the experiment

Label	Concentration (g/l)	Atrazila (ml)	Water (ml)
A_{10}	500	100	0
A_9	250	50	50
A_8	125	25	75
A_7	62.5	12.5	87.5
A_6	31.5	6.2	93.7
A_5	15.6	3.1	96.8
A_4	7.8	1.5	98.4
A_3	3.9	0.7	99.2
A_2	1.9	0.3	99.6
A_1	0.9	0.19	99.8

macroinvertebrates; sorting on the field after harvest was done through the 1 mm^2 mesh sieve. Macroinvertebrates were immediately transported to the laboratory in containers filled with water. Acute toxicity tests were performed in the laboratory in 41 300 ml capacity-jars filled with pure water used to dilute the pollutants.

Chemical agents used in this experiment are two herbicides atrazine (trade name Atrazila 500 g/l in concentrated lotion) and diuron (trade name ACTION 80, 800 g/kg). Mineral water (Lafi, Onea) was used for dilution as shown in Table 1. The chemical agent atrazine (Atrazila 500 SC) is a product of Shenzhen Baocheng Industry Co., Ltd. China, while Diuron is manufactured by SCPA Sivex International Paris, France.

Experimental design

The 3690 macroinvertebrates were transported to the laboratory and divided into groups of 30 and then placed in previously prepared solutions as shown in Tables 2, 3, 4a and 4b. Tables 2 and 3 represent different concentrations of atrazine and diuron respectively. From the starting concentration of 500 g/l for atrazine, 10 new concentrations were prepared with the dilution factor 0.5 (Table 2). For diuron, we considered the concentration mass/ mass of 800 g/kg of the trade product and diluted in mineral water to obtain a concentration of 800 g/l. From this initial concentration, 10 new levels with a dilution factor 0.5 were prepared (Table 3). Mixtures of the two products were developed by combining the first two lower concentrations (Table 4a) and second by combining the highest concentrations of atrazine and low concentrations of diuron and vice versa (Table 4b) .

In addition to these preparations, 30 macroinvertebrates were placed in 41 jars with mineral water as control treatment. Every 30 min, the jars containing insects + diluted herbicide were then emptied into the sieve and motionless beetles were counted; the operation lasted 12 h and was repeated three times. After each test, mobile living macroinvertebrates were re-introduced into the solutions (after stirring to prevent it from settling and maintaining a homogenous environment). For each of the three sets of experimental tests, the cropping of new macroinvertebrates was necessary; the temperature of the solution was maintained each time at 30°C (average temperature of the water in Bama reservoir where insects were harvested) via a stabilizing ambient laboratory temperature. In addition to harvesting macroinvertebrates, measures of water temperature, oxygen, conductivity and pH were reported to characterize the living environment of macroinvertebrates. These measurements were carried out using a multiparameter probe WTW 3430 MULTI-type

Table 3. Different concentrations of diuron (Action 80, wettable granules 800g/kg) that were used in the experiment

Label	Concentration (g/l)	Action 80 (g)	Water (ml)
D_{10}	800	100	100
D_9	400	50	100
D_8	200	25	100
D_7	100	12.5	100
D_6	50	6.2	100
D_5	25	3.1	100
D_4	12.5	1.5	100
D_3	6.2	0.7	100
D_2	3.1	0.3	100
D_1	15	0.19	100

Table 4a. Mixtures of different concentrations of atrazine and diuron (Mixture 1) ranking from the highest to the lowest concentrations values

Label	Concentration			
	[Atrazine (g/l) + Diuron (g/l)]	Atrazila (ml)	Action 80 (g)	Water (ml)
A_{10}/D_{10}	500+800	100	100	0
A_9/D_9	250+400	50	50	50
A_8/D_8	125+200	25	25	75
A_7/D_7	62.5+100	12.5	12.5	87.5
A_6/D_6	31.5+50	6.25	6.2	93.7
A_5/D_5	15.6+25	3.1	3.1	96.8
A_4/D_4	7.8+12.5	1.5	1.5	98.4
A_3/D_3	3.9+6.2	0.7	0.7	99.2
A_2/D_2	1.9+3.1	0.3	0.3	99.6
A_1/D_1	0.9+1.5	0.19	0.19	99.8

Table 4b. Mixtures of different concentrations of atrazine and diuron (**Mixture 2**) ranking from the highest to the lowest for atrazine and from the lowest to the highest (for diuron) concentrations values

Label	Concentration			
	[Atrazine (g/l) + Diuron (g/l)]	Atrazila (ml)	Action 80 (g)	Water (ml)
A_{10}/D_1	500+1.5	100	0.19	0
A_9/D_2	250+3.1	50	0.3	50
A_8/D_3	125+6.2	25	0.7	75
A_7/D_4	62.5+12.5	12.5	1.5	87.5
A_6/D_5	31.5+25	6.2	3.1	93
A_5/D_6	15.6+50	3.1	6.2	96.8
A_4/D_7	7.8+100	1.5	12.5	98.4
A_3/D_8	3.9+200	0.7	25	99.2
A_2/D_9	1.9+400	0.3	50	99.6
A_1/D_{10}	0.9+800	0.19	100	99.8

(Enterprise ZEISS, Germany) and taken in 5 stations at the puddle area and 5 others inside the deep water of the reservoir.

Data analysis

These tests being of short durations were analyzed using SigmaPlot 10.0 software to determine the effective concentration EC_{50} which is defined as the concentration of a toxicant that cause a 50% effect compared to the control (Bessi and El Alami, 2009). This value was investigated graphically for each herbicide; singly and synergistically.

RESULTS

Physico-chemical variables

The results of individual measurements of physico-

chemical variables are shown in Table 5 indicating that temperature, conductivity, pH and oxygen are not limiting factors: their values are in the range of average productivity in water environments (Ministry of Environment of Quebec, DENV, 2001).

Results of the different tests

After 12 h of exposure with 30 min interval, mineral water (control test) had no effect on *Crenitis sp* and all organisms survived. Tables 6, 7 respectively indicate the survival of the individuals introduced into different concentrations of atrazine and diuron. Tables 8a and b depicts the survival of individual organisms when the various mixtures of the two herbicides
were used. The triplicates were pooled to calculate the overall EC_{50}.

Graphical assessment of EC_{50} of each herbicide

The effective concentration (EC_{50}) for atrazine alone was 11.75 g / l; in Mixture 1 (equal mixture of diuron and atrazine), it was 7.33 g/l (Table 8a). For diuron, the EC_{50} value was 44.96 g/l and only 11.72 g/l in Mixture 1. In Mixture 2 (Table 8b), the EC_{50} was not obtained for atrazine (Figure 2) nor for diuron (Figure 3).

In both cases (diuron and atrazine), the mixture was more toxic (less EC_{50}) than the toxicity of each contaminants considered separately; especially Mixture 2 which induced massive immobility except for intermediate concentrations (
between 10 to 100 g/l) for which there were few survivors after 12 h.

For atrazine the addition of diuron in Mixture 1 had a limited effect (Figure 2); that is, the EC_{50} calculated for atrazine alone and in Mixture 1 was not statistically different (Figure 4) with changes in toxicity of the synergistic mixture equalling zero. In contrast, the results were not the same for Mixture 2: the induced effects were attributable to doses of diuron associated with low concentrations of atrazine.

The situation was not the same for diuron (Figure 3) with an observed steady decrease in the effective concentration when atrazine was added at high concentrations as well as low concentrations. In other words, diuron added to atrazine results in a very toxic mixture.

EC_{50} comparison of the two products alone and in mixture (Mixture 1)

EC_{50} were calculated graphically for each bio-essay (Table 9). There were thus three EC_{50} values for each treatment and a comparison among these triplicates with ANOVA (one factor) was performed. Probabilities were shown and the difference was significant only in the case of diuron (Figure 4; Table 9).

DISCUSSION

Harvesting of macroinvertebrates in puddles was to have a number of individuals needed for statistical analyzes. The physico-chemical variables are not limiting factors in Bama reservoir so it can be concluded that macroinvertebrates in puddles can be used instead of those of the deep water in the reservoir for these bioassays.

In this study, the effective concentration at which 50% of beetles was immobilized within 12 h of exposure to atrazine is lower than that of diuron (11.75 g/l against 44.96 g/l, respectively). This lower value denotes the high toxicity of atrazine compared to diuron whose use is endorsed by the FAO (2010) as a good herbicide in areas of agricultural production in the Sahel. Samuel and St. Lawrence (2001) suggest that exposure to chemical agents may modify the toxic effects. Also, Price et al (2002) showed that the toxicity of a chemical mixture is proportional to the sum of the toxicity of each individual contaminant. In this study, the mixture of the two herbicides generated a higher toxicity. When searching the additive effect of these herbicides by the addition of higher doses and following the decreased gradient towards the lower value (Table 4a), the EC_{50} of atrazine reduced from 11.75 g/l to 7.33 g/l and that of diuron from 44.96 g/l to 11.72 g/l. The difference between the two values for each product is only significant in the case of diuron (Figure 4). The toxicity of diuron was thus reinforced by the presence of highly toxic atrazine on the surroundings (Robert et al., 1986). Indeed, the survival of individuals was observed only with the mixture during the fourth and fifth dilutions (Figures 2 and 3) and mass motionless individuals were observed for extreme high concentrations of the both products combined compared to low concentrations each one, individually; consequently the curves obtained do not allow a graphic determination of EC_{50} values.

The results in these tests were obtained using very high concentrations of contaminants to pinpoint the expected effects of exposure in 12 h; an exposure time of within 24 to 48 h is recommended for acute toxicity tests (Bessi and El Alami, 2009). The bioassay model used in this study allowed for the revelation of evidence of pollution-resistance for *Crenitis sp* as this beetle can be used as a bioindicator of water quality.

CONCLUSION

Diuron is an herbicide whose toxicity to *Crenitis sp* (Coleoptera, Hydrophylidae) is increased when combined with the herbicide atrizine; the two products act synergistically to induce an acute toxicity. Indeed, the effective concentration that immobilize 50% (EC_{50})

Table 5. Measured chemical parameters at Bama reservoir and its puddles area where the beetles *Crenitis sp* were collected.

N	Bama reservoir				Puddles areas for collection of *Crenitis sp*			
	Temperature (° C)	conductivity (µS/cm)	pH	Oxygen (mg/l)	Temperature (° C)	conductivity (µS/cm)	pH	Oxygen (mg/l)
1	34.8	139.6	8.22	8.12	35.1	165.5	8.81	7.81
2	34.5	155.5	7.13	7.7	34.3	146.4	7.42	8.92
3	33.2	154.7	6.33	5.75	34.5	146.8	7.15	7.53
4	31.8	161.2	8.01	5.47	34.8	155.2	7.27	6.62
5	33.6	159.1	7.59	6.31	33.4	163.2	6.75	6.99
Moyenne	33.58	154.02	7.45	6.67	34.42	155.42	7.48	7.57

Table 6. Number of *Crenetis sp* motionless when exposed to different concentrations of atrazine

Exposure time	Number of motionless (test 1)										Number of motionless (test 2)										Number of motionless (test 3)									
	A_{10}	A_9	A_8	A_7	A_6	A_5	A_4	A_3	A_2	A_1	A_{10}	A_9	A_8	A_7	A_6	A_5	A_4	A_3	A_2	A_1	A_{10}	A_9	A_8	A_7	A_6	A_5	A_4	A_3	A_2	A_1
0 mn	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30 mn	30	25	0	0	0	0	0	0	0	0	30	30	5	0	0	0	0	0	0	0	30	18	4	0	0	0	0	0	0	0
1h		30	13	1	0	1	0	0	0	0			10	0	0	0	1	0	0	0		23	13	1	0	0	0	0	0	0
1h30			18	11	7	1	0	0	2	0			10	7	0	0	1	0	0	0		30	13	1	0	0	0	0	0	0
2h			18	11	12	3	0	0	3	1			17	17	11	0	2	0	3	1			13	5	3	1	0	0	0	3
2h30			18	11	12	3	1	0	3	1			18	17	12	0	2	0	4	1			14	5	3	1	0	0	3	3
3h			18	11	12	3	1	0	3	1			18	17	13	0	2	0	4	1			21	12	3	1	4	1	3	3
3h30			18	17	12	3	2	0	7	6			19	24	17	0	2	0	4	1			26	13	3	1	4	1	3	3
4h			20	21	14	3	3	0	7	6			19	25	17	9	3	0	4	1			26	13	4	1	4	1	3	3
4h30			20	21	14	6	8	0	7	6			19	26	17	9	7	0	4	1			30	13	8	5	6	1	3	3
5h			28	21	17	6	9	2	7	6			27	26	17	9	7	0	4	1				20	11	5	7	1	3	3
5h30			28	25	17	6	9	2	7	6			28	26	17	9	7	0	5	1				20	11	10	7	3	3	3
6h			30	26	17	6	9	2	8	6			28	26	17	9	11	0	5	1				26	12	10	7	3	4	3
6h30				26	17	6	9	3	9	6			28	26	18	13	11	1	5	1				26	19	10	7	5	4	3
7h				26	17	6	9	4	11	6			28	26	19	13	12	4	5	1				26	19	10	11	5	4	3
7h30				26	18	13	9	5	11	6			30	26	19	14	12	8	5	1				26	19	10	12	5	7	3
8h				28	18	13	9	8	11	6				28	19	14	12	10	5	1				29	19	11	12	5	7	3
8h30				28	18	13	9	8	11	6				29	19	14	12	10	5	1				29	28	11	12	5	7	3
9h				28	18	13	11	8	11	6				29	23	16	12	10	5	1				29	28	11	12	9	7	3
9h30				28	18	14	11	8	11	6				30	23	16	12	10	5	1				29	28	11	12	9	7	3
10h				28	18	15	11	8	11	6					24	16	12	13	5	1				30	28	18	12	9	7	3
10h30				28	19	16	11	8	12	6					24	16	12	13	5	1					28	18	12	9	9	3
11h				29	19	16	11	8	12	6					24	16	12	13	5	1					28	18	12	9	9	3
11h30				30	19	16	11	8	12	6					24	16	13	13	5	1					28	18	13	9	9	3
12h					19	16	11	8	12	6					24	16	13	13	5	1					30	18	14	9	11	3

Table 7. Number of *Crenetis sp* motionless when exposed to different concentrations of diuron

Exposure time	Number of motionless (test 1)										Number of motionless (test 2)										Number of motionless (test 3)									
	D_{10}	D_9	D_8	D_7	D_6	D_5	D_4	D_3	D_2	D_1	D_{10}	D_9	D_8	D_7	D_6	D_5	D_4	D_3	D_2	D_1	D_{10}	D_9	D_8	D_7	D_6	D_5	D_4	D_3	D_2	D_1
0 mn	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30 mn	30	30	1	0	0	0	0	0	0	0	30	21	6	1	0	1	0	0	0	0	24	30	2	0	0	0	0	0	0	0
1h			15	6	0	0	1	0	0	1		28	6	1	0	1	2	0	0	0	30		21	0	0	0	1	0	0	0
1h30			17	6	0	0	1	0	0	1		30	17	5	0	1	2	0	0	0			21	4	5	1	1	0	0	0
2h			17	6	1	2	1	0	0	1			17	5	0	1	2	0	0	0			23	8	5	1	1	0	0	0
2h30			18	8	1	2	1	0	0	1			17	5	0	1	2	0	0	0			23	10	5	1	1	0	0	0
3h			25	11	3	2	1	0	0	1			21	5	0	1	2	0	0	0			23	12	6	4	2	0	0	0
3h30			25	11	3	2	1	0	0	1			21	5	0	1	2	0	0	0			30	12	7	4	2	1	0	0
4h			26	11	3	2	5	0	0	1			21	10	1	1	2	0	0	0				13	7	4	3	1	0	0
4h30			29	11	3	2	5	0	0	1			30	10	1	1	4	0	0	0				13	7	4	3	1	0	0
5h			29	17	7	3	5	0	0	1				10	1	5	4	0	0	0				13	7	4	3	1	0	0
5h30			30	18	7	6	5	3	0	1				16	2	5	4	0	0	0				15	8	4	3	1	0	0
6h				19	8	6	5	3	0	1				16	3	5	4	0	0	0				15	10	5	3	1	0	0
6h30				20	9	6	5	3	0	1				16	9	6	4	0	0	0				24	10	6	3	1	0	0
7h				20	10	6	5	3	0	1				25	15	9	4	0	0	0				24	11	6	3	1	0	0
7h30				20	10	6	5	3	0	1				27	15	9	4	0	0	0				24	12	6	3	1	0	0
8h				20	11	6	5	3	0	1				27	15	9	4	0	0	0				24	12	6	3	1	0	0
8h30				21	11	6	5	3	0	1				27	15	9	4	1	0	0				29	14	8	3	1	0	0
9h				21	11	6	5	3	0	1				27	15	9	4	1	0	0				29	14	8	3	1	0	0
9h30				21	11	6	5	3	0	1				29	16	10	4	1	0	0				29	14	8	3	1	0	0
10h				22	11	6	6	3	0	1				30	18	10	4	1	0	0				29	14	9	3	1	0	0
10h30				22	11	6	6	3	0	1					18	10	4	1	0	0				29	14	9	3	1	0	0
11h				22	11	6	6	3	0	1					18	11	4	1	0	0				29	14	9	3	1	0	0
11h30				22	11	6	6	3	0	1					18	11	4	1	0	0				30	14	10	3	1	0	0
12h				22	11	6	6	3	0	1					18	11	4	1	0	0					14	10	3	1	0	0

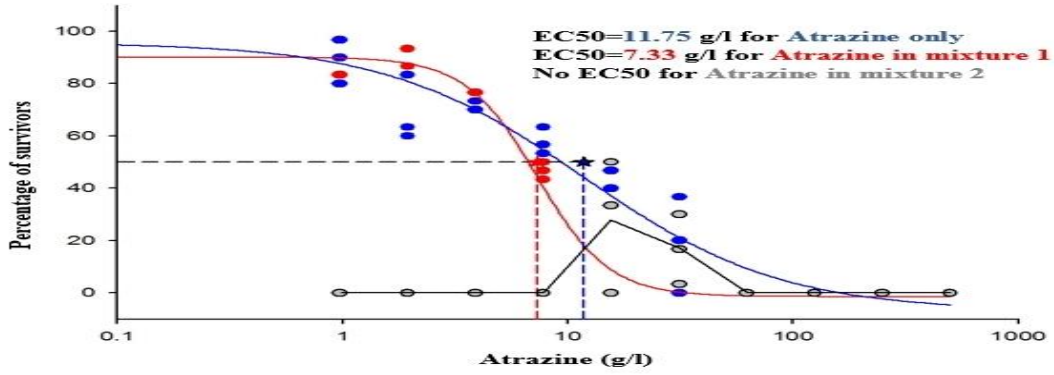

Figure 2. Graphical assessment of the effective concentration (EC50) for atrazine toxic to *Crenitis sp.*

Table 8a. Number of *Crenetis sp* motionless in a mixture of different concentrations of atrazine and diuron (called **mixture 1**).

Exposure time	No. of motionless (test 1)									
	A_{10}/D_{10}	A_9/D_9	A_8/D_8	A_7/D_7	A_6/D_6	A_5/D_5	A_4/D_4	A_3/D_3	A_2/D_2	A_1/D_1
0 m	0	0	0	0	0	0	0	0	0	0
30 m	30	30	30	22	16	6	0	0	3	0
1h				22	24	9	0	0	3	0
1h30m				25	25	9	0	0	3	0
2h				30	26	9	0	0	3	0
2h30m					29	9	0	6	3	0
3h					29	9	1	6	3	0
3h30m					29	12	1	6	3	0
4h					29	13	3	6	3	0
4h30m					29	13	3	6	3	0
5h					30	13	3	6	3	0
5h30m						20	3	6	3	5
6h						20	3	6	3	5
6h30m						20	3	6	3	5
7h						27	9	6	3	5
7h30m						27	15	6	3	5
8h						30	15	6	3	5
8h30m							15	6	3	5
9h							15	7	3	5
9h30m							15	7	3	5
10h							15	7	4	5
10h30m							15	7	4	5
11h							15	7	4	5
11h30m							16	7	4	5
12h							16	7	4	5

Exposure time	No. of motionless (test 2)									
	A_{10}/D_{10}	A_9/D_9	A_8/D_8	A_7/D_7	A_6/D_6	A_5/D_5	A_4/D_4	A_3/D_3	A_2/D_2	A_1/D_1
0 m	0	0	0	0	0	0	0	0	0	0
30 m	30	30	22	22	0	0	0	0	1	1
1h			22	24	17	7	0	0	2	1
1h30m			25	24	17	7	0	0	2	1
2h			30	24	21	10	0	0	2	1
2h30m				29	21	10	0	0	2	1
3h				29	30	10	0	1	2	1
3h30m				29		10	3	1	2	1
4h				30		10	4	1	2	2
4h30m						10	4	1	2	2
5h						19	4	1	2	2
5h30m						19	4	1	2	3
6h						19	4	1	2	3
6h30m						24	4	3	2	3
7h						24	6	3	2	3
7h30m						25	6	3	2	3
8h						25	6	8	2	3
8h30m						26	7	8	2	3
9h						27	9	8	2	3
9h30m						30	9	8	2	3
10h							14	8	2	3
10h30m							14	9	2	3
11h							15	9	2	3
11h30m							15	9	2	3
12h							15	9	2	3

Exposure time	No. of motionless (test 3)									
	A_{10}/D_{10}	A_9/D_9	A_8/D_8	A_7/D_7	A_6/D_6	A_5/D_5	A_4/D_4	A_3/D_3	A_2/D_2	A_1/D_1
0 m	0	0	0	0	0	0	0	0	0	0
30 m	30	30	30	29	9	3	0	0	0	0
1h				30	22	14	0	0	0	0
1h30m					25	14	1	0	0	0
2h					25	16	1	0	0	0
2h30m					25	16	2	0	0	0
3h					25	16	2	0	2	0
3h30m					25	16	4	0	2	0
4h					30	17	4	3	3	0
4h30m						19	9	3	3	0
5h						19	9	3	3	0
5h30m						24	11	3	3	0
6h						30	12	7	5	0
6h30m							12	7	5	2
7h							13	7	5	2
7h30m							17	8	7	2
8h							17	8	9	2
8h30m							17	8	10	2
9h							17	8	10	2
9h30m							17	8	10	2
10h							17	8	10	3
10h30m							17	8	10	3
11h							17	9	10	3
11h30m							17	9	11	3
12h							17	9	11	3

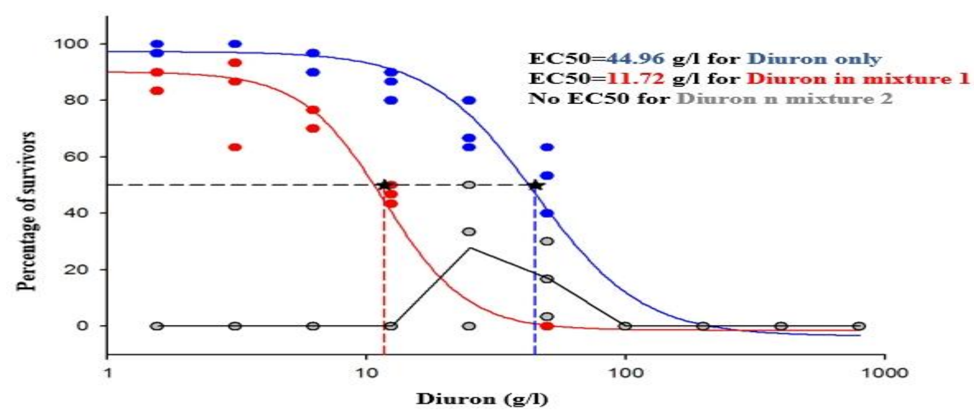

Figure 3. Graphical assessment of the effective concentration (EC50) for diuron toxic to *Crenitis sp*

Table 8b. Number of *Crenetis sp* motionless in a mixture of different concentrations of atrazine and diuron (called **mixture 2**).

Exposure time	Number of motionless (test 1)											Number of motionless (test 2)										Number of motionless (test 3)										
	A_{10}/D_1	A_9/D_2	A_8/D_3	A_7/D_4	A_6/D_5	A_5/D_6	A_4/D_7	A_3/D_8	A_2/D_9	A_1/D_{10}		A_{10}/D_1	A_9/D_2	A_8/D_3	A_7/D_4	A_6/D_5	A_5/D_6	A_4/D_7	A_3/D_8	A_2/D_9		A_1/D_{10}	A_{10}/D_1	A_9/D_2	A_8/D_3	A_7/D_4	A_6/D_5	A_5/D_6	A_4/D_7	A_3/D_8	A_2/D_9	A_1/D_{10}
0 mn	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0
30 mn	30	30	30	10	0	0	29	30	30	30		30	30	30	23	0	1	3	10	22		30	30	30	30	21	0	0	12	30	30	30
1h				10	0	0	30								23	3	1	22	17	26						21	0	0	19			
1h30				11	0	0									23	3	1	26	30	30						28	0	5	22			
2h				23	7	0									23	5	1	26								28	1	12	30			
2h30				30	7	0									27	5	2	26								28	2	12				
3h					14	0									27	5	3	26								29	9	12				
3h30					14	0									27	11	7	30								30	15	15				
4h					14	3									30	11	7										15	19				
4h30					14	3										11	7										15	19				
5h					14	3										19	7										15	19				
5h30					14	5										21	10										17	19				
6h					14	6										21	11										17	19				
6h30					17	6										21	11										17	24				
7h					25	6										21	11										23	26				
7h30					25	19										21	11										23	29				
8h					25	19										21	11										26	29				
8h30					25	19										21	13										26	29				
9h					25	19										21	13										26	30				
9h30					25	19										21	13										29					
10h					25	19										21	13										29					
10h30					25	19										21	13										29					
11h					25	19										21	13										29					
11h30					25	20										21	15										29					
12h					25	20										21	15										29					

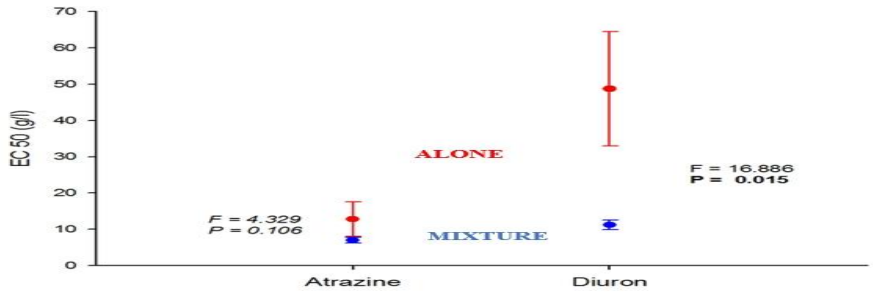

Figure 4 : Graphical comparison of EC_{50} values between atrazine and diuron each product alone (in read) and in synergy or mixture (in blue) used for the toxicity tests on the species *Crenitis sp.*

Table 9 : Statistical comparison of EC50 values between atrazine and diuron(each product alone and in synergy) used for the toxicity tests on the species *Crenitis sp*

		Atrazine		Diuron	
		Alone	**Mixture**	**Alone**	**Mixture**
Different bioessays	1	18	7.75	66.32	12.4
	2	11.57	7.2	36	11.5
	3	8.78	6.09	43.8	9.75
Mean		12.78	7.01	48.70	11.21
Standard deviation		4.72	0.84	15.74	1.34

macroinvertebrates using a single diuron is 44.96 g/l only, but drops to 11.72 g/l in the mixture; while the reduction for atrazine is 11.75 g/l single to 7.33 g/l in the mixture. The difference is significant in the case of diuron which becomes hazardous when used in combination with atrazine; such mixture of herbicides may jeopardize the water quality in hydro-agricultural environment. It is concluded that studies of ecotoxicology should consider these synergistic effects of herbicides to better describe the bioecological traits related to macroinvertebrates species in aquatic environments. These results serve to improve the development of bioindicators index for the constantly polluted hydro-agricultural systems.

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