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Full Length Research Paper

Toxicological effect of DDT in *Colpoda cucullus* and its potential application in forming environmental biosensor

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The main goal of this study was to determine the toxicological effect of DDT on the ciliate *Colpoda cucullus* and its potential use as a biological sensor for DDT-polluted soils. Toxicity bioassays were performed using different DDT concentrations, and an exposure time of 1 hour, with a wild strain of *C. cucullus*. Probit results indicate that the median lethal concentration of DDT for the *C. cucullus* strain is 6.68 x10^{-6} mol/l (M). This species does not show an observable morphological effect at a concentration of 1.41 x 10^{-6} M DDT, but mitochondria and nuclear membrane damage occurs at a concentration of 2.82 x 10^{-6} M and 5.64 x10^{-6} M DDT, and 100% of mortality was observed at concentrations higher than 1.12 x 10^{-5} M DDT. The change in membrane potential might be used as a signal receivable by a transducer; thus enabling the use of *C. cucullus*.

Keywords: *Colpoda cucullus*, DDT, bioassays, toxicity, LC_{50}.

INTRODUCTION

1,1,1-trichloro-2,2-bis(4-chlorophenyl)-ethane (DDT) is an anthropogenic compound that causes adverse effects on living beings. The pollutant may cause damage to human health when consumed in contaminated food, when DDT gains access to the food chain and is ultimately transported up to products consumed by humans (Table 1). In Mexico, it was detected that 82.7 mg/kg of DDT in superficial soil come from a paludic zone (ISAT, 2001). The studies of protozoa grow in relevance because these organisms are one of the links located at the beginning of the trophic chain in aquatic and terrestrial ecosystems (Pratt et al., 1997). A review of literature of research works carried out with pesticides on soils showed that the variability of the results depends on the species of ciliates, type of pesticide, concentration and exposure time, causing responses ranging from molecule synthesis impairment and deformation to the death of the organism (Lal and Saxena, 1980; Amanchi and Hussain, 2010; Trielly et al., 2006; Rehman et al., 2008). Thus, *C. cucullus* may be used as biological element to form a biosensor for DDT-polluted soils given its sensitivity to environmental changes due to anthropogenic pollutants present in soil, and further considering that it is easy to grow and handle in the laboratory. It is therefore possible to use the response generated by the *Colpoda cucullus* cells being exposed to DDT as a signal for a transducer contributing to the formation of a whole-cell biosensor. The objective of this work was to observe the toxicological effects of DDT on the soil ciliate *C. cucullus*, and to determine its potential use as an environmental biosensor.

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MATERIAL AND METHODS

Collection, Culture, and Morphological Identification of *C. cucullus* (Müller, 1773)

A wild strain of *C. cucullus* (Colpodidae) was collected from an agricultural soil from Hidalgo, Mexico, which was considered to be healthy. This soil had the following characteristics: a pH close to neutral (7.54), 8% of humidity, organic carbon content classified as very high (45 g/kg) for agricultural use (Rodriguez and Rodriguez, 2002) and a sandy-loam texture as per the results of a granulometric analysis. Following the methodology indicated by Bamfort (quoted by Allef and Nanniper, 1992), the excystment of *C. cucullus* was inducted by adding distilled water to boxes containing said soil. Then, the culture was incubated at 28°C (Incubator J.M. Ortiz, Mexico). The nourishing medium was an infusion of barley containing 10^5 living *E. coli* in 200 ml.

Vital staining with 1% methyl green and 1% neutral red were used for the morphological identification of the relevant organelles using a stereoscopic microscope (Motic SMZ-168 Germany). Silver impregnation was made with 0.5% silver nitrate (Klein, 1958; Da Silva, 2000) to reveal the infraciliature using a light microscope and contrast media, a magnification of 400X and a calibrated ocular micrometer coupled to a photographic camera, video recording system and color printer (Sony, Japan). Further, the sizes of 10 specimens of the *C. cucullus* were measured, and the required time for their asexual reproduction was determined under controlled laboratory conditions.

C. cucullus DNA Extraction Procedure for Molecular Identification by PCR

The genomic DNA extraction from pure cultures of *C. cucullus* was performed as follows: Cell lysis was achieved by concentrating 50 ml of culture medium that was centrifuged at 1760 g of medium containing the specimens. The resulting pellet was re-suspended by adding 200 µl of 10X TE (Tris-HCl 10 mM, pH 8.0 and EDTA 1 mM) and frozen at –80 °C for 15 minutes. Then, it was subjected to a boiling temperature (85 °C) for 10 minutes. Equal volumes (50 µl) of chloroform-isoamyl alcohol (24:1) and saturated phenol were added to the above mixture, centrifuged for 5 min at 1760 g, 4 °C, washed two times with 200 µl of chloroform-isoamylalcohol and further centrifuged for 1 minute at 1760 g, 4 °C. The aqueous phase, containing the genomic DNA was washed in a fresh tube; 1/10 volume of sodium acetate (3 M, pH 7.0) and 2 volumes of cold absolute ethanol were added, mixed and stored for 12 hours at –20 ºC. Thereafter, the mixture was centrifuged for 30 minutes at 13,805 g at 4 °C, the DNA pellet was double washed using 300 µl of cold ethanol (80%), and centrifuged again for 3 minutes at 13,805 g (4 °C). The pellet was dried thoroughly before its final re-suspension in 100 µl of deionized water, and was stored at –20°C.

Polymerase Chain Reaction

The 18S Ribosomal RNA gene was amplified using Colp-F (5´-ACCATACATATGCATGCTGT(C/A) AAACCT(G/A)ACTT) and Colp-R (5´-ATCATAGATGCTTGCACA(T/C)AAAGTCCCTC) primers for amplifying a length of 1190 bp. The PCR conditions were designed in an Eppendorf Mastercycler gradient thermocycler with the following program: initial denaturation at 94°C for 2 min, a total of 25 cycles, each of 94°C for 67 s, 58°C for 41 s, 72°C for 65 s, was followed by a final extension step of 72 °C for 7 min.

The master mix was 3 mM $MgCL_2$, 2U Taq Polymerase (Vivantis), 0.2 mM each dNTP, 4 mM each primer and 1 µl DNA for a 50 ml of total reaction volume. The electrophoresis was carried out in 0.8 and 1% agarose gel at 75 volt and was revealed using an ethidium bromide bath. The resulting image was taken with a Benchtop UVP photo-documentation system. The amplicon was cleaned up with QIAquick PCR purification kit, QIAGEN and sequenced in an automatized sequencer (Perkin-Elmer Applied Biosystems ABI PRISM 310 Genetic Analyzer).

Molecular identification

Morphological species identification was confirmed by molecular comparison of the resulting sequences of both the isolated wild strain and the American Type Culture Collection 30916 (ATCC) *Colpoda* strains (1130 base pairs or bp) against those available in the Genbank of the National Center for Biotechnology Information (NCBI), using the Basic Local Alignment Search Tool (BLAST) software to find the identity of the highest sequences to identify the specimens. Both sequences of the wild strain and ATCC were aligned with the CLUSTAL W software, BIOEDIT package, version 7.09 (Hall, 1999). The MEGA software version 4.0 (Tamura et al., 2007) was used to build a phylogenetic tree. The distance matrix calculations and the phylogenetic tree construction were made using the Neighbour-Joining method (Mihaescu et al., 2009) and the maximum likelihood. The Bootstrap values were generated with 1000 replicas.

Transmission electron micrographs of *C. cucullus*

Samples were fixed in 3% glutaraldehyde phosphate buffer and 1% osmium tetraoxide for two hours. Serial alcohol dehydration (50%, 75%, 95% and 100%) was carried out before embedding samples in an epoxy resin.

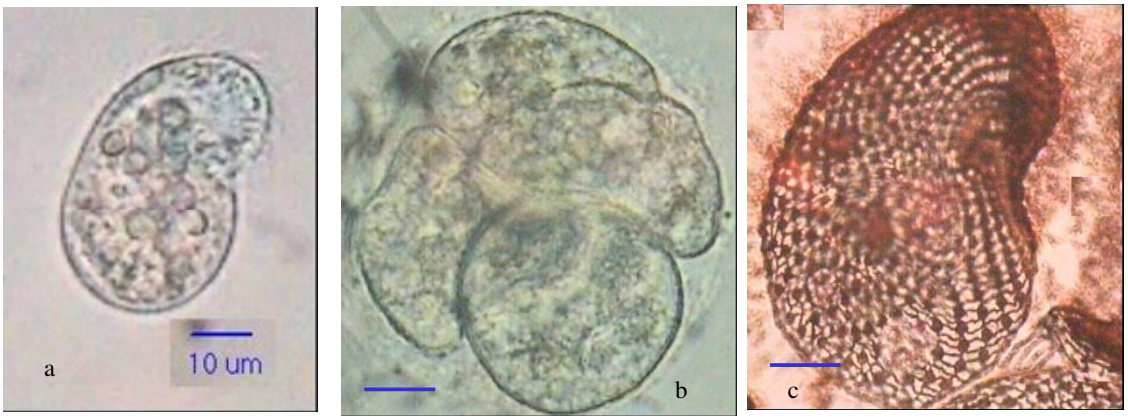

Figure 1. Micrographics of *C. cucullus* a) Digestive vacuoles observed with methyl green, 40X; b) Reproduction cyst, Phases contrast, 40X; and c) Silver lines system, 100X. Bar = 10 µm.

Ultra-fine slices were obtained using ultra–microtome (Leica UltraCut-R). The slices were mounted on racks having a diameter of approximately 3 millimeters. The samples were observed through a JEOL electron microscope (JEM 1200 EX-11).

Toxicity bioassays of the *C. cucullus* with DDT

Aqueous solutions containing 1.41 x 10^{-6} M, 2.82 x 10^{-6} M, 5.64 x 10^{-6} M, 8.46 x 10^{-6} M, 1.12 x 10^{-5} M and 1.41 x 10^{-5} M of DDT were prepared. DDT at 99.5% of purity (Sigma-Aldrich) had been previously dissolved in 10 ml acetone (analytic grade). Later, an aliquot of 2 ml was taken and diluted to volume with distilled water to obtain the required concentration. The control solution was prepared likewise, omitting the addition of DDT. The acetone was eliminated by evaporation and its complete elimination was verified by determining the osmolarity of the prepared solutions. The DDT dissolved into the acetone presented an osmolarity of 69 mOsm. After evaporation this value decreased to 39 mOsm. In the water control having no acetone, the osmolarity was 41 mOsm. It had been previously observed that the different solutions of acetone caused the immediate death of *C. cucullus.* Due to the fact that DDT precipitates in a short time (first 24 hours), it was necessary to carry out the intended bioassays immediately. These bioassays were set up using, for each concentration, three glass containers with a diameter of 1 cm containing 10 specimens of *C. cucullus* per concentration. Overall, 30 cells were exposed to DDT and 30 cells were assigned to the control solution. Assays with soil extracts were done in the same way, i. e., soil extract without DDT and with DDT. The observations and counts were made using a stereoscopic microscope during 1 hour exposure. This test was conducted with the aim of monitoring the concentrations at which the exposed cell died and to identify any other morphological change, including no damages.

Determination of the morphological effect of the DDT in *C. cucullus*

In order to obtain evidence of the effects of the DDT on the *C. cucullus* morphology, the specimens were only exposed to 6.68 x 10^{-6} M and 2.82 x 10^{-6} M of DDT. The respective samples were processed as mentioned above so as to obtain transmission electron microphotographs, using a JEOL JEM 1200 EX-11 microscope. The mortality criterion used was the time when cell lyses occurs at these concentrations.

RESULTS AND DISCUSSION

Morphological identification of the wild strain of *C. cucullus*

According to the performed assays, the wild strain of *C. cucullus* is 41.15 ± 5.23 x 60.81 ± 5.69 µm in size. The vital staining carried out with methyl green and neutral red allowed for the examination of digestive vacuoles, nucleus and ciliates. Also, the silver lines systems were evidenced by silver impregnation (Foissner, 1993). These observations lead to the conclusion that the isolated strain effectively corresponds to the description of *C. cucullus* (Figure 1a and c). Additionally, the asexual reproduction was observed after 6.5 hours, when a reproductive cyst originated four daughter cells of smaller size (Figure 1b). This observation was made in the cultures maintained under controlled laboratory conditions.

Consequently, the species classification, according to Lynn and Small (Lynn and Small, 2000) is as follows:

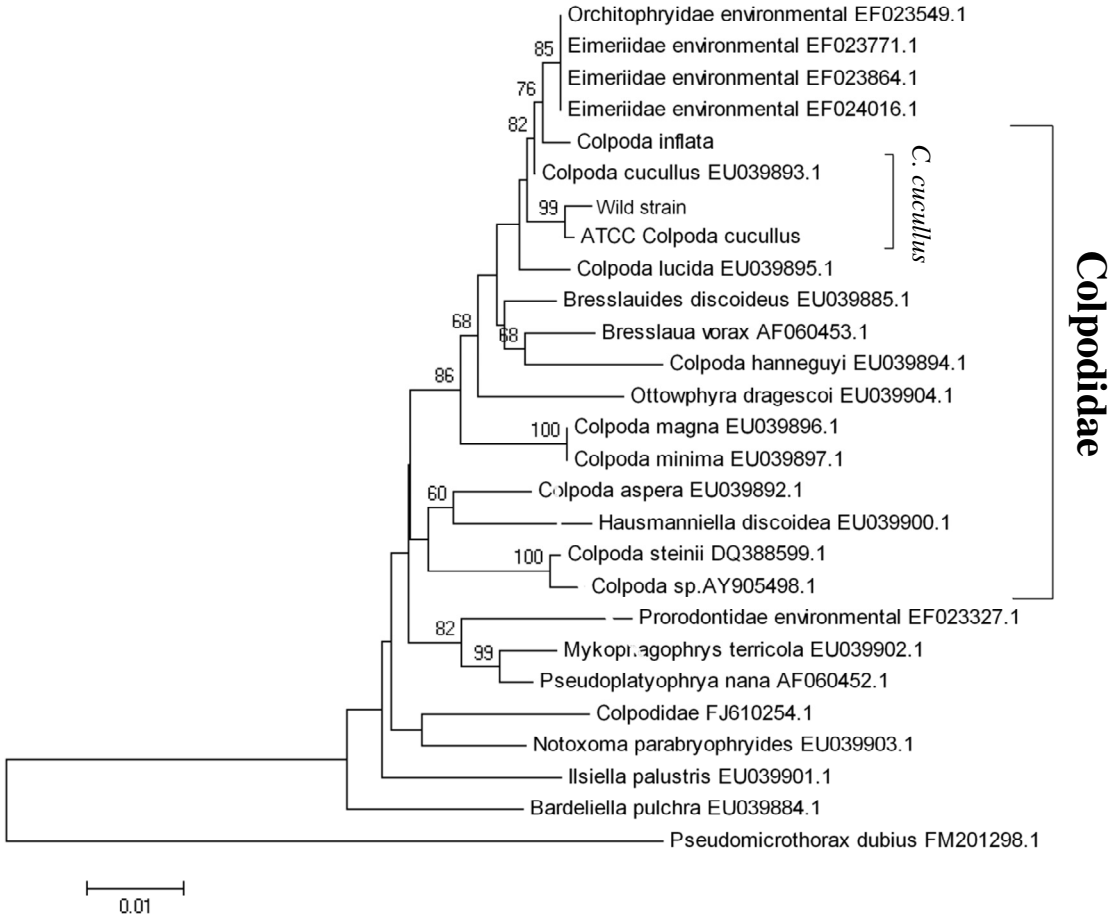

Figure 2. The phylogenetic tree of the 18S Ribosomal gene sequences of two pure strains amplified with the Colp-F Colp-R primers referred from the NCBI's Genbank. *Bootstraps* values over 50% are shown. Both sequences are related to the *Colpoda cucullus* reference (Access No. EU039893.1).

Domain: *Eucaryota*
Phylum: *Ciliophora* Doflein, 1901
Subphylum: *Intramacronucleata* Lynn, 1996
Class: *Colpodea* Small and Lynn, 1981
Order: *Colpodida* from Puytorac et al., 1974
Family: *Colpodidae* Bory from St. Vicent, 1826
Genus: *Colpoda* Müller, 1773
Species: ***C. cucullus*** Müller, 1773

Molecular identification

The resulting phylogenetic tree of the above data is show in Figure 2, in which the molecular biology analysis of the wild strain is shown to correspond to ***C. cucullus***.

Response of *C. cucullus* to the DDT exposure

The toxicity tests using *C. cucullus* exposed to different DDT concentrations (Table 1) indicate that the concentration, at which a survival rate of 100% was seen without any observable effects after 1 hour of contact, was 1.41×10^{-6} M of DDT. At a DDT concentration of 2.82×10^{-6} M, *C. cucullus* specimens showed a mortality rate of 33.3% after 30 minutes of exposure. Further, the cells are inactive and cell lysis is likely to occur. When using a concentration of 5.64×10^{-6} M, the mortality time for some specimens starts at 5 minutes of exposure, with a mortality rate of 63.3%. At 6.68×10^{-6} M, mortality rates raise to 50 %. Finally, at concentrations of 1.12×10^{-5} M and 1.41×10^{-5} M, a mortality rate of 100% occurs almost upon contact between the specimen and DDT (Figure 3 and 4). The total exposure time was of 1 hour for the full test. Probit results indicate that the mean lethal concentration (LC_{50}) of DDT for the *C. cucullus* strain is 6.68×10^{-6} M (Figure 5), while the toxicity units (TU) are 42.19 UT, thereby confirming that this is effectively a highly toxic compound for this species.

The correlation coefficient (R^2) obtained from the

Table 1. Concentrations at which DDT causes adverse effects to some organisms.

Organism	Concentration	Exposure Time (min)	Effects	Reference
Enterobacter aerogenes	0.1 - 5 mg/kg	ND	Degradation up to 3.1%	(Aislable et al., 1997)
Blepharisma intermedium	1 mg/ml	240	Bioaccumulation in over 90%	(Amanchi and Hussain, 2010)
Stylonychia notophora	100 mg/ml	60	Inhibition of cell growth and division	(Lal and Saxena, 1980)
Cheloniamydas, Eretmochelys imbricata	0.494 mg/ml	ND Not Determined	Emaciation of the eggs shell	(Cuevas et al., 2003)
Plants (seeds)	63.6 µg/kg	ND	Bioaccumulation	(Waliszewki and Infanson, 2003)
Mammals (bats)	<40 mg/kg	ND	Bioaccumulation, death	(Ministerio del medio ambiente, 2006)
Helichoerus gripes (grey seal)	420 mg/kg	ND	Bioaccumulation in lipids	(Badii et al., 2006)
Human being (milk)	1.11± 2.38 µg/g	ND	Intoxication of newborns and infants	(Prado et al., 2002)
C. cucullus	1.12×10^{-5} M (4.0 mg/ml)	Immediate	Death	This study
C. cucullus	6.68×10^{-6} M (2.3 mg/ml)	60	LC_{50}	This study
C. cucullus	2.82×10^{-6} M (1.0 mg/ml)	30	Morphological damages	This study
C. cucullus	1.41×10^{-6} M (0.5 mg/ml)	60	NOEL	This study

Probit chart with a value of 0.99 indicates that the observed effect on *C. cucullus* cells is caused by DDT. The mean lethal concentration value was obtained from the antilog of the $\log_{10}$ concentration, at which half of the cells exposed to DDT are killed (five out of ten cells in this particular study, corresponding to 2.3 mg/ml equivalent to 6.68×10^{-6} M of DDT.)

Morphological damage caused by the exposure of *C. cucullus* to DDT

The morphology of the mitochondria showed damage at a DDT concentration of 2.82×10^{-6} M (Figure 6). The main effects occurred on the mitochondrial crest, which disappeared due to cell lyses, and cytoplasm liquefaction was also observed.

At a DDT concentration of 6.68×10^{-6} M, corresponding to LC_{50}, both the damage to the nuclear membrane and cytoplasm lyses are more evident, possibly due to the change in the cell osmolarity. The mitochondria have practically lost their structure (Figure 6). Damage in the nuclear membrane due to cell lysis was also noted (Figure 7). This effect might be caused by the fact that DDT blocks the ion channels, causing the leakage of Na^+ and Ca^{++}, which aid the cell membrane to go back to its stable state (Ramirez and Mijangos, 1999). This occurs because DDT acts as an inhibitor of ATPase,

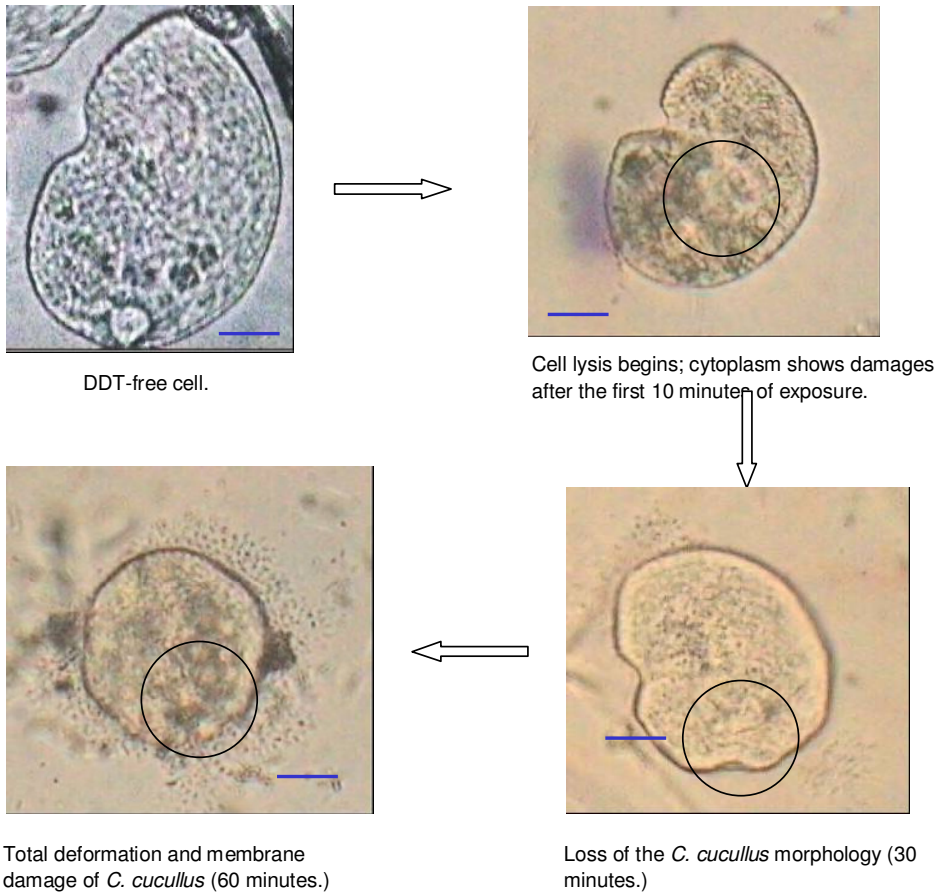

Figure 3. Sequence in which damages occur in the *C. cucullus* upon exposure to DDT. The circle shows the zone of damage in the cell at a concentration of $8.46x10^{-6}$ M. Bar =10 µm.

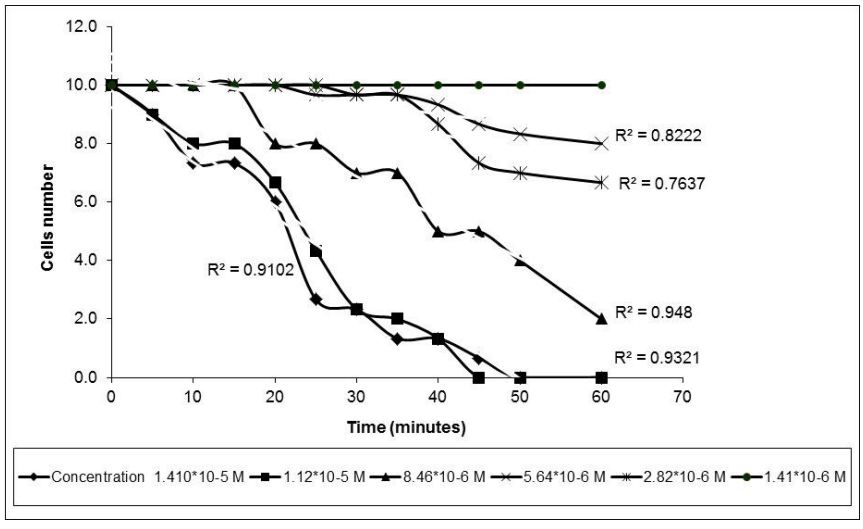

Figure 4. Data obtained from the acute toxicity tests at different DDT concentrations.

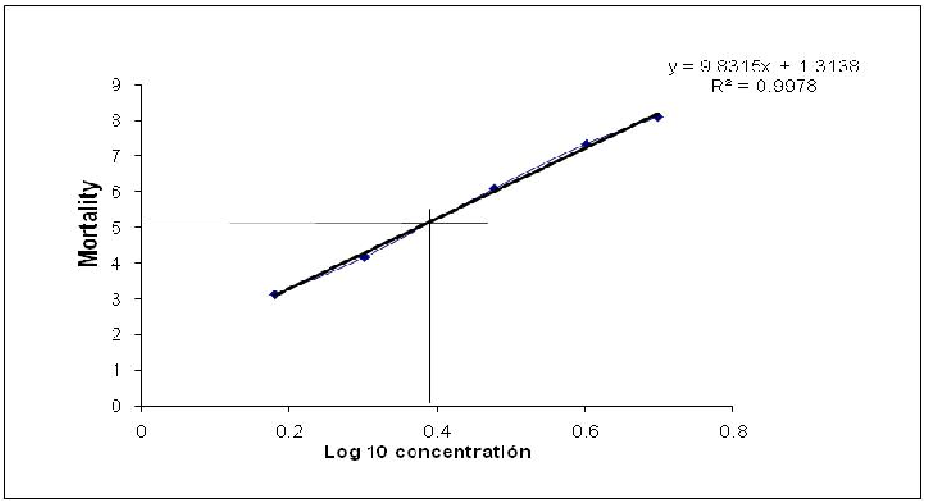

Figure 5. Result of the Probit analysis for calculating the LC_{50} of DDT on *C. cucullus.* The LC_{50} was 6.68 x 10^{-6} M DDT after an exposure time of 1 hour

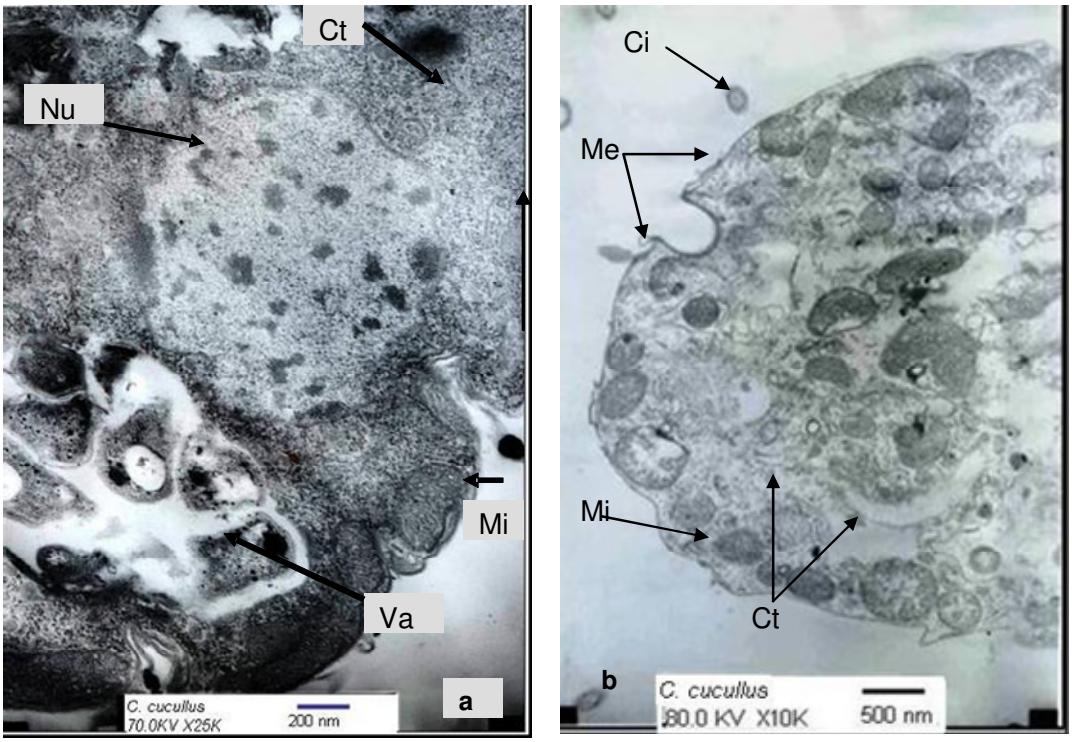

Figure 6. Transmission electron micrographs of *C. cucullus.* a) Healthy cell showing no damaged structures; and b) 2.82 x 10^{-6} M DDT or 1 mg/ml, damage is observed in mitochondria. Me=membrane, Ci = ciliate, Mi = Mitochondria, Nu = nucleus, Ct = cytoplasm, Va= digestive vacuole.

which regulates Ca^{++} ion in the cellular membrane, thus preventing a quick return to an equilibrium state (Barberá, 1989).

Upon DDT induction, the permanent opening of the ion channels of the *C. cucullus* cell membrane causes their depolarization due to ion leakage (Figure 8). This effect may be detected using fluorochromes, which constitute a signal detectable as luminescence by an optic transducer, and the intensity of which would be inversely proportional to the DDT presence. Finally, that signal would be processed to obtain a quantitative response of the DDT contents in the analyzed specimen, which had been previously extracted from the soil. An optoelectronic environmental whole-cell biosensor might thus be formed, in which the *C. cucullus* would be the biological element (Figure 9).

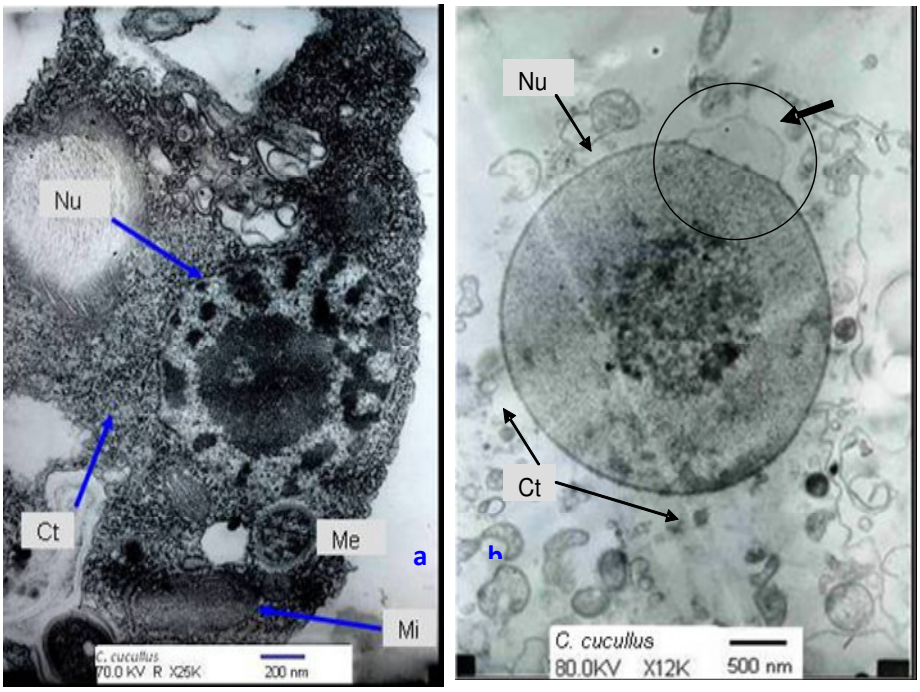

Figure 7. Micrographics of *C. cucullus* a) Control, DDT-free cell; b) Damage observed in the nucleus at [6.68 x 10^{-6} M or 2.3 mg/ml]. Me=membrane, Ci = ciliate, Mi = Mitochondria, Nu = nucleus, Ct = cytoplasm. The circle indicates damage in the nucleus.

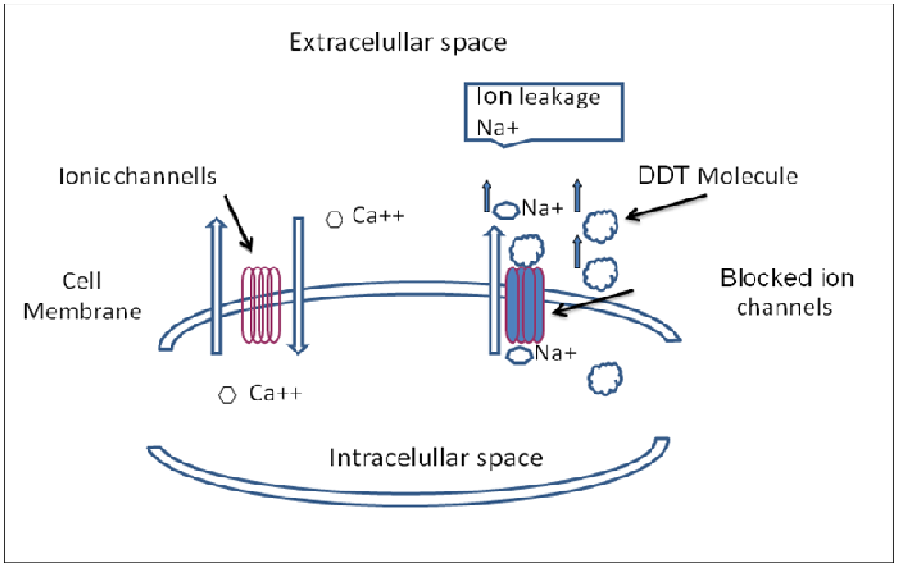

Figure 8. Possible effect of DDT on the cell membrane of *C. cucullus*

CONCLUSION

The performed tests to reveal the morphology and those based on molecular biology indicate that the wild strain of the isolated *Colpoda* corresponds to the *Colpoda cucullus* species. The bioassays carried out with *C. cucullus* with different DDT concentrations allow to establish that, for 1-hour exposure, there are no observable effects on the morphology at a DDT concentration of 1.41 x 10^{-6} M. Morphological damages

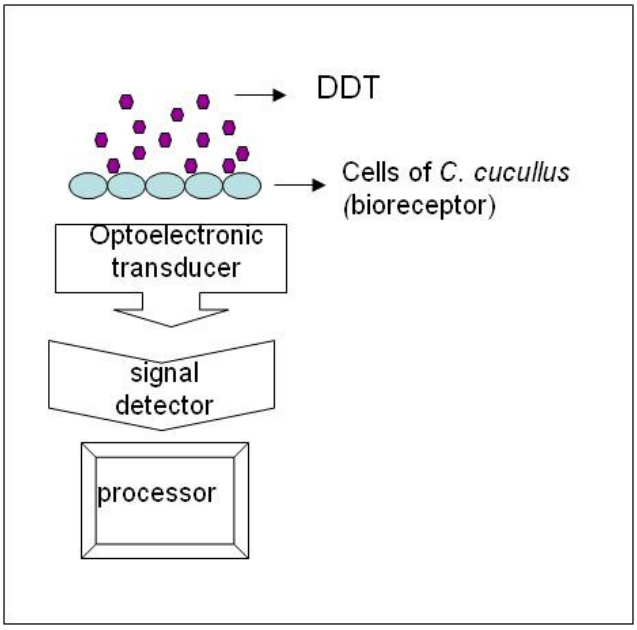

Figure 9. Diagram of the optoelectronic whole-cell biosensor.

are observed at a DDT concentration of 2.82 x 10^{-6} M, starting at 30 minutes of exposure. The transmission electron micrographs showed that at low DDT concentrations, cell physiological damage is significant since there is mitochondrial loss by lysis, possibly due to the change in cell osmolarity. The LC_{50} was a DDT concentration of 6.68 x 10^{-6} M. As DDT concentration increases, cells undergo damages in other important structures, such as the nuclear membrane and mitochondria. This means that *C. cucullus* may be used to monitor residual contamination of DDT in soils up to a DDT concentration of 2.82 x 10^{-6} M, hence providing a device that is able to measure membrane depolarization.

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