Study on The Effect of Ammonium Hydroxide on Survival, Growth, Reproduction and Cocoon Hatching of *Eisenia fetida*

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ABSTRACT

In this study, two chronic toxicity tests were conducted to assess, in vitro, the toxicity effect of ammonium hydroxide 'AH' on survival, growth, reproduction and cocoon hatching of *Eisenia fetida* worm. Adult earthworms and cocoons were exposed to an increasing AH concentration (0, 0.05, 0.1, 0.5, 1 and 5mg.g^{-1} of soil). Mortality, growth, cocoon production and juveniles' emerging were measured over 56 days to determine LC_{50}, EC_{50}, NOECs and LOECs values. Moreover, the percentage of hatching success and number of juveniles emerging were recorded. For the first test, our results show that AH caused a high mortality rate, decrease of: biomass, cocoon produced and hatchlings emerging trend with increasing of the AH concentration and exposure time. The calculated LC_{50} after 28-d was 0.85 and 0.83mg.g^{-1} after 56-d. Otherwise, the obtained EC_{50} for biomass changes after 28-d and 56-d were 1.64 and 0.82mg.g^{-1}, indeed, the EC_{50} of juveniles' production was 0.82mg.g^{-1}. The estimated NOEC value was similar for survival, growth and reproduction (0.5mg.g^{-1}). Otherwise, AH has a toxic character on *E. fetida* cocoon hatchability, the estimated NOEC value of cocoon hatching success was 1mg.g^{-1}.

KEYWORDS: Ammonium Hydroxide, *Eisenia fetida*, Growth, Reproduction, Cocoon Hatching

List of Abbreviations

T: Temperature (°C)
AH: Ammonium Hydroxide
-d: Days
EC: Environment Canada
EC_{50}: Effect concentration at which 50% effect (mortality, inhibition of growth, reproduction, etc) is observed compared to the control box.
ISO: International Standardization Organization
LC_{50}: Lethal concentration (The concentrations of the chemical that kills 50% of the test animals during the observation period).
LOEC: Lowest Observed Effect Concentration
NH_3: Ammonia
NH_4^+: Ammonium
NOEC: No Observed Effect Concentration
OECD: Organization for Economic Co-operation and Development
pH: Potential of Hydrogen

INTRODUCTION

Soil is a dynamic and complex system functioning as a habitat for microorganisms, flora, edaphic invertebrates, animals and humans [1]. Edaphic invertebrates such as earthworms have the potential to be a useful indicator of soil function and quality as they influence and are influenced by the physical and chemical properties of the soil [2]. Earthworms are a major component of the animal biomass of terrestrial ecosystems where they play several biological roles; as food for other organisms, interactions with plant roots and soil micro-organisms, chemical and physical functions while they play a crucial role in maintaining the structure and fertility of soils recycling nutrients, increasing aeration and drainage, eat and crush soil, mixing mineral layers and organic compounds to produce soil crumbs [3;4]. The ability of earthworms to perform these beneficial functions in the soil can be inhibited upon exposure to harmful substances like ammonia and ammonium hydroxide AH.

Agricultural NH_3 emission has become one of the major air pollution problems in recent years. Ammonia from animal wastes and fertilizers was believed to constitute about 90% or more of the anthropogenic NH_3 emission [5]. According to [6], ammonia in the atmosphere can be deposited to the surface of the earth by either wet or dry deposition; wet deposition is simply the uptake of ammonia into precipitation. Ammonia deposition is responsible for causing various negative environmental effects: eutrophication of nitrogen sensitive ecosystems and acidification of soils, and cause loss of biodiversity and other deleterious effects [7-9]. The atmospheric concentration of ammonia (NH_3) is often high in countries with intensive agriculture and animal husbandry [10]; consequently, the effects of its deposits on soil organisms, (e.g., earthworms) are more intensive in these areas. The ammonia deposition in the soil is also increasing during the anarchic spreading of poultry droppings which contributes to an

additional supply of this pollutant [11], this last can be more hazardous if agriculture didn't respect the storage period of poultry droppings. The ammonia is highly water-soluble; and the dissolution of ammonia gas NH_3 in water is called ammonium hydroxide (AH) solution [12; 13]. According to [14] earthworms are very sensitive to ammonia and cannot survive in organic wastes containing high levels of this cation (e.g., fresh poultry litter).

The need for protecting earthworms has become inevitable. As a good indicator of soil quality, earthworms were used as testing organisms by OECD in the early 1980s [15]; they represent 60-80% of the total soil animal/invertebrate biomass. Many ecotoxicological studies have used earthworms as biological models to assess potentially toxic materials and chemicals in soils e.g.: [16; 17; 18; 19; 20; 21; 22; 11]. Furthermore, by having chemoreceptors in the prostomium and sensory tubercles on their body surface, they can provide a high sensitivity to chemicals in soil [23]. According to [24], they are useful to model organisms because many aspects of their response to environmental perturbations can be assessed and connected to environmental outcomes, including their avoidance behaviour, growth rate, enzyme activity level, mortality, and reproduction patterns. They are a key indicator organism in acute and chronic laboratory tests for the detection of side effects of chemicals [25].

An acute toxicity test [26] has been used to determine the concentrations of chemicals that cause specific lethal and sub-lethal effects in the earthworm. [27] found a reasonable correlation between the results of acute toxicity tests and the effects observed in the field, moreover, the adverse effect of sub-chronic or chronic exposure is also important in ecological risk assessments [28; 29]. Nevertheless, according to [30], the use of edaphic invertebrates in acute ecotoxicological tests has shown some disadvantages: acute tests are not ecologically relevant when compared to chronic ones, because they do not provide insight into the effects on the population dynamics, while chronic tests last too long and are very labour intensive. Moreover, the endpoint of the earthworm acute toxicity test is mortality. However, mortality is unlikely to be either the most sensitive or ecologically relevant parameter for predicting effects on field populations. Reproductive and/or growth disturbances are far more likely to mediate population effects. To date, the growth and reproduction of earthworms have been important endpoints used in environmental eco-toxicity [31].

To our knowledge, little information regarding on toxicity of ammonia solution (AH) to soil-living organisms is available. Studies on AH toxicity have focused more on aquatic species and less on terrestrial invertebrates in soils; like earthworms.

Our study is one of the first where the chronic toxicity tests were adopted as the testing methods, in order to assess the effect of ammonium hydroxide (AH) on *Eisenia fetida* taxon from point of view: of survival (mortality), growth (biomass change), reproduction and cocoon hatchability. The main of this study is to get a more comprehensive understanding of the toxic effects of AH on earthworms and to provide information and baseline data to be used in ecological risk assessment on the soil ecosystem. In addition, this work sheds light on the potential exposure to the organic wastes containing high levels of this cation in livestock areas (e.g., poultry droppings) and the effects of potential exposure of earthworms to ammonia generated from agriculture (poultry farming), which return to the biosphere occurs through wet deposition in the form of aerosols of NH_4^+ into precipitation.

MATERIALS AND METHODS

Test Species

The tests were conducted with the earthworm *Eisenia fetida*. It is frequently used as a biological monitor for testing the effects of contaminants on soil biota, and it is the test species recommended by the Organization for Economic Co-operation and Development (OECD) and International Standardization Organization (ISO) [26, 32; 33; 34]. It is a prolific species and its rearing in a laboratory setting is simple. They were obtained from laboratory cultures (laboratory of Animal Ecology, Mentouri Brothers Constantine 1 University, Algeria), and kept at room temperature ($20 \pm 2^{\circ C}$). Earthworms were maintained in plastic boxes (10 L) covered with perforated lids. The culturing of earthworms in the laboratory was done according to the guidelines specified by [32]. The pH of the culture medium is adjusted to (6.0–7.0) with $CaCO_3$. Earthworms are fed according to demand, usually once a week, with wheat bran. The water content of the soil was measured each week and the moisture was adjusted to [35-40%] of the maximum water-holding capacity by adding distilled water as needed.

For the first test, only adult worms with well-developed clitella (sexually mature) were used, they were collected from synchronized cultures with homogeneous age structures and were approximately the same size and weighing between 250 and 600mg. While cocoons with homogeneous sizes were used in the second test and were collected just after the cocoons laying by the adults.

Test Chemical

Ammonium hydroxide (NH_4OH) refers to the basic aqueous solution of ammonia gas, the solution used for

this study contains near (30%) of this gas. The ammonia in the solution reacts with water to produce ammonium cations NH_4^+ and OH^- hydroxide anions in equal amounts; the equilibrium between these two forms is governed by a sensitive reaction to pH changes [35]. The release of hydroxide OH^- ions gives the weak basic character of ammonia solution.

Toxicity test methods

Survival, growth and reproduction test

The reproduction tests with *E. fetida* were carried out according to [36] guidelines with some modifications. Before carrying out the experiment, adult animals of similar size with a fully developed clitellum and an individual fresh weight between 250 and 600mg were preconditioned in the untreated soil before the dose-response test, for a period of 7th day with a similar condition of test (alimentation, lighting, temperature, humidity). Afterwards, each group of 2 animals was cleaned in water to remove soil particles, gently dried, weighed, and placed into the test container. The total numbers of adults were 120 earthworms distributed in 60 plastic containers. In each test container (19 x 14 x 6cm^3) 450g of soil were placed. The used soil is a forest soil collected from the Ichemoul region (35° 18′ 00″ N, 6° 29′ 00″ E) in the region of Batna (North-East of Algeria). It was used also for the second bioassay (cocoon hatching test). The soil was air-dried at 30 to 40°C, sieved through a 2 mm mesh, and stored until use. The physicochemical characteristics of this soil were: pH = 7.3, electric conductivity = 0. 22dS.m^{-1}, organic matter = 4.32%, total carbonates = 17.30 %, total nitrogen = 4×10^{-3} % (NH_3 2.5×10^{-4} % and NO_2 5×10^{-4} %). In this study, the soil was taken to conduct the soil test, is complemented with 30% of organic compost, it was homogenized before its use in experimentations.

In this test, control and five tests concentrations of AH were used: 0, 0.05, 0.1, 0.5, 1 and 5 (mg.g^{-1} of soil), the experimentation was carried out under laboratory conditions; hence, we have maintained a fixed rang concentration of AH during the test.

The dispersion in the concentration range was made for two main reasons (i) to determine the concentration range that resulted in 0 and 100% mortality respectively, these two values are sufficient to indicate between which limit is the LC_{50}, and (ii) to obtain the $EC_{50,}$ NOEC and LOEC values. The test solution (AH) was mixed thoroughly and homogeneously in the soil. The controls were prepared similarly but only moistened with deionized water and no-AH. Ten replicates were used for each tested concentration and the control.

The plastic containers were perforated at their base to ensure the evacuation of excess water and loosely covered with polypropylene lids, allowing an exchange of air, and stored at $20 \pm 2^{\circ C}$ with 80–85% relative humidity under 400–800 lux of constant light with a 12:12-h light: dark photoperiod. In toxicity tests, the water content was adjusted to 35% by the addition of deionized water. During the test, organisms were fed once a week, with 5g per box of wheat bran, and the soil moisture content was weekly monitored and adjusted whenever necessary.

Adult earthworms and the produced cocoons and juveniles persisted in the soil until 56 days have been completed. During this period, survival and biomass (at 1st day, 28th day and 56th-day intervals after treatment) were assessed. At the end of the test, living earthworms were gently extracted from the soil by hand sorting and were counted and then transferred to a Petri dish for weighing. Worms shall be washed prior to weighing (with distilled water) and the excess water removed by placing the worms briefly on filter paper. Any worms no found at this time are to be recorded as dead and decomposed prior to the assessment; an aliquot of the soil was taken for chemical analysis and was examined for the presence of cocoons. The remaining soil was wet sieved with a 2 mm mesh and then with a 1 mm mesh, and the total number of cocoon formations for each concentration was counted on the mesh, moreover, juveniles from each test container were counted and weighed. The number of hatchlings produced per worm was calculated using the following formula:

$$Number\ of\ hatchling\ produced\ per\ worm = \frac{Total\ number\ of\ hatchlings}{Total\ number\ of\ worms}$$

Moreover, other behaviour observations were noted for the earthworms in each concentration. Likewise, the mesocosms were monitored for moisture content (MC), T°, pH and electrical conductivity (EC) before and after the test.

Cocoon Hatching Test

This test was carried out in plastic bottles (6 cm diameter and 4 cm height), with *E. fetida* cocoons which were collected just after cocoons laying by the adults and have a homogenous size. Five cocoons were placed in each bottle. The bottles were perforated at their base to ensure the evacuation of excess water and were covered with a perforated lid. In total 18 bottles were used, and to each bottle, 80 g of soil mixture and compost were added, with the same AH concentrations used for the first test. Each concentration and the control were tested with three replicates; the plastic bottles were maintained at $20 \pm 2^{\circ C}$ with 80–85% relative humidity in the dark. The water content was adjusted to 35% whenever necessary.

After the 14th to 28th day of incubation, the number of remaining cocoons that did not hatch was counted

every day and the number of juveniles emerging and empty shells in each test container were also counted, recorded and removed. The percentage of hatching success and number of hatchlings emerging per cocoon was calculated using the following formula:

$$Hatching\ success\ rate\ (\%) = \frac{Number\ of\ cocoons\ hatched}{Total\ number\ of\ cocoons}\ x\ 100$$

$$Number\ of\ hatchling\ emerging\ per\ cocoon = \frac{Total\ number\ of\ hatchlings}{Total\ number\ of\ cocoon}$$

Statistical Analysis

In this paper we could not use parametric tests (ANOVA and t-test) as our data were not normally distributed, we, therefore, used instead of non-parametric tests. Differences in mortality, biomass, cocoon production, juvenile production and cocoon hatching between treatments were assessed and compared using the nonparametric Kruskal–Wallis H test. NOECs (no observed effect concentration) and LOECs (lowest observed effect concentration) values were calculated by Kruskal-Wallis ANOVA followed by post-hoc multiple comparisons between all treatments [37]. The non-parametric Wilcoxon (2 samples) test for paired data was used to compare the significance of the observed difference in the data pertaining to the effect of the period of the experiment on the growth, and mortality of the worms which were recorded after 28 days and 56 days. Results with P ≤ 0.05 (equivalent to 95% confidence) were considered significant with all statistical significance. A probit's analysis was used to calculate the median lethal concentration (LC_{50}) with 95% confidence intervals at 28 days and 56 days for the mortality parameter. For the biomass (growth) and reproduction (number of cocoons and juveniles), the median effective concentration (EC_{50}) values and its 95%-confidence limits were determined also with the probit analysis method. All statistics were performed using SPSS software (version 25).

RESULTS

Survival, Growth and Reproduction test

Effects on survival (earthworm mortality)

The difference in recorded survival rate after 28-d and 56-d is shown in (Fig. 1). Our results express a clear concentration-dependent relationship. No mortality occurred at 0, 0.05 and 0.1mg.g^{-1}AH concentrations during the 28-d exposure period. However, after 56-d mortality rate was 10%, 15% and 25%, at 0, 0.05 and 0.1mg.g^{-1}AH concentrations respectively with no observed significant difference ($p > 0.05$). For (0.5mg.g^{-1}) AH concentration, the mortality increased from 25% after 28-d to 35% after 56-d and no significant difference from control was detected, furthermore, it was increased from 55% to 70% for (1 mg.g^{-1}) AH concentration with significant difference from 0, 0.05, 0.1 and 0.5 mg.g^{-1} AH concentrations ($p < 0.05$) after 28-d. However, high mortality was recorded for earthworms inoculated in the highest contaminated soil (5mg.g^{-1} of AH) in which all earthworms remained on the soil surface and were died with 100% mortality after 28th days which was a significant difference from the other AH concentrations ($p < 0.05$) exception to 1 mg.g^{-1} AH concentration.

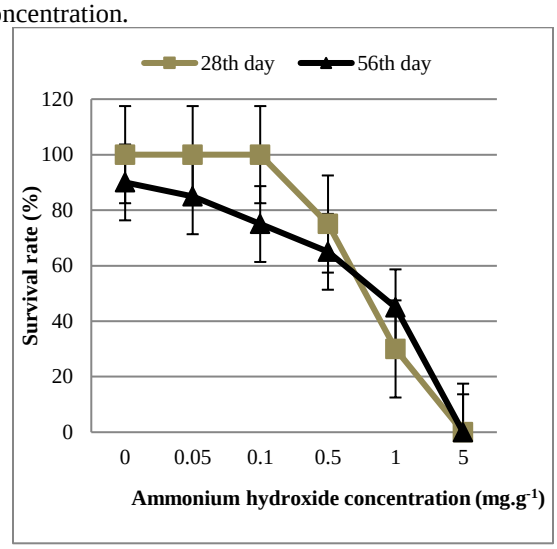

Fig. 1: The survival rate of *Eisenia fetida* in contaminated soil with an increasing series of AH concentrations (mg) after the 28th day and 56th day of survival, growth and reproduction test. Each point represents the mean of ten replicates, each comprising 2 worms at the start of the experiment (i.e., 20 worms per treatment).

Very high significant differences were detected between treatments (Kruskal-Wallis: $H = 43.53$, $p < 0.001$) after 4 weeks (28-d) of exposure to AH, and after 8 weeks (56-d) among tested concentrations ($H = 35.64$, $p < 0.001$) and period of exposure to AH (28 -d and 56-d) from the first day (test Wilcoxon, $p < 0.001$), therefore the post-hoc of test Kruskal-Wallis showed that the 28-d and 56-d NOEC and LOEC were 0.5 and 1mg.g^{-1}, respectively. Indeed, the 28-d LC_{50} was 0.85mg.g^{-1} and the 56-d LC_{50} was 0.83 mg.g^{-1} respectively.

Effect on growth (earthworm fresh biomass change)

Dose-response relationships for the effect of AH on *E. fetida* fresh biomass change are presented in (Fig. 2). The average fresh biomass of the tested earthworms for all concentrations is (770 ± 106mg), (787± 189mg) and (673 ± 269mg) on the first day, after 28-d and 56-d, respectively. Our results highlight a progressive

increase in earthworms' biomass after 28-d and a significant reduction in earthworms' biomass after 56-d for control and all concentrations with a very highly significant difference from the first day and the last day (test Wilcoxon, $p < 0.001$) and a highly significant difference ($p = 0.002$) between 28-d and 56-d of experimentation, thus earthworm body weight change and their responses to AH appeared at the end of the toxicity tests.

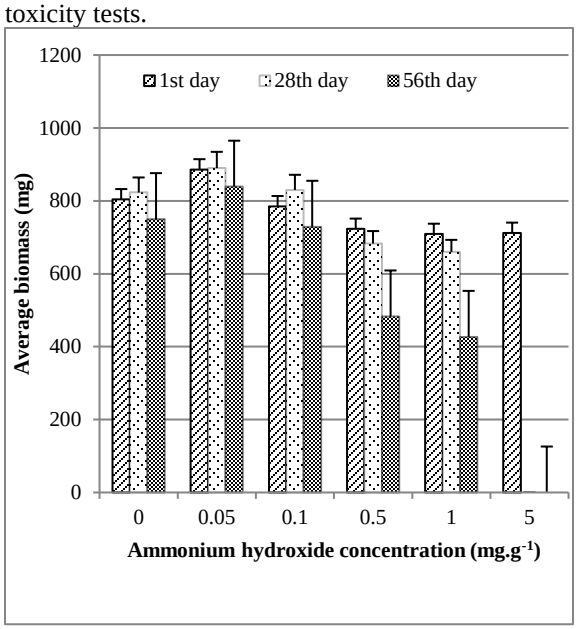

Fig. 2: Changes in fresh weights (biomass) of *Eisenia fetida* in contaminated soil with an increasing series of AH concentrations (mg) before the test, after the 28th day and 56th day of survival, growth and reproduction test. Each histogram represents the mean of ten replicates, each comprising 2 worms at the start of the experiment (i.e. 20 worms per treatment).

Control worms and those on the lowest concentration of AH (0.05 and 0.1mg.g^{-1}) had slightly increased in weights after 4 weeks (28-d). However, in subsequent weeks their weights decreased with a less significant difference ($p > 0.05$). In fact, the effect of AH concentrations on tested earthworms' biomass resulted in the maximum reduction in weights at the highest concentrations of AH (0.5 and 1mg.g^{-1}) between the 1st day and 28-d of exposure, and after 56-d compared to control and (0.05 and 0.1mg.g^{-1}) concentrations. A significant difference was detected between 1mg.g^{-1} and control after 56-d ($p < 0.05$). Biomass change data for 5mg.g^{-1} AH is not shown as to the absence of worms survived, consequently, a significant difference was found ($p < 0.05$) between the average weights of worms at 0, 0.05 and 0.1mg.g^{1} AH treatments and 5mg.g^{-1}.

Indeed, the Kruskal-Wallis H test revealed a very highly significant concentration effect ($H = 34.36$, $p < 0.001$) after 28-d and ($H = 31.84$, $p < 0.001$) after 56-d of exposure to AH, and the post-hoc multiple comparisons between all treatments shows that the obtained values of NOEC and LOEC for earthworms fresh biomass change after 28-d were (1 mg.g^{-1} and 5mg.g^{-1}) and (0.5mg.g^{-1} and 1mg.g^{-1}) after 56-d. The obtained values of (EC_{50}) for the AH effects on adults' biomass change, after 28-d and 56-d, were 1.68mg.g^{-1} and 0.82mg.g^{-1} respectively.

Effect of AH on reproduction

Effect on juvenile production (total number and fresh biomasses)

Results of the total number of juveniles produced and associated biomasses are presented in Figs. 3 and 4. For the control experiments, the total number of produced juveniles was 119 juveniles (approximately 12 juveniles per replicate) with average biomass of 130 ± 76mg. Results indicate that AH was less toxic to earthworms' reproduction at 0.05 and 0.1 AH concentrations with a slight decrease in the number of juveniles 67 and 55 respectively as per their biomasses and more toxic at 0.5mg.g^{-1} and 1mg.g^{-1} AH concentrations with 27 and 17 of produced juveniles respectively. Nevertheless, the toxicity was higher at 5mg.g^{-1} AH concentration where all adults earthworms died (0 juveniles).

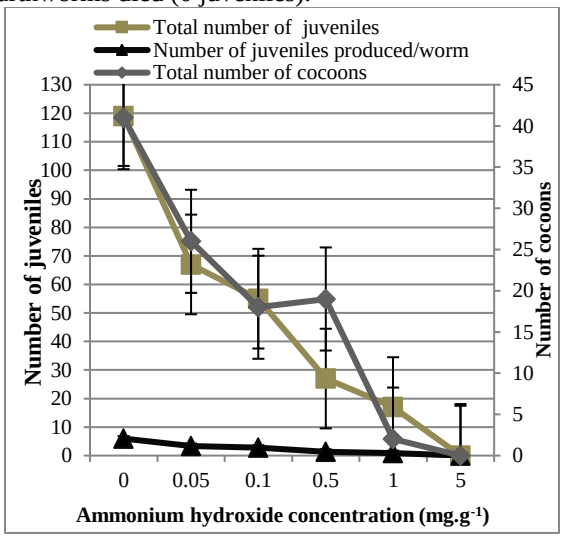

Fig. 3: Variation in the total number of cocoons and juveniles produced by adults earthworms of *Eisenia fetida* in contaminated soil with an increasing series of AH concentrations (mg) after the 56th day of survival, growth and reproduction test. Each point represents the mean of cocoons and juveniles production from ten replicates for each concentration.

Kruskal-Wallis H test revealed that the total number of produced juveniles and their biomass after 56-d were very highly significant differences between treatments ($H = 24.06$, $p < 0.001$) and ($H = p < 0.001$) respectively, and the post-hoc multiple comparisons found a significant difference ($p < 0.05$) between controls and (1 and 5mg.g^{-1}) AH concentration. Thus, the obtained

values of NOEC and LOEC were (0.5mg.g^{-1} and 1 mg.g^{-1}). Indeed, the obtained value of EC_{50} was 0.82mg.g^{-1}.

Number of juveniles per worm

Results revealed that the estimated number of produced juveniles per worm decreased with increasing AH concentrations (Fig. 3), and very highly significant differences were found between treatments in the average numbers of juveniles per worm (H= 24.60, $p < 0.001$). The post-hoc multiple comparisons show that the juvenile production at 1 and 5mg.g^{-1} AH concentrations were significantly different from the control ($p < 0.05$). The estimated average number of juveniles per worm in control was approximately 6 juveniles per worm; however it was 3 and 2 juveniles for 0.05mg.g^{-1} and 0.1 mg.g^{-1} AH concentrations, and approximately 1 juvenile for the AH concentrations (0.5mg.g^{-1} and 1mg.g^{-1}). Otherwise, the number of juveniles per worm was 0 for the highest AH concentration of 5mg.g^{-1} as all the adults earthworms died.

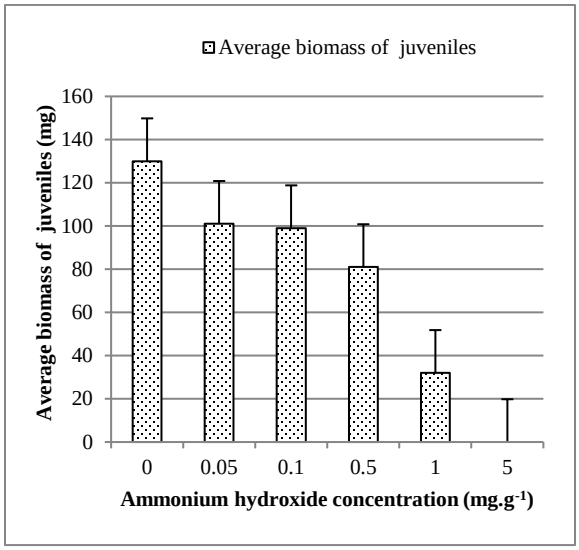

Fig. 4: Variation in fresh weights (biomass) of juveniles' earthworms of *Eisenia fetida* produced by adults after the 56th day of survival, growth and reproduction test in soil contaminated with an increasing series of AH concentrations (mg). Each histogram represents the mean fresh weight of juveniles produced in each concentration.

Effect on cocoon production.

The concentration-response relationship was also demonstrated in the form of cocoon production. The number of produced cocoons in the last days of the test (56th day) is presented in Fig.3. Results revealed that the number of cocoons formation at the end of the test decreased with the increase of ammonium hydroxide concentrations. The total number of cocoons per 10 earthworms varied from 2 to 25 cocoons. The maximum cocoons occurred at the controls boxes, and the lowest was observed in 1 mg.g^{-1} AH concentration. No cocoon formation was recorded at the highest AH concentration (5mg.g^{-1}).

A very high significant difference was obtained for the total number of cocoons produced at all AH concentrations (H= 36.99, $p < 0.001$) and significant differences were found between control and (1mg.g^{-1} and 5mg.g^{-1}) treatments. Thus, the obtained LOEC value was 1mg.g^{-1} and the NOEC cocoon production value was 0.5mg.g^{-1}, similar to the obtained NOEC of mortality and body weights. The EC_{50} value for AH effect on cocoon production was 0.11mg.g^{-1}.

Observations on earthworms' behaviour

In this test, some behavioural responses (reduction of activity, reduction of movements) and physiological symptoms (bleeding, burns) were observed on tested earthworms' *E. fetida.*

The tested earthworms placed in 0.5 and 1mg.g^{-1} concentrations have difficulty withstanding the presence of AH in the soil; therefore, it makes responses like the migration towards the bottom containers followed by the reduction of their activity. Moreover, at the highest AH concentration (5mg.g^{-1}); the earthworms initially exhibited avoidance behaviours and were observed climbing the walls of the container, and remaining on the soils surface. Unexpectedly, most of the earthworms appeared to have died after 24h due to the toxic effects of AH on breathing with signs of burns observed on their corps.

Cocoon hatching test

Hatching success and number of juveniles emerging per cocoon

Dose-response relationships for the effects of AH on cocoon hatching of *E. fetida* under different test concentrations are presented in Fig.5. The cocoon hatching reached its maximum (80%) of success for the control's boxes; 30 juveniles emerging with an average number of 2 juveniles emerging per cocoon. According to [38], the number of young earthworms hatching from each viable cocoon varies from 2.5 to 3.8 depending on temperature. Both AH test concentrations of 0.05mg.g^{-1} and 0.1mg.g^{-1} were the less toxic for cocoon hatching, with 67 % and 47% hatching success rate and 22 juveniles emerging from cocoons respectively, with an average number of 1.46 hatchlings juveniles per cocoon. However, both concentrations of 0.5mg.g^{-1} and 1mg.g^{-1} of AH have a negative impact on the cocoon viability with a percentage of 13% and 20% hatching success and a mean of 0.46 and 0.33 juveniles emerging per cocoon respectively. No juveniles emerging with the highest concentration of AH (5mg.g^{-1}).

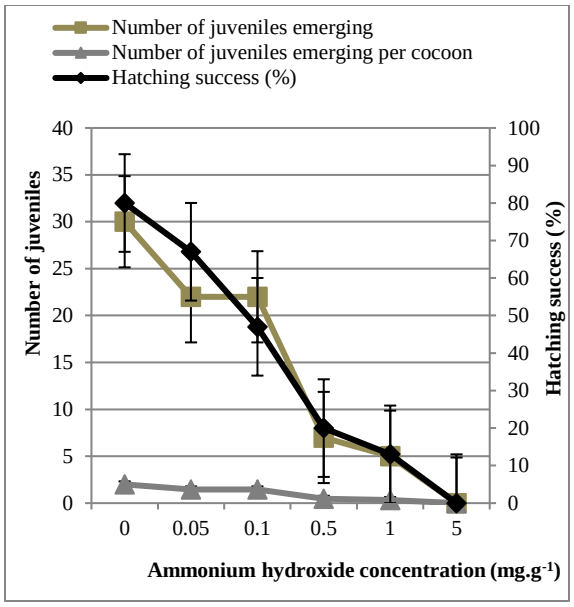

Fig. 5: Variation of cocoon hatching success rate, number of juveniles emerging per cocoon and the total number of juveniles emerging from all cocoons of *Eisenia fetida* placed in contaminated soil with an increasing series of AH concentrations (mg) after 28th days of cocoon hatching test. Each point represents the average hatching success rate and juveniles emerging from three replicates for each concentration

The number of cocoon hatching and juvenile emerging from 15 cocoons at all AH concentrations was significantly different between treatments ($H=14.75$, $p < 0.05$) and ($H= 16.04$ $p < 0.01$) respectively. The signification of the difference from the control was obtained for $5mg.g^{-1}$ AH concentration ($p < 0.05$). Thus, for this test the obtained values of NOEC and LOEC for hatching success and juveniles emerging were ($1mg.g^{-1}$ and $5mg.g^{-1}$) respectively.

DISCUSSION

The objectives of this study were to investigate and assess the potential toxicity of AH on the *Eisenia fetida* worms through the chronic toxicity tests (survival, growth and reproduction test) and (cocoon hatching test) in order to provide informative data for use in ecological risk assessment on soil ecosystem and to protect the earthworms from hazardous exposure to ammonia deposition generated by poultry farming; which return on soil in the form of aerosols of NH_4^+ into precipitation and also potential exposure to the organic wastes (poultry droppings) contains high levels of this cation particularly in poultry droppings storage areas.

Overall, we found in the present study negative effects of AH (ammonia solution) were measured on *E. fetida* earthworms (i.e., on mortality, growth and reproduction). The AH effects were increased progressively with increasing concentrations and exposure time. Additionally, the obtained results upheld our expectation that cocoon hatching impacted significantly AH concentrations.

According to [32], the chronic test was considered to be valid if adult mortality was less than 10% in the controls at the end of the test, which is similar to our case in the first test. The obtained LC_{50} after 56-d was lower than LC_{50} after 28-d (0.83 and $0.85mg.g^{-1}$ respectively). Otherwise, it has been obtained in a previous study conducted by [11] on the effect of AH for the soil acute test, that the LC_{50} value after 14-d was $1.05mg.g^{-1}$. Thus, the effect of AH on earthworms' mortality varied with exposure time, its toxicity was considerably harmful after 8 weeks of exposure compared to 2 and 4 exposure weeks.

According to [39], all earthworms are very sensitive to ammonia and did not survive long in organic wastes containing much ammonia (e.g., fresh poultry litter). Laboratory experiments showed that both ammonia and inorganic salts have a very sharp cut-off point between being toxic and non-toxic i.e. $< 0.5mg.g^{-1}$ of ammonia and < 5 % salts for earthworms, in the present study the 28-d and 56-d NOEC and LOEC were 0.5 and $1mg.g^{-1}$, respectively, which means that the significant effect starts from $1mg.g^{-1}$ after 28-d of exposure.

In a study conducted by [40], they have used ammonium chloride (NH_4Cl) as a toxicant, and ammonia (from NH_4Cl) was measured by an ion-selective electrode, and their results showed an increase in the ammonia concentration with increasing in NH_4Cl and found a correlation between high ammonia levels and high mortality. Significant amounts of ammonia are being added to the soil through microbial ammonification of dead worms, and at higher pHs, the form of the toxic free ammonia is in a greater proportion. Studies of [41], have shown that ammonia NH_3 is toxic and that ammonium NH_4^+ would not be toxic or that its effect would be insignificant. Since the concentration of free ammonia (NH_3) in solution depends strongly on pH, the toxicity of an effluent contaminated with ammonia is, therefore, a function of the pH. The toxic-free ammonia in the solution increases with increasing pH. A simple way to reduce the ammonia toxicity of an alkaline effluent would undoubtedly be to decrease its pH.

Our results confirm that the exposure time and the AH concentration affect the earthworms' biomass; it decreased with increasing AH concentrations. The initial increase in weights of control worms, was 0.05 and $0.1mg.g^{-1}$ AH concentrations were due probably to ingestion of soil and organic matter (compost). Although, worms were fed once a week during the test, with 5 g per container of wheat bran and pre-exposed to the soil for 7th days before being placed in the treatment containers. After 56-days, it was reported

that earthworms showed a dose-related biomass reduction. The observed earthworms' loss of weight can be attributed to the lack of compost (organic matter), and to the high level of AH which inhibits earthworms to ingest soil, also to the mortality of worms due to the AH effect. In a study conducted by [11], it was obtained that earthworms showed a dose-related biomass reduction after 14-d of exposure to AH without food. Although this conclusion was based on laboratory experiments, a similar earthworm' loss of weight can be expected from field systems. It is most likely that ingestion of organic waste and soil by earthworms stops when a critical level of ammonia solution material appears.

According to [42], the compost temperature and humidity are the most important environmental factors affecting the growth and development of earthworms. The proper humidity is 70 to 80% [43], which was adjusted in our study. Otherwise, according to [44], the *E. fetida* species is resistant to the changes in environmental conditions and has a high alimentation and reproduction rate. Ref. [14] proved that earthworms did not survive long in organic wastes containing much ammonia. However, organic wastes containing large amounts of ammonia can become acceptable after their removal after a period of composting.

For the last parameter which is reproduction, it was assessed on the last day of the experiment throw (total number of juveniles produced and their biomass, number of juveniles per earthworm and total number of cocoons produced), our results showed that cocoons and juveniles production were sensitive to AH concentrations, the number of juveniles produced after 56 days and of cocoons produced at the last day was significantly different between different treatments; 41 cocoons were collected in controls at the last day of the experiment. According to [32], the test was considered to be valid if at least 25 cocoons were produced in the controls at the end of the test. [45], recorded a production rate of 0.2 cocoons per worm per week in worms not supplied with animal manure, compared with the 1.2 to 2.0 cocoons per worm per week in those supplied with food. Edwards' studies in animal wastes showed that the maximum reproduction rate of *E. fetida* is 3.8 cocoons per adult worm per week [42]. In this regard, [46] reported 0.35 cocoons per adult worm per day. *E. fetida* produces 900 egg capsules per worm per year [43]. Each worm of this species produces an egg capsule every seven to ten days and there are two to twenty eggs in each capsule [47]. In our study, cocoon production after 56 days was low in all AH concentrations due to the mortality of adult earthworms resulting in insupportable exposure to AH, moreover, the number of juveniles produced and their biomass decreased with increasing AH concentration. What is more, results revealed that the number of juveniles with all AH concentrations was lower than controls, this significant reduction is considered as a sign of a toxic effect of AH on the reproductive success of earthworms and due probably to the effect of AH on hatchability of produced cocoons. This conclusion explains the need for such a test to study the cocoon hatchability in presence of AH.

Cocoons are tiny and roughly lemon-shaped with specific characteristics; their mean size is 4.85 x 2.82 mm, containing the fertilized eggs. Cocoon laying begins 48 hours after copulation, and the number of cocoon production is between 0.35-and 0.5 per day. Under optimal conditions, the hatching viability of *E. fetida* cocoons is 73-80%, and the incubation period which varies according to the earthworm species and environmental conditions, ranges from 18 to 26 days for this species. The number of young earthworms hatching from each viable cocoon varies from 2.5 to 3.8 depending on temperature. If the conditions of temperature and humidity are not favourable the capsules remain [38; 3; 48]. In this study, the cocoon hatching reached its maximum success for the control bottles with 80% hatch success and an average of 2 hatchlings emerging per cocoon, what else, [49] found 96% for cocoons incubated on artificial soil, and a mean value of 2.8 juveniles per cocoon using *E. fetida Andrei*, in 1989 [45] obtained a higher mean value of 3.5 juveniles in controls using *E. fetida Andrei* which were preconditioned for one week with 2% cow dung. Both AH concentrations of 0.05mg.g-1 and 0.1 mg.g-1 were the less toxic on cocoon hatchability. However, both AH concentrations 0.5mg.g^{-1} and 1 mg.g^{-1} were not favourable to the cocoon hatching. In the highest AH concentration 5mg.g^{-1} no juveniles emerged from cocoons in all replication and all cocoons capsules remained. It is concluded that the high concentrations of AH in culture substrate preclude the cocoons viabilities (i.e., the proportion from which juveniles emerged).

CONCLUSION

In conclusion, the result of the present investigation shows that a high concentration of Ammonium hydroxide (AH) affected the survival, growth, reproduction and cocoon hatching of the earthworms *E. fetida.* For the all parameter assessed, the low AH concentrations (0.05 and 0.1mg.g^{-1}) were less toxic on worms and cocoons compared to other AH concentrations, in adverse the higher AH concentrations 1 and 5 mg.g^{-1} was the most toxic for worms and cocoons. The 56 days' NOEC values for the effect of AH on survival, growth and reproduction were similar (0.5mg.g^{-1}) and show that survival, growth and reproduction are sensitive to AH,

otherwise, the obtained NOEC value for the effect of AH on cocoon hatching rate and juvenile emerging was higher to theses obtained for the first test ($1mg.g^{1}$) which indicate that survival, growth and reproduction are more sensitive to AH than cocoon hatching success. These obtained results can be used in environmental impact assessment, and therefore, provides important information on the ecological relevance of these types of toxicity data for use in ecological risk assessments or derivation of soil quality standards, more precisely, in agriculture regions well known by massive poultry husbandry and spreading of poultry droppings in an anarchic way.

ETHICAL ISSUES

Ethical issues have been completely observed by the authors.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

AUTHORS' CONTRIBUTIONS

All authors participated in the design and conduct of the study. All authors have made contributions to drafting, revising, and approving the manuscript.

FUNDING/ SUPPORT

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

REFERENCES

[1] Hund-Rinke K, Kordel W, Hennecke D, Eisentraeger A, Heiden S. Bioassays for the Ecotoxicological and Genotoxicological Assessment of contaminated soils (Results of a Round Robin Test): Part I. Assessment of a possible groundwater contamination: ecotoxicological and genotoxicological tests with aqueous soil extracts, JSS. 2002; 2 (1): 43-50.

[2] Velki M, Stepi S, Lon cari Z, Hackenberger BK. Effects of electroshocking and allyl isothiocyanate on biomarkers of the earthworm species *Eisenia andrei* Possible side-effects of non-destructive extraction methods, Eur J Soil Biol. 2012; 51: 15-21.

[3] Edwards CA, Bohlen PJ. Biology and Ecology of Earthworms. London: Chapman and Hall. UK; 1996. 426 p.

[4] Allen HE. Bioavailability of Metals in Terrestrial Ecosystems: Importance of Partitioning for Bioavailability to Invertebrates, Microbes, and Plants, New York: SETAC; 2002.176 p.

[5] Ni JQ, Heber AJ, Diehl CA, Lim TT. Ammonia, Hydrogen Sulphide and Carbon Dioxide Release from Pig Manure in Under-floor Deep Pits, J Agric Eng Res. 2000; 77 (1):53-66.

[6] Wiegand AN, Menzel S, King R, Tindale N. Modelling the aeolian transport of ammonia emitted from poultry farms and its deposition to a coastal waterbody, Atmos Environ. 2011; 45: 5732-41.

[7] Von Bobrutzki K, Muller HJ, Scherer D. Factors affecting the ammonia content in the air surrounding a broiler farm, Biosyst Eng. 2011; 8: 322-33.

[8] Zhang Y, Dore AJ, Maa L, Liu XJ, Maa WQ, Cape JN, Zhang FS. Agricultural ammonia emissions inventory and spatial distribution in the North China Plain, Environ Pollut. 2010; 158: 490-501.

[9] Wu SY, Hub JL, Zhang Y, Aneja VP. Modeling atmospheric transport and fate of ammonia in North Carolina—Part II: Effect of ammonia emissions on fine particulate matter formation, Atmos Environ. 2008:42: 3437-51.

[10] Paoli L, Pirintsos AS, Kotzabasis K, Pisani T, Navakoudis E, Loppi S. Effects of ammonia from livestock farming on lichen photosynthesis, Environ Pollut. 2010; 158 (6): 2258-65.

[11] Zeguerrou N, Adjroudi R, Si Bachir A, El Hadef El Okki M. Assessment of ammonium hydroxide effect on Eisenia fetida: acute toxicity and avoidance tests. International Journal of Agricultural Resources, Governance and Ecology, 2019; 15 (1): 27-44.

[12] Gary, R. 2006. Ammoniac: polluant acide de l'air, des sols et des eaux superficielles. Available from: http://www.eau-et-rivieres.asso.fr/media/user/File/Ammoniac_ERB%20juin%202006.pdf. Accessed 20 August, 2019

[13] Wolfe AH, Patz JA. Reactive nitrogen and human health: acute and long-term implications, Am J Hum Environ. 2002; 31 (2): 120-25.

[14] Edwards CA. Breakdown of animal, vegetable and industrial organic wastes by earthworms. In: Edwards CA and Neuhauser EF, editors. Earthworms in Waste and Environmental Management. The Netherlands, The Hague: SPB Academic Publishing BV; 1988.pp. 21-31.

[15] Abbiramy KS, Ronald Ross P. Determination of acute toxicity of potash to *Eisenia foetida* using a

simple paper contact method, Ann Biol Res. 2012; 3 (12): 5714-17.
[16] Chen C, Wang Y, Zhao X, Wang Q, Qian Y. Comparative and combined acute toxicity of butachlor, imidacloprid and chlorpyrifos on earthworm, *Eisenia fetida*, Chemosphere. 2014; 100: 111-15.
[17] Siddique S, Syed QY, Saleem A, Adnan A, Qureshi FA. Toxicity of avermectin b1b to earthworm and cockroaches, J Anim Plant Sci. 2015; 25 (3): 844-50.
[18] Zeriri I, Tadjine A, Grara N, Belhaouchet N, Berrebbah H, Djebar MR. Potential toxicity of an insecticide of the family of carbamates on a bioindicator model of the pollution the earthworm *Octodrilus complanatus* (Oligochaeta, Lumbricidae), Ann Biol Res. 2012; 3 (11): 5367-73.
[19] Landrum M, Cañas JE, Coimbatore G, Cobb GP, Jackson WA, Zhang BH, Anderson TA. Effects of perchlorate on earthworm (*Eisenia fetida*) survival and reproductive success, Sci Total Environ. 2006; 363 (3): 237-44.
[20] Schaefer M. Assessing 2, 4, 6-trinitrotoluene (TNT)-contaminated soil using three different earthworm test methods, Ecotoxicol Environ Saf. 2004; 57: 74-80.
[21] Bouguerra S, Gavina A, Ksibi M, Rasteiro M, Rocha-Santos T, Pereira R. Ecotoxicity of titanium silicon oxide (TiSiO4) nanomaterial for terrestrial plants and soil invertebrate species, Ecotoxicol Environ Saf. 2016; 129: 291-01.
[22] Van Hoesel W, Tiefenbacher A, König N, Dorn VM, Hagenguth JF, Prah U, Widhalm T, Wiklicky V, Koller R, Bonkowski M, Lagerlöf J, Ratzenböck A, Zaller JG. Single and Combined Effects of Pesticide Seed Dressings and Herbicides on Earthworms, Soil Microorganisms, and Litter Decomposition, Front Plant Sci. 2017; 8: 215.
[23] Reinecke AJ, Maboeta MS, Vermeulen LA, Reinecke SA. Assessment of lead nitrate and mancozeb toxicity in earthworms using the avoidance response, *Bull Environ Contam Toxicol*. 2002; 68: 779-86.
[24] Yeardley RB, Lazorchak JM, Gast LC. The potential of an earthworm avoidance test for evaluation of hazardous waste sites, *Environ Toxicol Chem*. 1996; 15: 1532-37.
[25] ECPA (European Crop Protection Agency, European commission) *Soil biodiversity and agriculture*. Available from: https://www.europeanlandowners.org/files/pdf/soil_bio_and_ag_009.pdf. Accessed 20 August, 2019.
[26] OECD (Organization for Economic Co-operation and Development) 1984. *Earthworm acute toxicity tests.* Guidelines for the testing of chemicals No. 207. Adopted 4 April, Paris, France.
[27] Heimbach F. Effects of pesticides on earthworm populations: comparison of results from laboratory and field tests. In Greig-Smith PW, Becker H, Edwards PI, Heimbach F, editors. Ecotoxicology of Earthworms. Andover, Hampshire: Intercept; 1992. pp.100-06.
[28] Jensen J, Diao XP, Scott-fordsmand JJ. Sub-lethal toxicity of the antiparasitic abamectin on earthworms and the application of neutral red retention time as a biomarker, Chemosphere. 2007; 68: 744-50.
[29] Liu S, Zhou QX, Wang YY. Ecotoxicological responses of the earthworm *Eisenia fetida* exposed to soil contaminated with HHCB, Chemosphere. 2011; 83: 1080-1086.
[30] Moriarty F. Ecotoxicology: The Study of Pollutants in Ecosystems. London, UK: Academic Press; 1983. 347p.
[31] Wang Y, Cang T, Zhao X, Yu R, Chen L, Wu C, Wang Q. Comparative acute toxicity of twenty-four insecticides to earthworm, *Eisenia fetida*, Ecotoxicol Environ Saf. 2012; 79: 122-28.
[32] OECD (Organization for Economic Co-operation and Development) 2004. *Earthworm Reproduction Test (Eisenia fetida/Eisenia andrei).* Guideline for testing of chemicals No. 222. Adopted 13 April, Paris, France.
[33] ISO (International Standard Organization) 1993. Soil Quality-Effects of Pollutants on Earthworms *(Eisenia fetida)*-Part I: Determination of Acute Toxicity Using Artificial Soil Substrate. Standard Number No.11268-1. Geneva.
[34] ISO (International Standard Organization) 1998. Soil Quality-Effects of Pollutants on Earthworms *(Eisenia fetida)*-PartII: Method for the Determination of Effect son Reproduction. Standard Number No.11268-2. Geneva.
[35] Barneaud A, Bisson M, Del grata F, Ghillebaert F, Guillard D, Tack K. (INERIS). Toxicological and Environmental Data Sheet for Chemical Substances: Ammonia. 2012. Availabe from: https://substances.ineris.fr/fr/substance/getDocument/2709. Accessed 20August, 2019.
[36] EC (Environment Canada) 2004. Biological test methods: tests for determine the toxicity of contaminated soils to earthworms (*Eisenia andrei*, *Eisenia fetida* or *lumbricus terrestris).* Available from: http://publications.gc.ca/collections/collection_2013/ec/En49-7-1-43-fra.pdf. Accessed 20 August, 2019
[37] Lock K, Janssen CR. Ecotoxicity of nickel to *Eisenia fetida*, *Enchytraeus albidus* and *Folsomia candida*, Chemosphere. 2002; 46: 197-00.
[38] Dominguez J. State-of-the-Art and New Perspectives on Vermicomposting Research. In: Edwards CA, editors, Earthworm Ecology. 2nd ed., Spain: CRC press LLC; 2004. 401-24.

[39] Srivastava RK. Beohar PA. Production of *Eisenia foetida* and vermicompost from poultry waste, Asian J Biol Sci. 2008; 3: 395-98.
[40] Yeardley RB, Lazorchak JM, Pence MA. Evaluation of alternative reference toxicants for use in the earthworm toxicity test, Environ Toxicol Chem. 1995; 14 (7): 1189-94.
[41] Vines HM, Wedding RT. Some effects of ammonia on plant metabolism and a possible mechanism for ammonia toxicity, Plant Physiol. 1960; 35 (6): 820-25.
[42] Edwards CA. Historical overview of vermicomposting. Biocycle, 1995; 36 (6): 56-58.
[43] Hashemi Majd K, Kalbasi M, Golchin A, Shariatmadari H. Identifying *"Eisenia foetida"*, a Native Compost Worm of Some North and Northwest Parts of Iran and Evaluation of its Ability in Vermicompost Production, Sciences and Technology of Agriculture and Natural Resources. 2004; 7 (4): 61-68.
[44] Coleman JC, Johnson DR, Stanley JK, Bednar AJ, Weiss CAJ, Boyd RE, and Steevens JA. Assessing the fate and effects of nano aluminum oxide in the terrestrial earthworm, *Eisenia fetida*, Environ Toxicol Chem. 2010; 29 (7): 1575-80.
[45] Van Gestel CAM, Van Dis WA, Van Breemen EM, Sparenburg PM. Development of a standardized reproduction toxicity test with the earthworm species *Eisenia andrei* using copper, pentachlorophenol and 2.4- dichloroaniline, Ecotoxicol Environ Safety. 1989; 18: 305-12.
[46] Reinecke AJ, Viljoen AA, Saayman RJ. The suitability of *Eudrilus eugeniae, Perionyx excavates* and *Eisenia fetida* (Oligichaeta) for vermicomposting in southern Africa in the term of their temperature requirements, Soil Biol Biochem. 1992; 24 (12): 1295-07.
[47] Smith K. Practical guide to raising earthworm (basic vermiculture information) K.W.rabbit and worm, Bioresour Technol.1998; 84: 191-96.
[48] Dominguez J, Edwards CA. Biology and ecology of earthworm species used for vermicomposting. In Edwards CA, Arancon NQ, Sherman R, editors. Vermiculture Technology. Florida: CRC Press Taylor and Francis Group; 2011. 249-61.
[49] Van Gestel CAM, van Dis WA, van Breemen EM, Sparenburg PM. Comparison of two methods for determining the viability of cocoons produced in earthworm toxicity experiments, Pedobiologia. 1988; 32: 367-71.