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Effect of Herbicides Used in Vegetable and Fruit Crops (2,4-D, Glyphosate and Nicosulfuron) on Earthworms (*Eudrilus eugeniae*)

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Abstract: Horticulture requires a significant use of chemical inputs due to the parasitic pressure to which it is exposed. Among these inputs are the herbicides that are the most used. Due to their mode of action and their selectivity, those based on 2,4-D, glyphosate and nicosulfuron are detected as being the most widely used. This massive use is not without consequences on the environment and particularly on soil. Herbicides are frequently implicated in the reduction of soil fertility and terrestrial biodiversity observed in agricultural areas. This situation requires the greatest caution and clear information on the environmental risks actually incurred by in situ studies. This study was initiated with the aim of evaluating the impact of herbicides on soil life by using *Eudrilus eugeniae* earthworms as bioindicators. To do this, each experimental plot on which microcosms of earthworms were deposited received a herbicide treatment based on 2,4-D, glyphosate or nicosulfuron at the concentrations recommended by the manufacturer. Parameters such as biomass, number of cocoons, hatching rate and number of individuals per cocoon were evaluated. After 35 days of exposure, no effect was observed on the biomass of earthworms. Reproductive parameters such as number of cocoons, hatching rate and number of individuals per cocoon were strongly reduced by 2,4-D herbicides. Those based on glyphosate and nicosulfuron only reduced to lesser degrees the number of cocoons laid per pair compared to 2,4-D. The acute avoidance reaction test was a function of the herbicide and its concentration used. At the end of this study, it appears that the treatment of 2,4-D, glyphosate or nicosulfuron at the manufacturer's concentrations poses a threat to the specific richness of earthworms; they therefore reduce soil fertility. Those based on 2,4-D exhibit severe toxicity to soil health.

Keywords: Horticulture, Herbicides, 2,4-D, Glyphosate, Nicosulfuron, Soil, Earthworms

1. Introduction

Côte d'Ivoire, like developing countries, is experiencing strong demographic growth with a very young population [1]. This population growth implies higher vital needs in care, space, food and drinking water. Human food, which is a major necessity, will see its needs increase exponentially, leading to an increase in agricultural production [2]. This increase in food needs requires new production techniques. Cultivation techniques once used by farmers that did not necessarily require the use of chemical inputs have now given way to so-called intensive agriculture. This type of agriculture aims to produce in large quantities on small surfaces using a lot of chemical inputs [3]. Among these are the herbicides which represent to date, the most used phytosanitary products in agriculture, for the year 2016 alone, the country imported a quantity of pesticides estimated at 20,000 tons, two thirds of which consisted of herbicides. [4]. In horticulture, 63.64% of the pesticides used are herbicides

[5]. They are applied there 2 to 3 times a week depending on the cultivation area due to the growth of weeds and the low price of herbicides [4, 5]. In addition, their use reduces the cost of labor for horticulturists and decreases the ardor of work [6]. Glyphosate and nicosulfuron herbicides have been recognized as the most widely used in horticulture [5]. 2,4-D herbicides are used in combination with glyphosate for best results and accounted for 41.04% of herbicides approved by the Ivorian government in 2012 [7]. These herbicides are used on the one hand for their effectiveness and on the other hand for their selectivity in the face of certain speculations.

However, the use of these products is not without consequences on the environment, in particular the soil. It is reported that nearly 95% of herbicides reach a destination other than the target species. They are aimed at non-targeted species of natural environments and elements such as air, water and soil [8]. Herbicides influence the mineralization of soil organic matter which is a key property in determining soil quality and productivity [9]. In addition, after their application, the amount of herbicides that penetrates the soil can harm living organisms such as earthworms involved in soil fertility [10]. These organisms can be exposed to herbicides through different routes, either through direct contact during field application or through food [11]. Many species are therefore at risk of disappearing in the short term [12]. The rate of extinction of species by phytosanitary products is 1000 times higher than the natural rate and the rate of destruction of ecosystems is increasing day by day [13].

Aware of this, the scientific community has been interested in finding more relevant methods to measure the degree of soil pollution and the harmfulness of these herbicides. Physico-chemical analysis remains a method for determining the nature and quantity of contaminants in polluted environments. However, this approach remains limited, because it does not provide information on the effect of pollutants on living organisms and the consequences generated on the functioning of ecosystems. The evaluation of the quality of environments by the physico-chemical approach has many limitations due to their punctuality in time and space of the analyses, the performance of the analytical methods, the absence of data on the bioavailability substances detected, uncertainties about the causes and extent of intra- and interspecific variability in the sensitivity of organisms [14]. In addition to these methods, the use of bioindicator species such as earthworms is necessary. These are effect bioindicators. They report on the state and uses of the soil ecosystem. In addition, they meet the four rules that define a good indicator, namely relevance, reliability, sensitivity and reproducibility [15]. Previous studies have used earthworms as bioindicator species to learn about the impact of herbicides on the soil [16, 17]. Most of these studies have been done largely in the laboratory, away from environmental conditions. However, environmental factors influence the behavior of herbicides during phytosanitary treatments [18]. The objective of this study is to evaluate the effect of 2,4-D, glyphosate and nicosulfuron on earthworms in microcosm.

2. Material and Methods

2.1. Study Area

This study was carried out in the village of Abbe Begnini located in the commune of Azaguié. The Azaguié area is located in the southeast of Côte d'Ivoire, about 40 km north of Abidjan. The choice of the site is justified by the fact that it is one of the most important regions in terms of market gardening and fruit growing in the country. The experimental plot is located between 05°37439'N and 03°59216'W.

2.2. Material

2.2.1. Biological Materials

The biological material was essentially composed of:

1. adult earthworms of the species *Eudrilus eugeniae*;
2. rabbit droppings.

2.2.2. Herbicide Products

Three herbicides were used. Those are:

1. Topextra 720 SL from Topex-ci. The active ingredient is 2,4-D formulated as a liquid soluble (SL) at 720 g (amine salt) per liter of commercial solution. The manufacturer has recommended the use of 50 mL to 100 mL for a 15 L water sprayer corresponding respectively to solutions of 1.92 g/L to 2.4 g/L.
2. Glycel 410 SL from Topex Agro-Élevage Développement SARL. The active ingredient is glyphosate at a rate of 410 g (41% isopropilamine salt) per liter of commercial solution. To obtain the expected effect, the manufacturer recommended diluting 200 mL of product in 15 L of water per ½ hectare of land, which corresponds to a concentration of 5.47 g/L.
3. Sofa 40 SC distributed by AF-CHEM SOFACO. The active ingredient is nicosulfuron. The formulation used is a suspension concentrate (SC) with 40 g of nicosulfuron per liter of commercial solution. The manufacturer recommended diluting 1 L of product in 60 L to 200 L of water to treat 1 ha, which corresponds respectively to concentrations of 0.66 g/L to 0.2 g/L.

2.3. Methods

2.3.1. Breeding of Earthworms Eudrilus Eugeniae

(i). Capture of Earthworms Eudrilus Eugeniae for Breeding

Worms used for cultivation were trapped and captured at the study site. Trapping consisted of depositing piles of rabbit droppings in shady places and then watering them. After two weeks, *Eudrilus eugeniae* and other species appeared in the manure. Individuals carrying a clitellum of the *Eudrilus eugeniae* species were recovered and weighed before being transferred to the breeding tanks. The average weight of individuals captured was 1,27 ± 0,35g.

(ii). Preparation of Breeding Environment

The culture protocol was based on the chemical analysis guide developed by the Organization for Economic Cooperation and Development and the Centre d'expertise en analyse environnementale du Québec [19, 20]. It consisted of transferring 8 kg of soil to the bottom of a tank, followed by 1.5 kg of previously dried and sieved rabbit droppings. To humidify the environment 4.5 L of water was added. Each rearing environment was pre-composted for seven days before adding the earthworms. The purpose of this operation was to reduce the high temperature of the environment due to the activity of other microorganisms to a temperature of 28 ± 1°C. Seven adult *Eudrilus eugeniae* worms were introduced into each vivarium. After four weeks of rearing, juvenile worms of about seven days of age from the broodstock were transferred to environment of the same composition as the broodstock, at a rate of 50 worms per environment. The adult worms were used for testing. The culture environment were prepared in plastic trays of 42 cm x 32 cm x 22 cm. The bottom of each tray was finely perforated to allow excess water to pass through. The lid of the culture tray was also perforated to allow good ventilation of the environment. Under these lids, a 500 µm diameter mesh screen was placed to prevent worms from escaping.

2.3.2. Acute Avoidance Reaction Test

This study was inspired by the protocol described by Environnement Canada and in ISO 17512-1:2008-Soil quality [21, 22]. In order to know the behavior of earthworms towards plant protection products, an experimental compartment was dedicated to each herbicide studied. A total of 9 compartment were used, with three experimental compartment for 2.4-D, glyphosate and nicosulfuron respectively. Approximately 300 g of dried and sieved soil were deposited in each experimental compartment. Herbicide slurries of 2.4-D, glyphosate or nicosulfuron were previously prepared according to the manufacturers' recommendations using fountain water. The concentrations of the prepared slurries were 1.92 g/L and 2.4 g/L for 2,4-D, 5.47 g/L for glyphosate, and 0.2 g/L and 0.66 g/L for nicosulfuron, respectively. This amount corresponds to the water holding capacity of the soils in the experimental compartment. The contaminated soils were alternated with the uncontaminated soils in each experimental enclosure. A total of three soil compartments were used for the control soils and three additional compartments for the contaminated soils. The control compartments received 150 mL of fountain water as a replacement for the herbicide slurries. Each compartment was labeled for identification. Ten adults *Eudrilus eugeniae* earthworms with an average mass of 1.14 ± 0.26 g were then deposited one at a time in the central stack of the experimental enclosure. To allow aeration of the earthworms, each environment was covered with a mosquito net (Figure 1). The test lasted 48 h after which the number of earthworms present in each compartment was recorded. In addition, no food was provided to the earthworms during the entire test period. The avoidance rate was calculated according to equation (1).

$$AR = \frac{(No.of\ worms\ (uncont\ soil) - No.of\ worms\ (cont\ soil))}{(Total\ No.of\ worms)} \times 100 \quad (1)$$

AR: avoidance rate.

No. worms (uncont): number of live worms found in all non-contaminated soil compartments at the end of the test;

No. of worms (soil cont): number of live worms found in all contaminated soil compartments at the end of the test;

Total no. of worms: total number of live worms found in all compartments, at the end of the test.

Figure 1. *Experimental enclosure.*

2.3.3. Chronic Effect of 2,4-D, Glyphosate and Nicosulfuron Herbicides on Earthworms Eudrilus eugeniae

(i). Preparation of the Environment

The experimental unit was a 900 mL clear plastic box containing 100 g of rabbit droppings added to 300 g of dried and sieved soil. The contents of the boxes were moistened with 200 mL of fountain water. The bottom and lid of the boxes were perforated beforehand (Figure 2). The purpose of this perforation was to ensure the circulation of water and air in the environment. The bottom of the holes in which each microcosm rested was lined with stones to ensure efficient water drainage. The soil level in the box coincided with the surrounding soil. The environment was prepared 24 h before the start of the test. The tests in this study were conducted in the undergrowth in order to promote moisture in the environment. Several parameters were evaluated such as biomass, number of cocoons laid, hatching rate and number of individuals per cocoon. In order to avoid any modification of the concentration in the environments, no food was added during the tests.

Figure 2. *Microcosm.*

(ii). Herbicide Treatments of Plots

The experimental plot was subdivided into four elementary plots with an area of 1350 m^2 5 m apart. Each elemental plot received a single herbicide treatment of 2,4-D, glyphosate or nicosulfuron. The fourth elemental plot was used as a negative control to assess the intrinsic toxicity of the environment, the health status of the worms and the validity of the test. The concentrations of the various herbicide porridges have been prepared on the basis of the lowest doses recommended by the manufacturer. These are 2.4 g/L for 2,4-D, 5.47 g/L for glyphosate and 0.66 g/L for nicosulfuron. The fountain water was used to prepare herbicide porridges. The treatments were carried out using a 16 L sprayer. The tests were carried out simultaneously.

(iii). Effect on Biomass

The test protocol was based on the OECD guideline for chemical testing; Environment Canada EPS 1/RM/43 and MA.500-VTL1.0, developed by CEAEQ [19-21].

For this experiment, three earthworms of the species *Eudrilus eugeniae* of 1.04 ± 0.13 g were added to each of the microcosms intended for testing the effect of herbicides on biomass. Fifty microcosms were placed on each elementary plot after the different herbicide treatments, and the mass of the worms was measured every week for five weeks. In order to avoid the effect of stress caused by the manipulations, the masses of the individuals of ten microcosms are measured per week. Once the mass is measured, these microcosms are removed so that measurements are not obtained on the same boxes.

(iv). Effect on Cocoon Production

The test procedure was based on ISO methods and the OECD guideline for determining the effects of contaminated soil on the reproduction of *E. andrei/fetida* [23-25].

Two individuals of worms approximately 35 days old with an average mass of 1.04 ± 0.13 g were introduced into microcosms for the cocoon production test. Forty microcosms were prepared for this test with 10 microcosms per elementary plot. After the herbicide treatments, the number of cocoons laid in each box was recorded every five days of the test. Cocoons counted were removed from the microcosms. The test lasted 35 days.

(v). Effect on Hatching Rate and Number of Individuals Per Cocoon

One cocoon from the rearing environment was used for each microcosm. Forty microcosms were prepared for this test with 10 microcosms per elementary plot. After 17 days after the herbicide treatments, the hatching rate was evaluated according to equation (2). The number of individuals per cocoon was counted at the end of day 25 to ensure that individuals were of sufficient size to be easily visible.

$$HA\ (\%) = \frac{NHC \times 100}{TNC} \quad (2)$$

Where
HA (%): hatching rate,
NHC: number of hatched cocoons,
TNC: total number of cocoons put in culture.

2.4. Analyses Statistiques

STATISTICA version 7.1 software was used to perform the statistical tests. One-factor analysis of variance (ANOVA I) was used to compare the means of the different parameters obtained for each treatment at the 5% significance level. In case of significant difference, multiple comparisons of means were performed using Tukey's test. Quantitative data organized in a matrix grouping individuals and measured parameters were processed by principal component analysis (PCA).

3. Results and discussion

3.1. Results

3.1.1. Avoidance Reaction Test

(i). Observation of the Experimental Enclosure at the End of the Test

On the first day of the test, after their introduction into the central stack, all earthworms directly avoided the 2,4-D contaminated soil compartments. Some worms, however, were able to enter the soil compartments contaminated with glyphosate and nicosulfuron. These observations were the same regardless of the concentrations used. After 48 h of exposure, galleries and castings were observed in the uncontaminated soil compartments of the 2,4-D and nicosulfuron test enclosure (Figure 3A). On the other hand, signs of relatively low activity were observed in the glyphosate-contaminated soil compartments compared to those present in the control soils (Figure 3B). In addition, turricules were also observed in the central stack of all experimental enclosure.

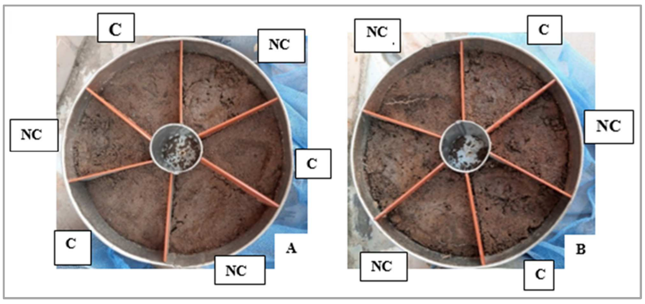

Figure 3. *Signs of earthworm activity.*

A: Galleries and castings present only on uncontaminated soil; B: Galleries and castings present on contaminated and uncontaminated soil.;
C: contaminated soil; NC: non-contaminated soil.

(ii). Determination of Avoidance of Herbicide-contaminated Environment by Earthworms

The results of avoidance, as well as the average number of earthworms that entered each compartment at the beginning of the test and those living there at the end of the test, are detailed in Table 1.

At the end of the test, all earthworms were found in the uncontaminated soil compartments of the 2,4-D experimental enclosure regardless of the solution concentration used. The avoidance rate was therefore 100% for the 1.92 g/L and 2.4 g/L concentrations. The nicosulfuron-contaminated enclosures caused almost all earthworms to migrate to the control soils. Avoidance rates were 100% and 80% for the 0.66 g/L and 0.2 g/L concentrations, respectively. For glyphosate, the avoidance rate was 20%. In addition, no mortality was recorded in any of the experimental enclosure during the test.

Table 1. *Earthworm avoidance rates as a function of herbicide concentrations used.*

Soil type by herbicide		NE start of the test	NL end of test	Avoidance rate (%)
2,4-D 1,92 g/L	UCS	10	10	100
	CSC	0	0	
2,4-D 2,4 g/L	UCS	10	10	100
	CSC	0	0	
Glyphosate 5,4 g/L	UCS	3	6	20
	CSC	7	4	
Nicosulfuron 0,66 g/L	UCS	10	10	100
	CSC	0	0	
Nicosulfuron 0.2 g/L	UCS	7	9	80
	CSC	3	1	

$NE_{start\ of\ the\ test}$: Average number of worms that entered each compartment at the beginning of the trial;
$NL_{end\ of\ test}$: Average number of live earthworms in each enclosure at the end of the trial;
UCS: uncontaminated soil compartment;
CSC: contaminated soil compartment.

3.1.2. Chronic Effect of 2,4-D; Glyphosate and Nicosulfuron on Earthworms

(i). Observation of the Microcosms at the End of the Tests

The effectiveness of the 2,4-D herbicide was clearly demonstrated after seven days. Almost all plants had lost their leaves. The stems had dried out. Those treated with glyphosate had yellowish looking leaves two weeks after treatment. These plants died after three weeks. No effect of the nicosulfuron herbicide was observed on the weeds in the experimental plot until the end of the test (Figure 4A).

The microcosms were removed from the experimental plots after 35 days. Signs of activity such as galleries, castings were observed in all experimental units of the treated plots (Figure 4B). No mortality was observed during the test.

A: galleries; B: castings

Figure 4. *Signs of earthworm activity.*

(ii). Effect on Biomass

Statistical analyses revealed no significant difference at the 5% level between the mass of *Eudrilus eugeniae* worms obtained for each treatment performed. The mass of earthworms present in the contaminated microcosms increased in the same way as the mass of worms present in the control microcosms (Table 2).

At the end of the test, the average mass of earthworms in the microcosms of the 2,4-D, glyphosate and nicosulfuron treated plots were 1.13 ± 0.15 g, 1.1 ± 0.18 g and 1.09 ± 0.14 g respectively compared to 1.15 ± 0.14 g in the microcosms of control plots. The differences in mass from the mass at the beginning of the test were 0.09 ± 0.02 g for 2,4-D, 0.06 ± 0.05 g for glyphosate and 0.05 ± 0.01 g for nicosulfuron. Those of worms in the control microcosms were 0.11 ± 0.01 g.

These results show that herbicide treatments of 2,4-D; glyphosate or nicosulfuron under field conditions do not affect earthworm mass.

(iii). Effect on Cocoon Production

For this test, the number of cocoons laid by earthworms varied by treatment type. The one-factor ANOVA test revealed a very highly significant difference at the 5% threshold between the numbers of cocoons obtained for each treatment ($p < 0.001$). *Eudrilus eugeniae* pairs in microcosms treated with 2,4-D, glyphosate, or nicosulfuron laid an average of 6.4 ± 5.12; 15.4 ± 5.12; and 15.8 ± 3.12, respectively, compared with 24.2 ± 4.02 in control microcosms (Table 2). The presence of herbicide in the environment causes a reduction in the number of cocoons laid. Multivariate comparison by turkey HSD test revealed three homogeneous groups (Table 2).

The 2,4-D herbicide was more damaging than glyphosate and nicosulfuron. This herbicide reduced the number of cocoons by one third.

(iv). Effect on Cocoon Hatching Rate

Cocoon hatchability was dependent on the nature of the environment. Statistical analyses reveal a very highly significant difference between hatching rates and herbicide treatments ($p < 0.001$). The hatching rate of cocoons from the control and glyphosate and nicosulfuron treated environments was 100% while that of cocoons from the 2,4-D contaminated microcosms was 60%, a 40% reduction in hatching rate (Table 2).

Thus, glyphosate and nicosulfuron herbicide treatments had no effect on hatch rate, while 2,4-D treatments significantly reduced cocoon hatch rate.

(v). Effect on the Number of Individuals Per Cocoon

The microcosm test on the effects of 2,4-D, glyphosate, and nicosulfuron on the number of individuals per cocoon revealed a very highly significant difference at the 5%

threshold (p < 0.001). The number of individuals per cocoon was a function of the environment. The number of individuals from cocoons obtained from the 2,4-D treated plots was 1 ± 0.8 individuals, those from cocoons laid on the glyphosate and nicosulfuron treated plots were 4.3 ± 0.54 and 4.4 ± 0.44 individuals respectively compared to 4.4 ± 0.54 individuals from cocoons on the control plot (Table 2).

With respect to the control and plots treated with glyphosate and nicosulfuron herbicides, 2,4-D herbicides significantly reduced the number of earthworm individuals per cocoon (p<0.001). Turkey's HSD test revealed two homogeneous groups. The means obtained showed that glyphosate and nicosulfuron herbicides had no effect on the number of individuals per cocoon.

Table 2. *Statistical tests of the studied parameters according to the treatments.*

Parameters	Treatments			Statistics		
	2,4-D	Glyphosate	Nicosulfuron	Control	F	p
Biomass (g)	1,13 ± 0,15	1,1 ± 0,18	1,09 ± 0,14	1,15 ± 0.14	0,37	>0.05
Number of cocoons per pair	6,4 ± 5,12 [a]	15,4 ± 5,12 [b]	15,8 ± 3,12 [b]	24,2 ± 4,02 [c]	131,3	<0.001
Hatching rate (%)	60 [a]	100 [b]	100 [b]	100 [b]	125,3	<0.001
Number of individuals per cocoon	1 ± 0,8 [a]	4,3 ± 0,54 [b]	4,4 ± 0,44 [b]	4,4 ± 0,54 [b]	147,8	<0.001

Values on the same line with the same letter are statistically identical at the 5% level according to Tukey's HSD test. F: Fisher's value; p: Probability.

(vi). Comparative Effect of Herbicides on Earthworms

Correlation results from principal component analysis (PCA) indicate that axis 1, which extracts more than 68% of the results, is positively correlated with the number of cocoons per pair (NC/C), hatching rate (TE), and number of individuals per cocoon (NI/C). Axis 2, which extracts more than 25% of the information, is positively correlated with earthworm biomass (BMS) (Figure 5A).

The organization of the populations on the plane of axes 1-2 shows a division of individuals into three distinct groups (Figure 5B). Group 1 (in the positive portion of axes 1 and 2) is predominantly composed of control environment. Group 2 (in the negative portion of axis 2) gathers the glyphosate and nicosulfuron treated microcosms. These groups are characterized by a high number of cocoons per pair, a high hatching rate and a high number of individuals per cocoon. Glyphosate and nicosulfuron showed results approximately equal to the controls.

The distribution of the treatments in the factorial plane revealed a demarcation of the environments contaminated with 2,4-D. The individuals of this 3rd group (in the negative part of axis 1 and positive part of axis 2) are characterized by a relatively low number of cocoons per pair, hatching rate and number of individuals per cocoon.

In addition, herbicide treatments of 2,4-D, glyphosate and nicosulfuron had no effect on earthworm biomass.

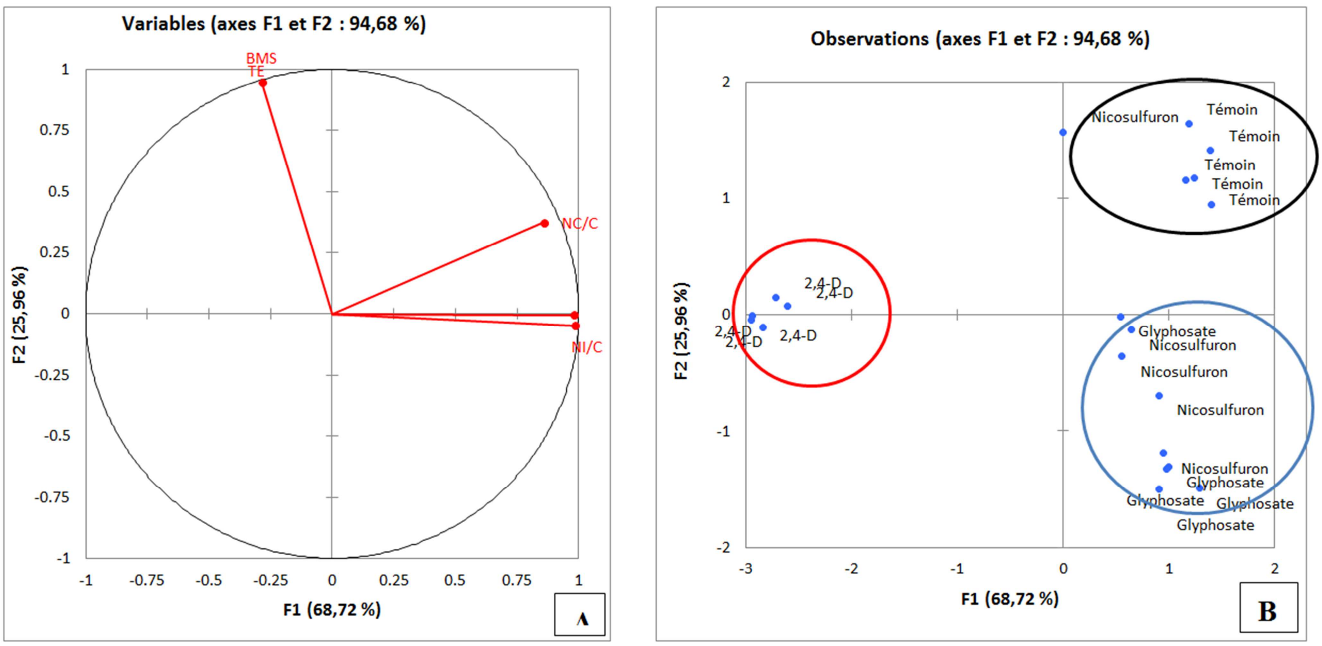

Figure 5. *Principal Component Analysis (PCA) of reproductive parameters and biomass.*

A: correlation circle; B: projection of individuals in the factorial plane of axes 1 and 2; BMS: biomass; NC/P: number of cocoons per pair; HR: hatching rate; NI/C: number of individuals per cocoon.

3.2. Discussion

This study revealed a more or less migratory behavior of the earthworms *Eudrilus eugeniae* exposed to the usual concentrations of 2,4-D, glyphosate and nicosulfuron. Avoidance was 100% with the 1.92 g/L and 2.4 g/L concentrations of 2,4-D. For glyphosate, the manufacturer's recommended concentration of 5.47 g/L resulted in 20% avoidance. Nicosulfuron induced 100% and 80% avoidance at 0.66 g/L and 0.2 g/L respectively. These avoidance reactions could be due to the sensitivity of earthworms to the different concentrations of herbicides used. Indeed, work has revealed that the lowest concentrations of pesticides are able to cause several morphological abnormalities such as bloody lesions, loss of skin coloration, strangulation of the posterior part of the body and even death in earthworms [26]. Furthermore, other studies revealed the ability of *Eisenia foetida* to detect and avoid low concentrations of Mancozeb in favor of lead nitrate contaminated soil [27]. The results obtained are supported by other authors who revealed a 65% avoidance response by *Aporrectodea caliginosa caliginosa* earthworms in the presence of lambda-cyhalothrin [28].

In terms of biomass, the herbicides 2,4-D, glyphosate and nicosulfuron produced no remarkable effect during the test. This result could be explained by the fact that the concentration recommended by the manufacturer during the treatments is low enough to cause a noticeable effect on earthworm biomass. Indeed, following the application of herbicide products in the environment, they are largely infiltrated or degraded, which could contribute to the reduction of their harmful effects. These results are in agreement with those of other authors who showed that 2,4-D and glyphosate did not cause any significant effect on the biomass of earthworms *Eudrilus eugeniae* exposed to 20 mg/L and 2000 mg/L respectively [17].

The reduction in the number of cocoons by 2,4-D, glyphosate and nicosulfuron herbicides could be explained by the stress that earthworms undergo due to the presence of pollutants in the environment. Under stressful conditions, earthworms would reduce their reproduction and their energy is redistributed into survival mechanisms [29]. Some studies have shown that metal-stressed earthworms enhance detoxification pathways, DNA damage repair processes or mucus production [30]. The reduction in the number of cocoons of *Eisenia foetida* earthworms by 2,4-D, glyphosate herbicides has also been observed in the laboratory [31]. In addition, exposure of *Achatina fulica* snails to the same concentrations of 2,4-D, glyphosate and nicosulfuron used in this study induced a reduction in the number of eggs laid [32].

In addition, it was found that the hatching rate was significantly reduced by the presence of 2,4-D herbicide. This could be explained by embryonic abnormalities due to the bioaccumulation of this herbicide in the eggs of *E. eugeniae*. Indeed, the octanol/water partition coefficient (log kow) is 2.5 for 2,4-D, -5.4 for glyphosate and 0.61 for nicosulfuron. The comparison of these values reflects the high lipophilic character of 2,4-D compared to other herbicides. The results obtained are in agreement with those of [33]. These authors revealed a 29.4 ± 1.04% reduction in the hatching rate of earthworms *Eudrilus eugeniae* exposed to a concentration of 20 mg/L of 2,4-D in the laboratory.

The reduction in the number of individuals per cocoon observed in microcosms in 2,4-D treated plots could be explained by alterations in cell multiplication and differentiation that 2,4-D may cause during embryogenesis [33]. The studies Fry have also highlighted the adverse effect of 2,4-D on the embryo of birds [34]. The reduction in the number of juveniles by 2,4-D herbicides was also observed in the laboratory [31]. It was greater than that caused by glyphosate herbicides on *E. foetida*.

In this study, the 2,4-D herbicide was more toxic than glyphosate and nicosulfuron. Glyphosate and nicosulfuron herbicides showed almost equal toxicity to earthworm reproduction. The difference between these effects could be due to the nature of the herbicides used.

In animal cells, 2,4-D acts mainly on the mitochondria, increasing the formation of activated oxygen derivatives, which are responsible for membrane peroxidation and depletion., resulting in increased apoptosis, leading to cell death [35]. This harm of pure 2,4-D to animal cells coupled with that of the co-formulants would be responsible for the high values of avoidance rate, number of cocoons, hatching rate and number of earthworm individuals per cocoon.

The target of glyphosate is an enzyme called 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) which is only present in plants and in some bacteria. Therefore, this molecule has no affinity with the biological systems of animals. The effects observed in the rate of avoidance and the number of cocoons of glyphosate would come from the co-formulants used in the preparation of herbicides. Their toxicities are organism dependent [36].

Nicosulfuron, although acting on acetolactate synthase in plants and microorganisms, is also an inhibitor of acetylcholinesterase. Studies revealed a negative impact of nicosulfuron on soil insects due to the inhibition of acetylcholinesterase [37]. Significant inhibition of this enzyme was also shown in the brain of goldfish *Carassius auratus* following exposure to a sublethal dose of nicosulfuron. It has also been shown that exposure of goldfish to different doses of nicosulfuron affects certain behavioral characteristics such as swimming, aggregation and escape [38]. The effect of adjuvants and nicosulfuron would therefore be responsible for the high rate of avoidance and effects on cocoon production. The concentration of 0.2 g/L induced a lower rate of avoidance compared to 0.66 g/L due to the amount of herbicides used for the spray. The avoidance rate increased as the concentration increased.

4. Conclusion

Herbicides based on 2,4-D, glyphosate, and nicosulfuron used in planting, negatively influence the life of earthworms *Eudrilus eugeniae*.

Earthworms flee from contaminated environments depending on the nature and concentration of the herbicide used in the treatment. Exposure of earthworms for five weeks to 2,4-D, glyphosate and nicosulfuron did not cause any effect on biomass. However, a toxic effect was observed in the microcosms of the reproduction tests. Herbicide treatments with 2,4-D, glyphosate and nicosulfuron caused a significant reduction in the number of eggs laid. The 2,4-D treatment also caused a 40% reduction in hatching rate and a decrease in the number of individuals per cocoon.

In view of the above, it should be noted that the phytosanitary treatments of 2,4-D; glyphosate and nicosulfuron have deleterious effects on soil life. The 2,4-D herbicide treated at the manufacturer's recommended concentrations has a more pronounced toxicity.

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