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Survival and reproduction of *Cyclops abyssorum* (freshwater copepod) exposed to spirotetramat and 2,4-D

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Abstract

The study was carried out to investigate the possible effects of spirotetramat and 2,4-D at relatively low concentrations as single or mixed pollutants to Cyclosps abyssorum. Acute toxicity tests were conducted in glass cups and chronic toxicity effets were assessed using both glass cups and microcosms. The acute toxicity of both products is more important to nauplial forms than to adults. The LC50 value was 34.85 mg/L and 96.79 mg/L to nauplii, respectively for spirotetramat and 2,4-D. On adults, it was 37.42 mg/L and 101.7 mg/L respectively. The chronic exposure showed that irrespective of the concentrations, both the single and mixed contaminants affected the survival and reproduction of C. abyssorum. Extension of the embryonic period with high mortality of nauplii was observed. The number of eggs laid was also affected. The sex ratio in adulthood showed a significant feminization of the population, about double that of obtained in the absence of pollutants. Effects of both contaminants mixed are greater than those of pollutants studied singly.

Keywords: acute toxicity, freshwater copepod, pesticide, risk assessment, sex ratio

1. Introduction

Agricultural development worldwide is highly correlated with the use of several crop protection products, mainly insecticide, herbicide, and fungicide (BOSCHETTO, [1]). These products, once in the soil, under the influence of environmental conditions, get scattered in various ecosystems, including aquatic environments (LALANLETTE, [2]). This has various effects on aquatic communities (PESCE & al [3]) and results, among others, in transient or irreversible adaptations (SABATER & al [4]). The challenge in ecotoxicology is therefore to understand and predict the impacts of chemical contaminations on natural communities (RELYEA & HOVERMAN [5]).

Spirotetramat and 2,4-D are respectively insecticide and herbicide which are used at great rates as crop protection tools. Spirotetramat is an inhibitor of lipid biosynthesis in target insects (BRUCK & al [6]). Its characteristics give it an increase potential for leaching (AGBOHESSI & al [7]). But it is an emerging insecticide, so it is important that we be aware of its impacts on various organisms, primarily in the aquatic environment (typically, the final pollutant collector).

As far as 2,4-D is concerned, it is a widely-used herbicide both on land and in the aquatic environment (SANTE CANADA [8]). It has shown a negative effect on the growth of marine algae suitable for zooplankton reproduction and has a stimulating effect on the undesired algae (ARZUL & al [9]). It is also appropriate to assess the effects on zooplankton compartments in this study. Since the study of plankton organisms allows an early prediction of disturbances to the ecosystem (HOUSSOU & al [10]), it is important to have access to pollutants toxicological data pertaining to that community.

It is particularly important to know the degree of sensitivity as well as the early reactions of aquatic organisms to hazards in their environments since they are close to the base of the food web. So, planktonic organisms such as zooplanktons are excellent bio-indicators that are used in several studies to monitor the aquatic environment (JEPPESEN & al, MIALET & al, TUCCI, HOUSSOU & al [11, 12, 13, 14]). In zooplanktons, copepods are very sensitive and easy to manipulate in an investigation. Several studies have evaluated their sensitivity to various chemical contaminants in order to use them as bio-indicators in their environment (MEDINA & al, GUSTAFSSON & al, GUTIERREZ & al, XU & LIU [15, 16, 17, 18]).

Nowadays, it is accepted around the world that the sensitivity of a species to a given pollutant is highly variable depending on other environmental factors (such as the geographical characteristics of the area and the type of anthropogenic factors at play). So, to bio-monitor an environment, it is important to identify the indicator species together with their specific responses to a number of environmental stressors. *Cyclops abyssorum* is a freshwater copepod having a very wide distribution. In the eutrophied Gheorgheni I natural lake of Cluj-Napoca in Romania, *C. abyssorum* is the main species, being both abundant and widely dominant in the zooplankton compartment. While spirotetramat and 2,4-D are widely used in that country, no study has evaluated their effects on this indicator species in that environment.

Currently, the aquatic ecotoxicological challenge is not only to understand the effects of each chemical on community's organisms, but also to understand the effects of the combined chemicals on those environments. The natural environment is rarely subjected to a singular pollution. Currently, very few studies have evaluated the impact of the spirotetramat insecticide and 2,4-D herbicide on a zooplankton species either singly or mixed. This study aims at evaluating the impact of these pesticides, either singly or mixed, on *C. abyssorum* and at deducing the possible damage done at the scale of the ecosystem.

2. Materials and Methods

2.1. Test organisms: Culture and rearing

In the Gheorgheni I natural lake (46° 46'N, 23° 37'E) in Cluj-Napoca, Romania, *Cyclops abyssorum* represents 47.72% of the total zooplankton population and 74.6% of the total copepods. This determination results from a preliminary sampling prior to this study (two samplings in spring and two more in summer 2014 using a 50-µm net). A planktonic sample was taken in spring 2014 and cultured in laboratory conditions. The culture medium was the Bold solution (Table 1). Culture temperature was 20.75 ± 0.1 °C and the pH was 7.6 ± 0.3. The photoperiod was 16/8 hours (light/dark). This pre-culture lasted 30 days and was subcultured twice. *C. abyssorum* was isolated after 10 days of culture.

2.2. Chemicals and test solutions

Two chemical compounds were used in different treatments in this study. One is an insecticide (spirotetramat) and the other is a herbicide (2,4-D).

These two pesticides were selected based on their wide usage in Romanian agriculture. The test solutions were prepared by dissolving various concentrations of both pesticides, either singly or mixed, in isolated samples of the Bold culture mediums; this is done in order to have different known concentrations of each active principle. The original Bold solution was previously prepared and stocked at 4°C for stabilization for 72 hours. The test solutions were immediately distributed in the experimental designs for each treatment.

Table 1. Composition of stock solutions of the culture medium of Bold

Solution A		Solution B		Trace Solution	
Products	Concentration (g/L)	Products	Concentration (g/L)	Products	Concentration (g/L)
$NaNO_3$	25	K2HPO4	7.5	H3BO3	2.4
$CaCl_2,2H_2O$	2.5	KH2PO4	17.5	MnCl2,4H2O	1.8
$MgSO_4,7H_2O$	7.5	NaCl	2	(NH4)6Mo7O24, 4H2O	0.02
FeEDTA	2			ZnSO4,7H2O	0.22
				CuSO4,5H2O	0.08
				CoNO3	0.09

2.3. Acute toxicity test

This study has made the determination of the short-term effects of spirotetramat and 2.4-D on *C. abyssorum* possible. These effects can be used as a basis for the study of any chronic effect on population dynamics both in this investigation and in others. Toxicity at 48 hours was determined for adults (females + males) and for nauplii less than 24 hours of age. The experiments were carried out in glass cups of 50 mL each. For each treatment, 30 mL of prepared test solution were distributed in each cup with three replications for each test concentration. Concentrations were established between 1 mg/L and 50 mg/L for spirotetramat and between 30 mg/L and 150 mg/L for 2-4-D. A total of 30 individuals of *C. abyssorum* was used for each treatment, 10 individuals per replica. The culture media were not renewed. Copepods were not fed during the exposure (USEPA [19]. Culture temperature was 20.2 ± 0.2°C and the photoperiod was 16/8 hours (light/darkness). The mortality of individuals was controlled daily by lack of movement after a mild stimulus. The 48-hours LC_{50} was estimated by using the probit method.

2.4. Hatching and nauplii survival test

This test was an evaluation of the effect of chronic exposure to *C. abyssorum* to low doses of both pesticides (spirotetramat and 2,4-D) in either single or mixed contamination. Two doses were tested for each active principle: 10% and 30% of the LC_{50} to nauplii. Testing was also done for the mixed chemicals at the lowest dosage (10%). The various test solutions were prepared and distributed in glass cups of 50 mL each. Each cup was filled to 30 mL of the solution, in three replications per treatment. Five *C. abyssorum* gravid female were introduced into each cup, making a total of 15 individuals per treatment. The culture medium was renewed every 48 hours, shortly after feeding with a concentration of *Monoraphidium griffithii* (71 ± 0.5 cells/L), *Coscinodiscus sp* (43 ± 0.1 cells/L), and *Brachionus quadridentatus* (25.3 ± 0.3 ind/L). Photoperiod was 16/8 hours (light/darkness) and the temperature was 20.4 ± 0.3° C. Nauplii hatching and mortality in each cup was recorded daily. The test lasted for a total of 10 days.

2.5. Microcosm test

This test allowed approximating the conditions to the natural environment. For that purpose, Bold culture medium were distributed into 12 aquariums receiving 10 liters each. The medium was then seeded with inoculum derived from the purified culture of phytoplankton (*M. graffithii* and *Coscinodiscus sp*). After four days, *B. quadridentatus* (42 ind/L) was introduced. A week later, 9 aquariums were contaminated with the pesticides (both spirotetramat and 2,4-D). Three concentrations of each active principle were tested (0 (control), 10%, and 30% of the LC_{50} to nauplii). The 10% LC_{50} of each substance was also used to make up the mixed contaminant. Each test was performed with three replications. Microcosms were then inoculated with *C. abyssorum* gravid females (20 ind/L of culture solution). The chosen individuals were of approximately equal age (from nauplii pre-culture). The average amount of eggs per female in the inoculum used was determined using 30 females after isolation, separation, and eggs count under a microscope. After 30 days of culture, sex ratio and fertility of copepods in each treatment was evaluated (average of gravid females per liter and average number of eggs produced per female). The culture media were renewed each 48 h. Photoperiod was 16/8 (light/dark) and average temperature was 20.7 ± 0.2°C.

2.6. Statistical treatment

To stabilize the variances, all biological data were transformed by log (x + 1) according to FRONTIER [20]. Statistical tests and derived figures were established using STATISTICA, v7 software to monitor the parameters. Changes in the rate of egg hatching, population fertility, and rotifer and phytoplankton growth following the various treatments were evaluated using one-way ANOVA and the HSD tukey post-hoc test.

3. Results and discussion

3.1. Acute toxicity

The median acute toxicity value (LC_{50}) and the corresponding 95% confidence interval of the two pesticides to nauplii and *C. abyssorum* adults are presented in table 2. At the nauplial life stage, the 48-hour median lethal concentration (LC_{50}) was 96.79 mg/L and 34.85 mg/L for 2,4-D and spirotetramat, respectively; whereas, it was 101.7 mg/L and 37.42 mg/L at the adult stage for 2,4-D and spirotetramat, respectively.

Table 2. Median lethal concentration (LC50) at 48 hours for 2,4-D and spirotetramat on nauplii and C. abyssorum adults and their 95% confidence limits.

	Pesticides	LC50 (mg/L)	95% confidence interval	
			Upper	Lower
Nauplii	2,4-D	96,79	90,65	104
	Spirotetramat	34,85	32,51	37,89
Adults	2,4-D	101,7	95,53	110,5
	Spirotetramat	37,42	34,99	41,89

3.2. Effects on nauplii hatching and survival

Eggs hatchability (Fig. 2) in the various culture media has significantly varied across pollutant concentrations ($p < 0.05$). In the absence of a contaminant, 70% of the estimated eggs have hatched. Both chemicals mixed have affected hatching down to 32.1%.

Embryonic time and nauplii survival in polluted conditions are shown in Table 3. In the control, all females hatched on the second day. In the polluted treatment, the hatching point got extended to the 4th day. On the 10th day, 14.4 ± 1.5% of the hatched nauplii were dead in the controls. In the culture exposed to spirotetramat, 21.6 ± 0.4% and 31 ± 1.1% of nauplii mortality were observed at 10%LC_{50} and 30%LC_{50}, respectively. In the case of 2,4-D, mortality was respectively 23.6 ± 1.3% and 38.6 ± 1.9% at 10% and 30% of LC_{50}. Regarding the mixture, 29 ± 3.1% of the hatched nauplii were dead.

Table 3 Embryonic time duration and mortality of hatched Cyclops abyssorum nauplii exposed to different polluted conditions.

			J1	J2	J3	J4	J5	J6	J7	J8	J9	J10
Control		Hatching	-	5/5								
		Death %	-				12 ± 1	1.6 ± 0	0 ± 0	0 ± 0	0 ± 0	0.8 ± 0.1
Spirotetramat	10%LC_{50} (A)	Hatching	-	2/5		3/5						
		Death %	-				19.7 ± 0.3	1.1 ± 0.0	0 ± 0	0.1 ± 0.3	0 ± 0	0.7 ± 0.2
	30%LC_{50}	Hatching	-			5/5						
		Death %	-				23.6 ± 0.3	1.5 ± 0.1	5.1 ± 0.1	0.8 ± 0.7	0 ± 0	0 ± 0
2,4-D	10%LC_{50} (B)	Hatching	-	4/5	1/5							
		Death %	-				20.3 ± 1.4	0 ± 0	3.3 ± 0.8	0 ± 0	0 ± 0	0 ± 0
	30%LC_{50}	Hatching	-		1/5	4/5						
		Death %	-				33.1 ± 1.9	4.7 ± 1.1	0.1 ± 0	0.5 ± 0.2	0 ± 0	0.2 ± 0
Mixture	A+B	Hatching	-	1/5	3/5	4/5						
		Death %	-				16.8 ± 2.1	10.2 ± 1.9	0.6 ± 0.1	1.2 ± 0.2	0.1 ± 0	0.1 ± 0

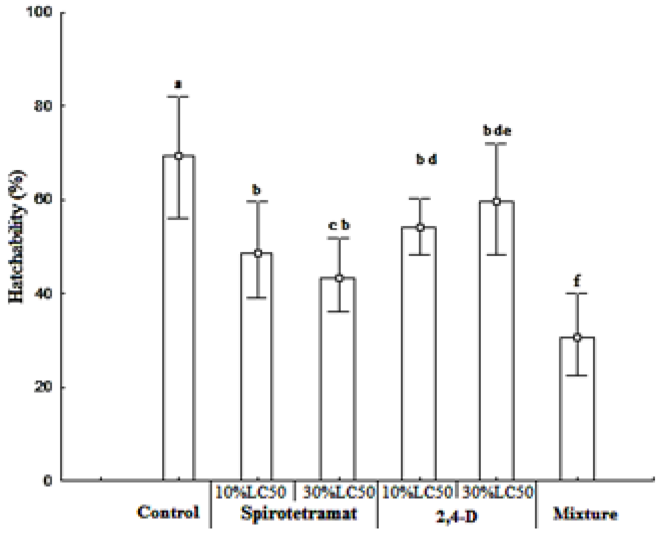

Figure 2. Rate of *Cyclops abyssorum* hatching according to the different conditions.
Moustaches with no common letter are significantly different (ANOVA 1, Tukey post-hoc, $p < 0.05$)

3.3. Effects on the sex ratio: microcosm

The females-to-males ratio is shown in Figure 3. It is observed that the tested pollutants at the different doses are favorable to females. In the absence of pollution, the ratio was one male for about 6 females, but this ratio increased to 1:12, 1:10, 1:12, 1:14 and 1:11 for the low and high concentrations of spirotetramat, low and high concentrations of 2,4-D, and the mixture, respectively.

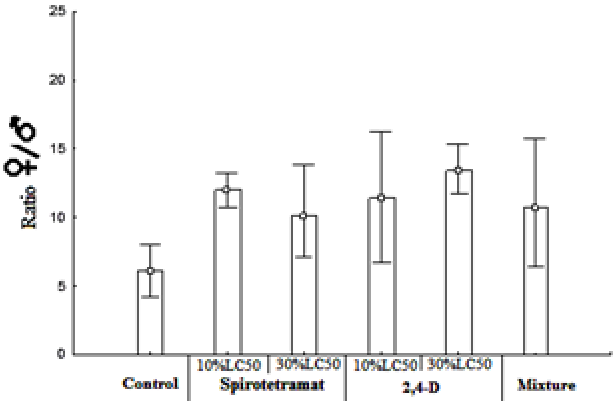

Figure 3: Sex ratio of *Cyclops abyssorum* adult of at the end of the exposure

3.4. Effect on fertility: microcosm

The parameters pertaining to the fertility of *C. abyssorum* are reported in table 4. The rate of population growth has been significantly varied among the treatments ($p < 0.05$). All treatment with pollutants gave lower densities, compared with the control treatment (1014 ± 1.1 ind/L). The 2,4-D herbicide was the least impactful, then came spirotetramat with its population density of 503 ± 3.1 ind/L, followed by the mixture treatment with a population density of 467 ± 4.4 ind/L. On the basis of the relative abundance of gravid females, except in the treatment with 10%LC50, no significant difference was observed among the control treatment (30 ± 1.2 ind/L) and the polluted cultures. As for the number of eggs laid per female, the profile among treatments showed that all polluted cultures have been significantly affected, compared to the control culture (132 ± 2.4 eggs/female).

Table 4: Population growth, gravid female abundance, and number of eggs per female according to the different treatments.

	Control	**Spirotetramat**		**2,4-D**		**Mixture**
		10%LC50	30%LC50	10%LC50	30%LC50	
Population growth (ind/L)	**20**/1014 ± 1,1*	**20**/832 ± 1,4**	**20**/503 ± 3,1***	**20**/791 ± 2,4**	**20**/642 ± 0,8**	**20**/467 ± 4,4***
Relative abundance of gravid females (%)	30 ± 1,2*	26 ± 0,9*	42 ± 0,3*	15 ± 1,9**	28 ± 1,1*	37± 0,1*
Number of eggs/female	132 ± 2,4* (30)	92 ±1,0** (30)	87,3 ± 0,5** (30)	75,7 ± 3,1** (30)	82,3 ± 3,0** (30)	78 ±1,1** (30)

The values in the same row carrying different numbers of asterix are significantly different (ANOVA 1, Tukey post-hoc, p <0.05)

4. Discussion

In order to study the dynamics of the population, it is essential to analyze the effects of each environmental factor on each life cycle parameter: namely, reproduction, development, and mortality (DEVREKER & al [21]).

The results of this study show a higher sensitivity to pesticides at both the nauplii and adults life stages. The median lethal dose (LC_{50}) in 48 hours, are lower for nauplii than adults. This includes both pollutants, which are parameters limiting the recruitment of the species in its ecosystem. The concentrations affecting adults could therefore impact significantly the early stages of life. The estimated LC_{50} at 48 hours are still high for both spirotetramat and 2,4-D, reflecting their relatively low toxicity (SAGE PESTICIDE, INRS [22, 23]). In general, 2,4-D appear to be less toxic to *C. abyssorum* than spirotetramat does. The greater sensitivity of the nauplii and the relative tolerance of cyclopoid species to pollutants have, in fact, been shown in several studies (MEDINA & al, GUTIERREZ & al, VAN DEN & al, SAIZ [15, 17, 24, 25]).

The studied pesticides, either singly or mixed, have affected both the survival and the reproduction of *C. abyssorum*. Species development stages are highly affected by environmental conditions. Embryonic development time is a factor that has a significant impact on the egg laying potential of a species (DEVREKER & al [21]). The extension of the embryonic period observed in this study is due to the absence of favorable conditions because copepod eggs typically go dormant for several days to several years (ALEKSEEV & LAMPERT, AVERY, RAZOULS & al [26, 27, 28]). It appears that the organic pollutants that spirotetramat and 2,4-D are, either singly used or mixed, affect hatching of *C. abyssorum* in aquatic environments.

The nauplii stage is very sensitive to the surrounding environment (MELAO & ROCHA [29]). Nauplii survival is very crucial to the recruitment and population replacement. Some authors have shown that the highest mortalities are in the embryonic and nauplii stages (DEVREKER & al, CAROTENUTO & al [21-30]). The reasons vary widely and involve environmental condition, predation stress, available food reserves at the hatching stage (impact on egg sizes), and also a maternally-induced impact on the viability of nauplii (IANORA & al [31]). These parameters, except the last and the predation, clearly explain the high mortality recorded for the different tests in this study. Because the species were bred in a micro biotope where food sources were more or less varied, the food allowed both predation and filtration. The presence of pesticide has certainly affected its quality. The feed used is abundant and came from the original location where the copepod is the predominant species. In this sense, the effect of food joins that of the environment through a direct or indirect mechanism. The transfer of certain pollutants to zooplankton predator is then more by bioaccumulation (KAISER [32]).

FORGET & al [33] asserted that some pesticide mixtures can affect copepod reproduction with a change in the egg-laying and in the sex ratio of the second generation. This explains the large variation observed in egg-laying of females bred in polluted medium and in their sex ratio. Authors have showed that a female copepod can have several clutches in its life, but eggs production is not constant and tends to decrease with age. The best productions are in the top third of its adult life (DEVREKER & al, JAMIESON & SANTER [21-34]). In this study, eggs production estimated at first spawning is not a perfect production; however, it is one of the most important in the lives of these individuals. The maximum lifespan of a female copepod is around 60 days (PAFFENHÖFER [35]) while MELAO and ROCHA [29] observed a maximum life of 98.7 days for *Tropocyclops prasinus* females and 90.9 days for *Mesocyclops longisetus* females at 20°C.

The sex ratio obtained at the end of the experiment shows a feminization of the population. Even in the absence of contamination it was more than six females for one male. This explains the predominance of the species in its natural environment, meaning that a male can fertilize several females. In the various cases of contamination, the degree of feminization was more than double that observed in the control groups. This poses an environmental problem: the lifespan of male individuals being lower than that of the female, in the absence of males all the eggs produced by the female can not hatch (GAUDY & PAGANON [36]). So, eventually, the species will go extinct due to excessive feminization, even though it may some times sound as if a population bloom were occurring (suggesting a proper use of the males). The sex ratio obtained at the end of the post-embryonic development of a population is highly variable depending on the species. It is rarely 1:1. The factors influencing the determination of the sex ratio are still very little known among copepods. However, some authors have suggested that temperature could play an important role (DEVREKER & al, LEE & al, DEVREKER [21-37-38]). This study shows that these two pesticides in aquatic ecosystems can play an important role in the feminization of copepods, particularly *C. abyssorum*.

4. Conclusion

If fertilization is not limiting, the time between two successive egg layings is determined by the time of embryonic development of eggs and the time required for the maturation of new eggs in the oocytes. Here, with spirotetramat and 2,4-D in the environment, the fertilization in *Cyclops abissorum* can become limiting due to the excessive feminization of the population. Also, all phases related to individuals recruitment (fertility, reproduction, and survival) would at times be disturbed. Both pesticides are major disruptors in pelagic ecosystems, affecting all the food chain.

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