Bioavailability and Genotoxicity of glyphosate treated soil on *Archachatina marginata*

Angela C. Udebuani[1], Ethelbert U, Ezeji[1], Reuben C. Agu[1], Ikechukwu N. E. Onwurah[2] and Priscilla N. Abara[3]

[1]Department of Biotechnology, Federal University of Technology, P.M.B. 1526, Owerri Nigeria
[2]Department of Biochemistry, University of Nigeria Nsukka, Enugu State Nigeria
[3]Department of Biology, Federal University of Technology, P.M.B. 1526, Owerri, Nigeria
ucheezeji@gmail.com

Abstract: The study of the effects of contaminants of emerging concern such as glyphosate on non-target organisms such as African snail (*Archachatina marginata*), with emphasis on the genomic DNA profile, is of great toxicological importance in terms of risk assessment. Until recently, genetic effects were largely inferred from observations of genotype. With the ability to perform DNA sequencing and use of PCR, it has become possible to determine genetic variations occasioned by environmental toxicants through the Science of Genomics. *Archachatina marginata's* nature and way of life offers an excellent model animal for the study of the sensitivity of some terrestrial animals to environmental stress induced by chemical pesticides such as glyphosate. In this study, the African giant snail was exposed to soil samples spiked with different concentrations of glyphosate for several weeks and their genomic DNA extracted and subjected to electrophoresis. Amplification of the various DNA bands was carried out using an OMnE-PCR System programming. Our result shows that the AP-PCR genotypic profile with oligonucleotide prima OPA (gaaacgggtg) on DNA of *Archachatina marginata* exposed to glyphosate for a period of 7 days gave varying number of fragments with different amplification bands, corresponding to different molecular weights or sizes. Similarly, in comparing the length of time of exposure to glyphosate, it was observed that newer DNA fragments with higher intensity and larger molecular sizes were obtained from DNA amplification as against the control that remained the same, except for the unique band (100-400bp) that was observed in all the snails (exposed and unexposed). It can be inferred from this study that since glyphosate caused increased number of DNA fragments and significantly varying molecular sizes in the African giant snail after an exposure, it has the potential mutagenicity that could induce genotoxicity on non-target organisms.
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1. Introduction

Pollution from agrochemicals, such as herbicide application in agricultural areas, is among the critical factors changing life forms in the environment. Herbicides are large group of compounds mostly used in agricultural, residential, and industrial areas to eliminate unwanted weeds. Its use in agricultural farm has allowed crop seeds to grow without competition from weeds, thereby increasing agricultural productivity and product quality (McDougali, 2010). However, due to their chemical compositions, high toxicity, and mode of action, they have been declared as persistent and bio-accumulative pollutants (Kreuger, 1998; Dorigo et al., 2007).

Among them, glyphosate is a non-selective, broad-spectrum herbicide used to control most annual and perennial weeds (William et al., 2002). It works by inhibiting the enzyme 5-enolpyruvil shikimate 3-phosphate synthase (EPSPS), a key enzyme in the shikimate acid pathway of plants, for the synthesis of aromatic amino acids (DellaCioppa et al., 1986). This inhibition property leads to a shortage in aromatic amino acids, quinones and cofactor biosynthesis. The inhibition of amino acids can also induce non-target indirect effect, such as proteolysis and increase in free amino acids (Zulet et al., 2013; Faus et al., 2015).

Results of previous studies have consistently shown diverse toxic effects of glyphosate and glyphosate-based herbicides on organisms in various environmental compartments (Akcha et al., 2012; Tsui and Chu, 2003; Giesy et al., 2003; de Liz Oliveira Cavalli et al., 2013 and Astiz et al 2012). However, there is paucity of information on the effects of glyphosate on non-target terrestrial organisms, and this may be attributed to the limited number of studies in this area. Also toxicological studies of the effects of glyphosate and its related compounds on organisms have shown cytotoxicity (Li et al., 2013; Marc et al., 2004a), reproductive toxicity (Dallegrave et al., 2007; Romano et al., 2012) and neurotoxicity effects. DNA damage following glyphosate exposure at high levels has been reported in various species of organisms such as rat, tadpole, bovine, drosophila, goldfish, caiman, eel and humans (Bolognesi et al., 1997; Cavas and Konen, 2007; Clements et al., 1997; Gasnier et al., 2009; Guilherme et al., 2010).

However, the potential genotoxic effects of glyphosate on a non-target organism such as *Archachatina marginata* is yet to be fully evaluated.

Biological alterations such as DNA strand breakage or chromosomal alterations due to exposure effects have been reported to be useful in monitoring environmental pollution levels (Jha, 2004; Theodorakis et al., 2011). The test organism is a terrestrial mollusk inhabiting moist areas and strives with its foot in agricultural soil where glyphosate is applied. *Archachatina marginata* can serve as a good environmental bio-indicator of chemical pollution from pesticides.

2. Material and Methods

Test Sample: Glyphosate (Roundup) was procured from an agro-chemical store at Owerri main market in Imo State, Nigeria.

Collection of soil samples: Soil samples were collected from different farmland located at Nekede Owerri Southeastern Nigeria. The Soil samples were then pulled together to have a homogenous sample for easy analysis. The soil samples were collected from three sites (farm land planted with *Manihot esculentus*, vegetable garden and yam farm). Depths of collection of samples were from 0 – 15 cm and collection was made easy with spade. The soil samples were taken to the laboratory for various physical and chemical analyses.

Sample preparation: The Soil analysis was carried out in the Soil Science Laboratory, School of Agriculture and Agricultural Technology, Federal University of Technology Owerri. The Soil Samples were air dried for 7 days, after which they were passed through a 2mm sieve and all larger particles discarded. The fractions that passed through a 2mm sieve were the fine earth, which were used for subsequent analysis.

Blending of Soil with Glyphosate: Different volumes of glyphosate were mixed with 20 g of agricultural soil and were developed at 4 levels each with the following concentrations of glyphosate to soil: 250 mg/g, 500 mg/g, 750 mg/g and 1000 mg/g. Each treatment was replicated thrice and this brought the total experimental pots to 12 (3x4). They were arranged in a completely randomized design (Gomez and Gomez, 1984). The samples were mixed thoroughly inside a plastic container and stored in a green house in School of Agriculture and Agricultural Technology, Federal University of Technology Owerri. Thereafter four sampling dates (1, 7 and 14 days) after application were taken and on each date 5g of each sample was taken to the laboratory for analysis.

Soil analysis: Soil particle size analysis was determined by hydrometer method (Gee and Or 2002), Percentage water content on saturation (%WCS) was calculated as weight of wet soil divided by weight of dry soil multiplied by 100. Soil pH was measured using pH meter in a soil water ratio of 1:2:5 (Hendershot et al., 1993), Cation exchange capacity was determined by ammonium acetate method (Soil Survey Staff, 2003), Organic carbon was estimated by wet digestion method (Nelson and Sommer, 1982) and organic matter was calculated by multiplying the percentage organic carbon by 1.724 which is the Vandenmelen's correction factor. Glyphosate was determined after extraction with Ammonium Molybdate Tetra hydrate and the absorbance of the phosphomolybdateheteropolyblue complex was measured against water on spectrophotometer at 830nM.

Determination of the effect of glyphosate on Snail after exposure

Procurement of Snail: A total of twenty (20) giant African snails *Archachatinamarginata* were procured from snail Nigeria. The reason for buying snails from a known farm was to avoid using already contaminated snail. The snails were picked manually and transported to the laboratory in pre-labeled plastic containers.

Animal husbandry: The Snails (*Achachatina marginata*) used in this study housed in mosquito net screened (to prevent flying insects and avoided snail escape) wooden cages (Cobbinah 1994). The cages were positioned into pens measuring 42cm x 28cm x 20cm. They were elevated 50cm off the ground with legs permanently placed in used engine oil to prevent crawling insects and termites.

Feeding: The snails were fed with plant materials such as leaves of *Amaranthus hybridus*, water leaf and leaves of *Manihot esculanta, Carica papaya*, fruits such as ripe pineapple, banana, and pawpaw. Water was sprinkled on the snails every evening on daily bases.

Acclimatization of Snails: The snails were exposed to the composited uncontaminated soil sample just to get them acclimatized.

Exposure of Snail to glyphosate: Three snails of known weight were exposed to different concentrations of glyphosate in 20grams of soil as mentioned earlier in experimental design.

GROUP A: 0 glyphosate + 20g of soil (0%, w/w) A

GROUP B: 500mg of glyphosate + 20g of soil (25%, w/w) B

GROUP C: 10,000mg of glyphosate + 20g of soil (50%, w/w) C

GROUP D: 15,000mg of glyphosate+ 20g of soil (75%, w/w) D.

The replicate experimental groups were as follows:

GROUP A: GA_1 GA_2 GA_3 (0%)
GROUP B: GB_1 GB_2 GB_3 (25%)
GROUP C: GC_1 GC_2 GC_3 (50%)
GROUP D: GD_1 GD_2 GC_3 (75%)

Group A (GA_1, GA_2, GA_3) serve as control for this study and therefore were not exposed to glyphosate.

Genomic DNA extraction from *Archachatina marginata:*

The genomic DNA of *Archachatina marginata* was extracted using the method of Stothard et al (1996). Each snail was removed with 70% ethanol and soaked in TAE buffer, pH 7.4, (10mM, TrisHCl and I mM EDTA) overnight so as to remove the remaining ethanol. Tissues from each snail were placed in a sterile 1.5ml micro-centrifuge tube with 500µl of CTAB solution (0.2% 2-mercaptoethanol, 2% hexadecyltrimethyl-ammonium bromide (CTAB); 100mM Tris (hydroxymethyl) amino methane; 16mM EDTA; 1.4M sodium chloride) and the tissue homogenized. Proteinase K solution (10µl at 20mg/ml) was added and the digests incubated at 55^{o}C for 1hr with occasional gentle mixing. Genomic DNA was extracted from the digests by adding an equal volume of Chloro-form/isoamyl alcohol (24:1) to each tube. The organic and aqueous layer were gently mixed for 5mins and spun at 13,000 rpm for 20min. For each sample, the upper aqueous layer was removed into another sterile micro-centrifuge tube and an equal volume of 100% ethanol was added, mixed and the whole incubated at – 20^{0}C overnight in order to enhance DNA precipitation. The precipitate was spun at 13000rpm for 20 min and the pellet was washed with 70% ethanol and spun for another 20 min. The supernatant was removed and the pellet was dried at room temperature. When completely dry, the pellet was re-suspended in 50µl of purified water.

Agarose electrophoresis of extracted DNA:

Agarose powder (0.75g) was added to 80ml, Tris acetate EDTA (electrophoresis buffer) in a conical flask and then heated in a microwave oven until the agarose completely dissolved. After cooling the solution to 60^{O}C, it was poured into a casting tray, which has the comb in place, to solidify at room temperature (45min). After solidification, the comb was removed. The gel still in its plastic tray was placed into the electrophoresis chamber and then covered with Tris- Acetate. EDTA (TAE) buffer the electrophoretic chamber was covered with the lid and the power terminals connected to the power pack. Current was 35 volts for 24 hours Migration was from negative pole to positive pole. This was followed by photo documentation.

PCR Amplification and Quantification of extracted DNA

The DNA fragments were amplified using the primer OPA07 (5^{1}-GAAACGGGTG-3^{1}). The primer and cycling condition were as described in Kane et al. (2008). Both the OPA 07 products of the exposed and unexposed were run on 0.8% agarose gel followed by photo-documentation. The DNA fragments were excised from an agarose gel using a scalpel blade and purified employing a QIA quick Gel Extraction Kit (Qiagen). Each sample was quantified using a Nano-drop ND – 1000 spectrophotometer (Nanodrop Technologies Inc.)

3. Results and Discussion

Results of some soil properties are shown in Table I. Variations were observed in the percentage composition of silt, clay and sand for glyphosate-treated soil and control. Dureja and Tanwar (2012) reported that soil texture (percentage sand, silt and clay) and structure play a large role in transport processes of pesticides. Soils generally were high in sandy fractions and they are distributed as follows 7 days (900 gkg^{-1}), 14days (890 gkg^{-1}), 21 days (912 gkg^{-1}). Glyphosate did not affect the sandy nature of the study site; earlier report by Onweremadu et al. (2007) showed that soils of this study area are sandy. Soil that are sandy in nature, allows liquid formulated pesticides like Roundup to move both vertically and horizontally, through them quickly.

Table 1: Selected soil properties (0 – 15 cm depth) (mean values) (n = 12)

Soil properties	7 days	14 days	21 days	Control
Silt (g kg^{-1})	34	32	29	24
Clay (g kg^{-1})	61	49	41	38
Sand (g kg^{-1})	90	89	91	96
MC (g kg^{-1})	150	150	141	138
pH	4.7	5.2	6.2	6.8
CEC (cmol kg^{-1}	11.2	10.7	8.7	7.7
OM (g kg^{-1})	8.74	7.24	5.53	3.62

Percentage moisture content was higher in glyphosate treated soil than in the untreated soils. Herbicides has been reported to be less persistent in soil with high moisture content Durejan and Tanwar (2012). Reduction in soil pH observed in glyphosate-contaminated soil (4.7) compared to the control (6.8) suggests that application of glyphosate may result in producing acidic soils. Acidification of soil increases the durability of pesticides in soil. Maqueda et al. 1998; Veiga et al 2001 reported that glyphosate is highly absorbed in natural fulvic acid. However, pH values in glyphosate-contaminated soil increases as day's progresses, showing possible response of degradative organisms proliferating in applied glyphosate. Higher values of organic matters were observed in contaminated soil compared to the

control. This could be attributed to the stimulatory effects of microbial population in the glyphosate-contaminated soil. Araujo et al. (2003) reported glyphosate displaying stimulatory effects on microbial proliferation in agricultural soil.

The degradation of the glyphosate applied to the soil samples at different concentrations, over a period of between 7 to 21 days was studied, and the result is presented in Table 2. The result showed that at 7 days 6.10±0.20, 5.64±0.17 and 4.10±0.17gkg^{-1} of glyphosate remained in the contaminated soil compared to the uncontaminated. However, at 14 days, the concentration of glyphosate reduced (4.19±0.08, 4.74±0.09 and 4.55±0.09gkg^{-1}) of soil for the three different mixture of glyphosate in glyphosate-contaminated soil. Also at 21 days, the concentration of glyphosate in glyphosate-contaminated soil continued to decrease. The lowest values of glyphosate concentration obtained at 21 days (2.35±0.02, 2.19±0.05, 2.05±0.03gkg^{-1} were significantly different from the values obtained at the first 14 days of the treatment. The results show that, the highest concentrations of glyphosate were obtained in early times of treatment (in 0-14 days) while the lowest concentrations of glyphosate were obtained at 21 days.

Table 2: Changes in glyphosate concentration in soil with time

Glyphosate concentration in soil (mg/kg)				
Days of Exposure	Control	15% (A)	10% (B)	5% (C)
0	0	5.0	10.0	15.0
7	0	4.10 ±0.17	5.64 ±0.17	6.10 ±0.20
14	0	0.55 ±0.09	0.74 ±0.09	0.91 ±0.08
21	0	0.11 ±0.06	0.27 ±0.07	0.40 ±0.05

Between 40 – 60% transformed in 7 days

Effects of Glyphosate on Genome of *A marginata*

Figure 1(a) shows the AP-PCR with oligonucleotide primer OPA (gaaacgggtg) genotypic profile of *A. marginata*. Lane 1, 2, and 3 are DNA extracts from unexposed snails (positive control with primer), which did not undergo any PCR before gel electrophoresis and hence very faint bands were observed. Lane 7 also is a negative control, which was amplified but without a primer, hence no band was observed after electrophoresis. Lane 4, 5, and 6 are respectively, amplified DNA from snails exposed to 25, 50, and 75% glyphosate- contaminated soil samples for 7days. DNA bands in lanes 8, 9, and 10 represent respectively exposure to 25, 50 and 75% glyphosate-contaminated soil at 14days after amplification.

Figure 1(b) also shows the AP-PCR genotype profile with oligonucleotide primer OPA (gaaacgggtg) on some DNA of *A. marginata* after 7, 14 and 21 days. Lane 1-4 represents control in which no glyphosate was applied and no amplified DNA from snails exposed to 25, 50 and 75% glyphosate-contaminated soil for 21days. In this case, the bands were less prominent and few than what was observed in Fig. 1(a) thus signifying decrease in toxic effect of glyphosate.

Several DNA bands observed in the exposed snails are indicators of genotoxicity as against the control, which gave discrete sharp bands. Most studies documented the occurrences of environmental contaminants such as PAHs have been reported to produce such effects. This is made manifest by increased DNA amplification products. This was observed through several DNA bands of different lengths and sizes in *A. marginata* exposed to different concentrations of glyphosate than in the unexposed snail. This observed increased number of DNA bands could be due to stress response. *A marginata* in the bid to tolerate adverse stress due to exposure to glyphosate must have produced excess DNA stains, which was helping to tolerate stressful condition. The result obtained in this study, tends to agree with Ranjendran et al., (2007) who reported that excess DNA strains are produced to help tolerate stressful condition in lower animals.

Similar findings have been reported by several researchers over increased production of novel proteins or increase damage of already existing genetic material due to exposure to pesticide: Ranjendran et al., (2007), Weber and Jung, (2002) and Bolognesi et al., (1993).

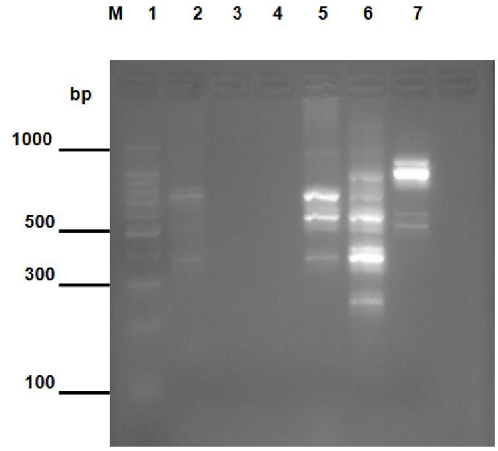

Figure 1(a): AP-PCR DNA profile of *A. Marginata* in agarose gel electrophoresis. M – Marker: 1 – 6: Lanes in which DNA were applied.

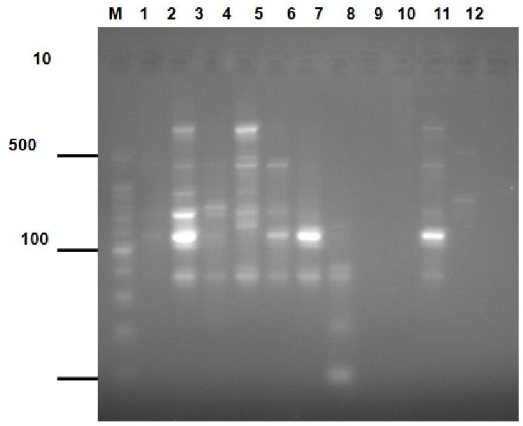

Figure 1(b): AP-PCR DNA profile of *A. Marginata* in agarose gel electrophoresis. M – Marker, 7 – 16: Lanes in which DNA were applied.

A decrease in DNA fragment / bands was observed in the present investigation after 21days exposure and this could be attributed to more severe effects of the pesticide, especially at higher concentration Kumar et al., (2011). Hence genotoxic effects are due to concentration and time dependent stress.

Qualification of DNA bands

The various bands of DNA separated on agarose gel were quantified as described in section and the result is presented in Table 3: Molecular weights (ng) of DNA bands separated on agarose gel electrophoresis at different days of exposure of *A. Merg* to varying concentration of glyphosate contamination soil. Results in Table 5 show that there is no significant difference ($p<0.05$) in DNA content in the control groups from 7 days to 21 days of exposure, while there were significant differences on the DNA contents of the exposed snails. The significant differences were highest in 50% and 75% contamination, especially at 14 days of exposure. However, the effect of the glyphosate was found to decrease in the exposed snails at 21 days of exposure, thus suggesting probable adaptation or repair mechanism of the damage DNA.

Table 3: Molecular weights (ng) of DNA bands

Exposure Period (Days)	Control	25%	50%	75%
7	107.33 ± 1.46	120 ± 1.00	175 ± 2.52	267 ± 3.00
14	108 ± 1.00	211 ± 1.73	282 ± 3.00	236 ± 1.53
21	106 ± 1.73	116 ± 1.73	114 ± 1.73	175 ± 5.00

* Highly significant different values

Conclusion

Although glyphosate is commonly used in agricultural farms to eliminate unwanted weeds, its genotoxic effect on some terrestrial non-target organism should be of great concern on ecotoxicology and human health. The giant snail, *A. marginata* is widely consumed as a source of protein in some parts of Nigeria, and hence the potential toxicity to indigenes that consume this organism as food cannot be over-ruled. Hence the application of glyphosate in agriculture should be done with greater caution especially when applied to sandy soil that is more liable to transfer the glyphosate away from the site of application rapidly into water bodies.

References

1. Acquavella JF, Bruce H, Alexander BH, Mandel JS, Gustin C, Baker B, Champan P, Bleeke M. 2004. Glyphosate biomonitoring for farmers and their families: results from the farm family exposure study. *Environ. Health Perspect*., 112, 321–326. Akcha F, Spagnol C, Rouxel J. 2012. Genotoxicity of diuron and glyphosate in oyster spermatozoa and embryos. *Aquatic Toxicology,* 106-107: 104 – 113.
2. Aktar M W, Sengupta D and Chowdhury A (2009) Impact of pesticides use in agriculture: Their benefits and hazards. *Inter disc Toxicol*., 2: 1-12.
3. Araujo ASF, Montaro RTR, Abarkeli RB. 2003. Effect of glyphosate on the microbial activities of two Brazilian soils. *Chemosphere*, 52: 799 – 804.
4. Astiz M, de Alaniz MJ, Marra CA. 2012. The oxidative damage and inflammation caused by pesticides are reverted by lipoic acid in rat brain. *Neurochemistry international*, 61, 1231-1241. Balthazor TM, Hallas LE. 1986. Glyphosate degrading microorganisms from industrial activated sludge. *Appl. Environ. Microbiol,* 51:432 – 434.
5. Bolognesi C, Parrium M, Reggiardo F, Bonassi S. 1993. Monitory of workers exposed to pesticide int. *Arch Occup Env Health,* 65 (5): 185 – 187.
6. Bolognesi C, Carrasquilla G, Volpi S, Solomon KR, Marshall EJ. 2009. Biomonitoring of genotoxic risk in agricultural workers from five Colombian regions: association to occupational exposure to glyphosate. *J Toxicol Environ Health,* 72: 986-997.
7. Cavas T, Konen S. 2007. Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassiusauratus*) exposed to a glyphosate formulation using the micronucleus test

and the comet assay. *Mutagenesis,* 22: 263-268.
8. Clements C, Ralph S, Petras M. 1997. Genotoxicity of select herbicides in Ranacatesbeiana tadpoles using the alkaline single-cell gel DNA electrophoresis (comet) assay. *Environ Mol Mutagen,* 29: 277-288.
9. Clive J. 2009. Global status of biotech/GM Crops: 2008. ISAAA Brief 39. Cobbinah RJ. 1994. Snail farming in West Africa: A practical guide. Technical center for Agriculture and Rural Cooperation, CTA, Hong Kong pp. 49.
10. Contardo-Jara V, Klingelmann E, Wiegand C. 2008. Bioaccumulation of glyphosate and its formulation Roundup in Lumbriculus variegates and its effects on bio-transformation and antioxidant enzymes. *Environ. Pollut*., 157, 57–63. Dallegrave E, Mantese FD, Oliveira RT, Andrade AJ, Dalsenter PR, Langeloh A. 2007. Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats. *Arch Toxicol*, 81: 665-673.
11. de Liz Oliveira Cavalli VL, Cattani D, Elise Heinz Rieg C, Pierozan P, Zanatta L, Benedetti Parisotto E, Wilhelm Filho D, Regina Mena Barreto Silva, F, Pessoa-Pureur R, Zamoner A. 2013. Roundup Disrupted Male Reproductive Functions By Triggering Calcium-Mediated Cell Death In Rat Testis And Sertoli Cells. *Free RadicBiol Med*., 65:335-46 Della-Cioppa G, Bauer SC, Klein BK, Shah DM, Fraley RT, Kishore GM. 1986. Translocation of the precursor of 5enolpyruvylshikimate 3-phosphate synthase into chloroplasts of higher plants in vitro. *Proc. Natl. Acad. Sci. USA,* 83: 6873-6877.
12. Dorigo U, Leboulanger C, Berard A, Bouchez A, Humbert JF, Montuelle B. 2007. Lotic biofilms community structure and pesticide tolerance along a contamination gradient in a vineyard area. *Aquatic Microbial Ecology,* 50: 91- 102
13. Dureja P, Tanwar RS. 2012. Pesticide residue in soil invertebrate: In Rathore H S and Nollet LML. 2012. *Pesticides evaluation of environmental pollution*. CRC press Boca Raton London New York. pp 393 - 454
14. Ebiloma SO, Nwonkoro SO, Okoro SPA. 2009. Calories and protein requirement of *Archachatina marginata* in capacity. Proceeding of the Third World Organization for Women in Science (TWOWS) African Regional Conference Abuja Nigeria. 639 – 650.
15. Faus I, Zabalza A, Santiago J, Nebauer SG, Royuela M, Serrano R, Gadea J. 2015. Protein kinase GCN2 mediates response to glyphosate in Arabidopsis. *Plant Biology,* 15: 14 – 26.
16. Gasniera C, Dumontb C, Benachoura N, Claira E, Chagnonb M, Séralinia G, (2009) Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology,* 262: 184 –191 Gee GW, Or D. (2002) Particle size analysis: In Dane JH, GC. Topps (eds). Methods of soil analysis Part 4, physical methods. Soil Sci. Soc. An Book series No. 5. A SA and SSSA, Madison, WI pp. 255-293.
17. Giesy, J, Dobson S, Solomon K. 2000. Ecotoxicological risk assessment for Roundup (R) herbicide. In *Reviews of Environmental Contamination and Toxicology*, Pp. 35 - 120. Guilherme S, Gaivao I, Santos MA, Pacheco M. 2010. European eel (Anguilla anguilla) genotoxic and pro-oxidant responses following short-term exposure to Roundup--a glyphosate-based herbicide. *Mutagenesis,* 25: 523-530. Guitart R. 2012. Pesticides residues in birds and mammals: In Rathore H S and Nollet L M. L. 2012 *Pesticides evaluation of environmental pollution*. CRC press Boca Raton London New York. pp 393 – 454.
18. Hendershot WHH, Lalande K, Duguette M. 1993. Soil reaction and exchangeable acidity. In carter, M. R. (ed). *Soil sampling and methods of soil analysis*. Can. Soc. Soil Sci. Lewis Pub. London. Pp 141-145.
19. IFEN, 2006. Les pesticides dans les eaux. *Dossier* 5, 15–20.
20. Jha AN. 2004. Genotoxicological studies aquatic organisms. *Mutat Res-Fund Mol M* 552(1-2): 1 – 17.
21. Jraungkoorskul W, Upatham ES, Kruatrachue M, Sahaphong S, Vichasri-Grams S, Pokcthitiyook P. 2003. Biochemical and histopathological effects of glyphosate herbicides on Nile tilapia (*Oreochromisniloticus*). *Environ. Toxico*l. 18: 260-267.
22. Kiely T, Donaldson D, Grube A. 2004. Pesticides industry sales and usage, 2000 and 2001 market estimate, US Environmental Protection Agency: Washington, DC. http:/www.epa.gov/pesticide/pestsales/01pestsales/ market_estimate 2001.pdf (accessed 28 January 2015).
23. Kishore GM, Jacob GS. 1987. Degradation of Glyphosate by Pseudomonas sp PG 2982 via a sarcosine intermediate. *J. Biol. Chem.* 262: 12164 – 12168.
24. Kumar N, Kumar RN, Bora A, Amb MK. 2011. An evaluation of pesticides stress induced protein in three cyanobacterial species, A. fertilissima, Aulosirafertilissima and Westiellopsisprolifica using SDS-PAGE. *World Academy of Science, Engineering and Technology.* 75: 770 – 773.
25. Li Q, Lambrechts MJ, Zhang Q, Liu S, Ge D, Yin R, Xi M, You Z. 2013. Glyphosate and AMPA inhibit cancer cell growth through inhibiting intracellular glycine synthesis. *Drug Des DevelTher*. 7, 635- 643 Marc J, Belle R, Morales J, Cormier P, Mulner-Lorillon O. 2004. Formulated glyphosate activates the DNA-response checkpoint of the cell cycle leading to the prevention of G2/M transition. *Toxicol Sci,* 82, 436-442.

26. Maqueda C, Morillo E, Undabeytia T, Martin F. 1998. Sorption of glyphosate and Cu (II) on a natural fulvic acid complex mutual influence. *Chemosphere*, 37:1063 1072.
27. McDougall P. 2010. The cost of new agrochemical product discovery, development and registration in 1995, 2000 and 2005-8: R & D expenditure in 2007 and expectations for 2012. Final Report for Crop Life America and the European Crop Protection Association. Phillips McDougall, Midlothian, UK, Pp 1–31.
28. Nelson DW, Sommer LE. 1982. Total carbon, organic carbon and organic matter: In: page, A. L., R. H. Miller, and D. R. Keeney (eds). Methods of soil analysis, Part 3 chemical methods ASA and SSSA, Madison, WI. Pp 539 – 579.
29. Onweremadu EU, Akamigbo FOR, Igwe CA. 2007. Lithosequencial variability between erodability and sodium concentration in soils of a rain forest. *Research Journal of Forestry,* 1(2): 73 – 79.
30. Pipke R, Schulz A, Amrhein N. 1987. Uptake of glyphosate by an *Arthrobactersp Appl. Environ. Microbiol*., 53: 974 -978.
31. Rajendran UM, Elango K, Anand N. 2007. Effect of a fungicide, insecticide and a Biopesticide on *Tolypothsixscytonemoides*. *Pest Biochem, Physiol* 87: 164 – 171.
32. Rocco L, Frenzilli G, Zito G, Archimandritis A, Peluso C, Stingo V. 2010. Genotoxic effects in fish induced by pharmacological agents present in sewage of some Italian water-treated plant. *Environ. Toxicol.* 27 (2) 18 – 25.
33. Romano MA, Romano RM, Santos LD, Wisniewski P, Campos DA, de Souza, PB, Viau P, Bernardi MM, Nunes MT, de Oliveira CA. 2012. Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. *Arch Toxicol,* 86: 663-673.
34. Shugart LR, Theodorakis C, Bickham JW. 2010. Evolutionary toxicology. In: Dewoody J A, Bickham J W, Michler C H, Nichols K M, Rhodes O E, Woeste K E (eds) Molecular approaches in natural resources conservation and management. Cambridge University Press, New York, pp 320 – 362.
35. Soil Survey Staff. 2003. Keys to soil taxonomy Ninth Edition United State Department of Agriculture Pp. 332.
36. Sullivan TP, Sullivan DS. 2003. Vegetation management and ecosystem disturbance: Impact of glyphosate herbicide on plant and animal diversity in terrestrial systems. *Environ. Rev*. 11: 37 – 59.
37. Theodorakis C W. 2008. Mutagenesis. In: Jorgensen S E (ed) Ency-clopedia of ecology. Elsevier, Amsterdam, Pp 2475 – 2484.
38. Theodorakis CW, Bickham JW, Donnelly KC, McDonald TJ, Willink WP. 2011. DNA damage in cichlids from an oil production facility in Guatemala *Ecotoxicology,* 21: 496 – 511.
39. Tsui MT, Chu LM. 2003. Aquatic toxicity of glyphosate-based formulation: Comparison between different organisms and the effects of environmental factors. *Chemosphere,* 52: 1189 – 1197.
40. Veiga F, Zapata JM, Fernandez Marcos ML, Alvarez E. 2000. Dynamics of glyphosate and aminomethylphosphonic acid in a forest soil in Galicia, North – West Spain. *Science Total Environment*71: 135 – 144.
41. Weber A, Jung K. 2002. Profiling early osmostress-Dependent Gene Expression in E. coli using DNA microarrays. *J. Bacteriol,* 184: 5502 – 5507.
42. Williams GM, Kroes R, Munro, IC. 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. *Regul Toxicol Pharmacol,* 31, 117-165. Yu M, Tsunoda H, Tsunoda M. 2011. Environmental Toxicology: Biological and health effects of pollutants. CRC press Boca Raton London New York. Pp 30 – 34.
43. Zulet A, Gil-Monreal M, Villamor JG, Zabalza A, van der Hoorn RAL, Royuela M, 2013. Proteolytic pathways induced by herbicides that inhibit amino acids biosynthesis. *Plos One* 8(9): 73847-73856.

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