*International Journal of Environment Sciences*
*Vol. 5 No. 1 (January-June, 2019)*

*Received: 01st September 2018 Revised: 15th October 2018 Accepted: 30th October 2018*

**Research Article** **ISSN 0976 – 4402**

# Pesticide-Resistant Bacterial Strain

Osama el gialani elsaid[1] ,Azhari Omer Abdelbagi[2], Elsiddig Ahmed Elmustafa Elsheikh[3]
1-Faculty of Agricultural Technology and Fish Sciences, Al-Neelain University, Khartoum, Sudan
2- Faculty of Agriculture, University of Khartoum, Khartoum, Sudan
3-- Faculty of Agriculture, University of Khartoum, Khartoum, Sudan

## ABSTRACT

Mutant strains of bacteria were isolated through consecutive exposure to elevated concentration of endosulfan under carbon free media. The results showed that the most tolerant bacteria were *Bacillus sp*. The comparative degradation of endosulfan by mutant and their wild types was studied under soil conditions. Results showed that wild types (present in large number) showed faster decrease in half lives compared to the mutant strains (few in numbers). However mutant strains might have greater potential if they find a chance to propagate in massive numbers.

**Key Words:** Endosulfan, degradation, soil, microorganism, Sudan

## 1. Introduction

The chlorinated cyclic sulfite diester endosulfan (Thiodan, bicyclo-[2.2.1]-2-heptene-5,6-bisoxymethylene sulfite) is a broad-spectrum insecticide that has been used extensively for over 30 years on a variety of crops. Endosulfan is often classified as a cyclodiene and has the same primary action and target site as other cyclodienes (**Casida,**1993). However, it has chemical and physical properties significantly different from other cyclodiene insecticides that affect both its environmental and biological fates. In particular, endosulfan has a relatively reactive cyclic sulfite diester group (Van Woerden, 1963) and, as a consequence, its environmental persistence is lower than that of other cyclodienes, albeit still higher than that of many other insecticides. Since the deregistration in many countries of most cyclodiene insecticides, the ongoing availability of endosulfan has become important as an alternative option in resistance management strategies of pest species. Additionally, compared to many other available insecticides, it has low toxicity to many species of beneficial insects, mites, and spiders (**Singh,** e.t. 1990). However, endosulfan is extremely toxic to fish and aquatic invertebrates and it has been implicated increasingly in mammalian gonadal toxicity (Turner, e.t. 1997 and Chaudhuri, e.t. 1999), genotoxicity (Pauland Balasubramaniam. 1997), and neurotoxicity. These environmental and health concerns have led to an interest in post application detoxification of the insecticide

Biodegradation of persistent compounds is an important mechanism for their dissemination in the environment (Alexander, 1981; Marcae, 1990; Wallnofer& Engelhardt, 1990). In predicting the persistence of synthetic chemicals in soil, sediment and natural water, it is necessary to determine the role of endogenous microorganisms in the over all degradation process. Microorganisms play an important role in the conversion of cyclodiene insecticides in soil to nontoxic products. In the natural environment microorganisms may provide some protection against toxicity of endosulfan. Pure culture of a range of soil microorganisms have been reported to transform endosulfan to a nontoxic diol metabolite

in unsealed liquid cultures (Elzorgani & Omer, 1974 and Marten, 1976). Endosulfan can be completely degraded in about two weeks to nontoxic metabolite under anaerobic conditions (Guerin & Kennedy, 1999). Microbial degradation of endosulfan was also reported by Shivaramaiah and Kennedy (2006). They ,also, identified endodiol as the major degradation product in an undefined mixture of microorganisms obtained from soil suspension. Tariq *et al*. (2000) reported that degradation of endosulfan occurred in contaminant with bacterial growth when endosulfan was used as only source of sulfur in the culture, while no growth occurred in the absence of endosulfan. As part of the development of an overall strategy to manage organochlorine pesticide residues, the present study aimed to study the relative capability of tolerant stains (compared to their wild types) degrading endosulfan in the soil.

## 2. Material and Methods

### 2.1 Mutant strains procedure

From stock cultures (isolated microorganism form soil by selective media) one ml was taken and placed in sets of sterilized test tubes containing MPB (Meat Peptone Agar**)** media. The media were prior treated with 200 mg/l endosulfan. The test tubes were incubated for seven days at 30˚ C. The growth of microorganisms in these test tubes was observed by locking for turbidity of the media. Then counts of microorganisms in each test tube were estimated. One ml was taken from each of the seven days incubated test tube and transferred to sterilized set of another test tubes each containing ten milliliter of MPB treated with higher concentration of endosulfan (400 mg/l ). Test tubes were incubated for another seven days, growth and count of microorganisms were observed and recorded. Microorganisms were subject to further consecutive elevated concentrations of endosulfan (600, 800 or 1000 mg/l) and effects on growth and counts were determined.

### 2.2 Identification of microorganisms

The microorganism tolerant to high concentrations of the endosulfan were identified as follows.

#### 2.2.1 Culture of sample on nutrient agar media

One ml was taken by sterilized pipette from tests tubes containing the most tolerant microorganisms (test tube containing 1000 mg/l endosulfan) and placed in a Petri dish containing sterilized nutrient agar. The inoculated plates were then incubated at 37˚ C for 24 hours. This procedure was replicated four times. The plates were checked for shape, color and other general characteristics of the colonies growth.

#### 2.2.2 Gram stain

The colonies obtained in the four plates were then subjected to Gram stain test as described in Brough (1999). One drop of distilled water was added to sterilized slides, and then small portion of colony was taken by the loop on a drop of water, and then was spreaded over the slide. The drop was allowed to dry by exposure to air at room temperature. Then the smear was fixed by heating on a flame and stained by three types of stains (Crystal violet stain for 60 seconds, lugols iodine for 60 seconds, and decolorized by alcohol for 10 seconds). After each stain the smear was rapidly washed by water. Lastly the smear was dried by exposure to air and examined under oil by microscope 100 x magnifications. The slides were examined

for Gram positive rod with central and terminal to sub terminal spores and results were recorded.

### 2.2.3 Inoculation in Mannitol salt agar

Small portion of colonies grown in nutrient agar plates were taken by sterilized loop and inoculated in Mannitol salt agar. They were incubated at 37˚ C over night. Shape and color of colonies were recorded.

### 2.2.4 The comparative degradation experiment

The purpose of this experiment was to study the relative capability of mutant stains (compared to their wild types) degrading endosulfan in soil. A pre cleaned and sterilized conical flask (1000 ml) containing 500 g soil was prepared. The flask with its contents was sterilized in an oven at 160˚ C for three hours. The flask was allowed to cool at room temperature. Two hundreds ml distilled water containing 150 mg endosulfan were added to sterilized soil. The treated soil was sub-divided into 30 sets each composed of 10 g in sterilized flasks (50 ml). Flasks in triplicate were then treated with endosulfan (500 mg/kg ) and inoculated with one ml of bacteria as follows;

1) Organic nitrogen bacteria (wild types).
2) Organic nitrogen bacteria exposed to 200 mg/l endosulfan
3) Organic nitrogen bacteria exposed to 600 mg/l endosulfan
4) Organic nitrogen bacteria exposed to 1000 mg/l endosulfan

All test tubes and flasks were arranged in a completely randomized design with three replicates and incubated at 30 $C^{\circ}$ for a total of 60 days. The level of starting material and endosulfan sulphate generated was checked at 15 days interval

.

### 2.2.5 Extraction and analysis

All flasks were incubated at 30˚ C for 60 days and residues of endosulfan were extracted and analyzed using GLC every 15 days.

### 2.2.6 Extraction of endosulfan from soil sample

Ten grams of dried soil from each flask was placed in a jar. Redistilled hexane (80 ml) and acetone (20 ml) were added. The jar was tightly closed and placed in an end over shaker for two hours. The sample was then left to stand for a while to enable the soil particles to settle down and then filtered in a round button flask through 240 mm filter paper containing 100 mg of anhydrous sodium sulphate to absorb the moisture from the filtrate. The round button flask with its content was placed in a rotary evaporator to reduce the filtrate volume to about ten ml. the extract was then kept in vials, tightly closed and stored in refrigerator at 5˚ C for the analysis.

### 2.2.7 GLC analysis

Agilent 6890N gas liquid chromatograph equipped with flame ionization detector (FID) and fused silica capillary column of 30 m and 0.25 mm i.d. was used for analysis of the extracts. The stationary phase (0.25 mm thickness) used was 5% phenyl. methylpolysiloxane. Oven, detector and injector temperature were 250˚ C, 270˚C and 230˚ C respectively. Nitrogen ($N_2$)

was used as carrier gas at flow rate of one ml\minute. Analysis of sample was done by injection of 0.2 micro liter. Five concentration of the standards (mg $L^{-1}$, 2 mg $L^{-1}$, 3 mg $L^{-1}$, 4 mg $L^{-1}$, and 5mg $L^{-1}$) were injected in the GLC used for constructions of the stander curves. Reanalysis of standard solution were repeated every morning to check for the performance of the machine. Septum was changed when necessary.

## 3. Results and discussions

### 3.1 Identification of organic nitrogen bacteria tolerate to high concentration of endosulfan

The organic nitrogen bacteria were cultured according to the methods described before (Brough, 1999). Results of various steps of culturing leading to the identification as *Bacillus sp*. was summarized in table 1. the only bacteria tolerant to the highest level of endosulfan (1000 mg/l) was *Bacillus sp*. Other unidentified types of bacteria were observed at lower concentration. And It is clear that the various types of bacteria have different tolerance to elevated level of endosulfan

### 3.2 Comparative degradation of endosulfan by mutant strains and their wild types

Results in Tables 3 and 4 show the half lives of endosulfan and the counts of microorganism. The wild types of bacteria (from stock culture) were the most effective in reducing the half lives of both $\alpha$ and $\beta$-endosulfan. The wild types bacteria strains caused 83% reduction in half lives of Both $\alpha$ and $\beta$- endosulfan The colony forming units(CFU/ml of wild types isolates of bacteria were high ( $80.3\times10^4$ ) compared to The colony forming units(CFU/ml of the mutant strains ( $0.9\times10^4$ ). Generation of endosulfan sulphate from degradation of endosulfan was monitored for a total of 60 days. In the control sets endosulfan sulphate starts appear before 10th day and gradually increases with time and did not appear to decrease until 60 days. The highest level of endosulfan sulphate observed was 0.2 mMl/l. However incubating endosulfan with various isolates of tolerant strains caused a clear change in the level and fate of endosulfan Sulphate.

**Table 1:** Identification tests for tolerant organic nitrogen bacteria

| **Test sequence** | Culture test | Observations |
|---|---|---|
| I st test | Culture in nutrient agar media | Dry, white and creamy colonies |
| 2 nd test | Gram stain | Gram positive rod with central and terminal to sub terminal spores. |
| 3 rd test | Inoculation in Manito salt agar. | Yellow, flat dry colonies. |

**Table2.** General counts Organic Nitrogen bacteria tolerant to the highest concentration of endosulfan (1000 mg/l )

| Types of microorganisms | | Concentration (mg/l ) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Zero | 200 | 400 | 600 | 800 | 1000 |
| Bacteria | *Bacillus Sp.* | + + + | + + + | + + + | + + | + + | + |

Where
+ = Growth covering less than 25 % of plate
++ = Growth covering between 25 – 50 % of the plate
+++ = Growth covering above 50 % of the plate
- = no growth observed

**Table 3**. Half live (days) and percentage reduction in half live of α-endosulfan incubated with microorganisms isolated from elevated concentration of endosulfan in soil.

| Microorganisms | $R^2$ | Slope | $\tau_{½}$(days) | Reduction in $\tau_{½}$ % | CFU/ gm of soil |
|---|---|---|---|---|---|
| Organic Nitrogen Bacteria from stock culture | 0.7173 | 1.5 | 14.4 | 82.7 | $80.3\times10^4$ |
| Organic Nitrogen Bacteria isolated from 200 mg/l endosulfan | 0.8947 | 0.99 | 39.7 | 52.2 | $4.9\times10^5$ |
| Organic Nitrogen Bacteria isolated from 600 mg/l endosulfan | 0.8097 | 1.2 | 28.7 | 65.4 | $6.7\times10^4$ |
| Organic Nitrogen Bacteria isolated from 1000 mg/lendosulfan | 0.8862 | 1.4 | 33.3 | 59.9 | $0.9\times10^4$ |
| Controls | 0.9529 | 0.54 | 82.9 | 0.0 | 0.0 |

Where R2 = Determination coefficient and $\tau_{½}$ = Half live

**Table 4**. Half live (days) and percentage reduction in half live of β-endosulfan incubated with microorganisms isolated from elevated concentration of endosulfan in soil.

| Microorganisms | $R^2$ | Slope | $\tau_{½}$(days) | Reduction in $\tau_{½}$ % | CFU/ gm of soil |
|---|---|---|---|---|---|
| Organic Nitrogen Bacteria from stock culture | 0.7753 | 1.54 | 15.4 | 82.5 | $8.0\times10^5$ |
| Organic Nitrogen Bacteria isolated from 200 mg/l endosulfan | 0.8331 | 0.89 | 35.5 | 59.6 | $4.9\times10^5$ |
| Organic Nitrogen Bacteria isolated from 600 mg/l endosulfan | 0.8754 | 1.54 | 18.1 | 78.9 | $6.7\times10^4$ |
| Organic Nitrogen Bacteria isolated from 1000 mg/L endosulfan | 0.9737 | 1.69 | 30.4 | 65.4 | $0.9\times10^4$ |
| Controls | 0.9921 | 0.53 | 87.9 | 0.0 | 0.0 |

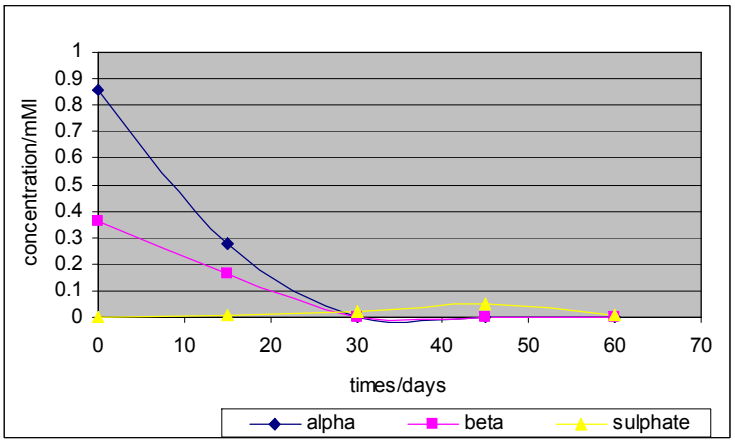


**Figure 1** Degradation of Endosulfan (α, β) and generation of sulphate upon incubation of the chemical (100 mg/l) with wild types of organic nitrogen bacteria (Isolated from stock culture free from endosulfan) in soil.

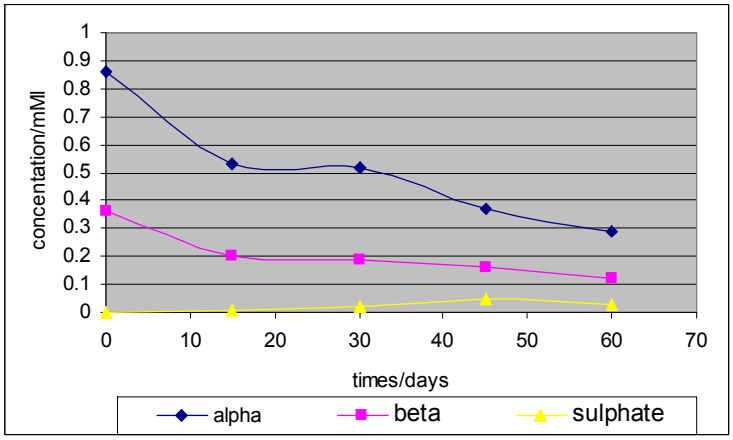


**Figure 2** Degradation of Endosulfan (α, β) and generation of sulphate upon incubation of the chemical (100 mg/l) with parent strain of organic nitrogen bacteria (exposed to 200 mg/l of endosulfan) in soil.

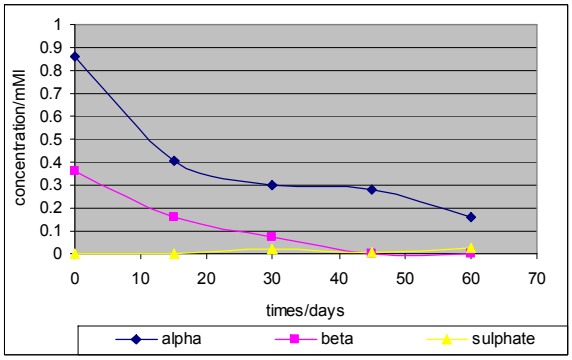

**Figure 3**. Degradation of Endosulfan (α, β) and generation of sulphate upon incubation of the chemical (100 mg/l) with parent strain of organic nitrogen bacteria (exposed to 600 mg/l of endosulfan) in soil.

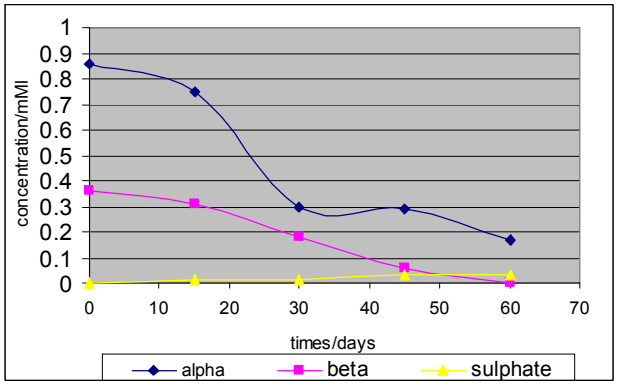


**Figure 4.** Degradation of Endosulfan (α, β) and generation of sulphate upon incubation of the chemical (100 mg/l) with parent strain of organic nitrogen bacteria (exposed to 1000 mg/l of endosulfan) in soil.

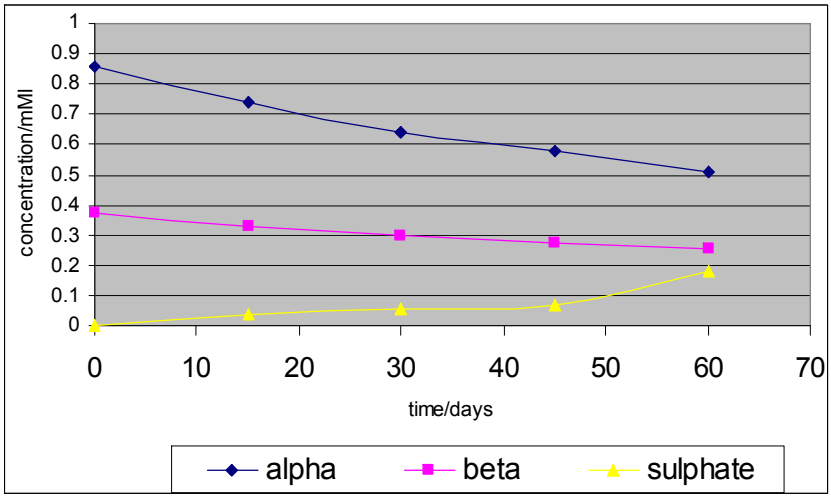


**Figure 5** Degradation of Endosulfan (α, β) and generation of sulphate upon incubation of the chemical (100 mg/l ) in sterilized soil ( control) .

## 4. Discussion

Mutant strains of bacteria from the soil of Ras Elfeel pesticide store were isolated through consecutive exposure to elevated concentration of endosulfan. Following the criteria listed in Brough (1999) the most tolerant Bacteria was identified as *Bacillus sp.*

The comparative degradation of endosulfan by mutant strains and their wild types was studied in soil. Results indicated that wild types strains (present in more number) caused faster decrease in half lives compared to mutant strains (found in lower number). Although the most mutant isolates appear relatively less efficient, (compared to wild types) but relating the counts with the capability in reducing half lives it appeared that they can be of a great potential if they had a chance to propagate in massive numbers. This explanation could be supported by the work of Tariq et al. (2000) who indicated that increasing the number of microorganism caused better activity and more degradation

The Sudanese isolates of microorganism could be of great potential in reducing the level of endosulfan in highly polluted storage soils. The use of microorganism for bioremediation requires better and more understanding of all the physiological and biochemical aspects involved in chemical transformations. This work is attempt to put a corner stone for some aspects needed for bioremediation of polluted sites. However further studies are needed prior to start any bioremediation process in such sites.

## Acknowledgement

Thanks are extended to those who contributed in one way or another to this work. They include Prof. Osman Ibrahim Gameel, Prof. Gafar Zorgani, Dr. Awad Galal, Dr. Imad Ali, Ismael Siddeg, Tarig Elsir and Triza . My strong appreciation is extended to Lift Engineering Company for financing this work

## 5. References

Alexander, M. (1981). Biodegradation of chemical of Environmental Concern, *Science* 211: 132-138.

Brough M.C. (1999). Medical Laboratory Manual for Tropical Countries. Jordan Hill, Oxford. Butrous (1999). Evaluation and assessment of obsolete and banned pesticides in five agricultural schemes. Report pp 83-89, Sudan.

Casida, J. E. (1993). Insecticide action at the GABA-gated chloride channel: recognition progress, and prospects. Arch. Insect Biochem. Physiol.pp 22**:**13

Chaudhur, K., Selvaraj, S., and Pal, A. (1999). Studies on the genotoxicology of endosulfan in bacterial system. *Muttat. Res*. pp 439:63-67

El Zorgani G,A, and Omer M.E.H (1974) metabolism of Endosulran Isomers by *Aspergillus niger*-bull. Environ, *Contam, Toxical.*

Goebel, H., S. Gorbach, W. Knauf, R. H. Rimpau, and H. Huttenbach**.** (1982). Properties effects, residues and analytics of the insecticide endosulfan. Residue Rev.83 pp 40-41.

Guerin, T. F. and Kennedy (1999) The Anaerobic Degradation of Endosulfan by indigenous microorganisms from Low oxygen soils and Sediments. *Envirn Pollution.* 63: pp 689-697

Macrae, I.C. (1990).The microbial Degradation of pesticides and related compounds. *Residue Reviews* 88,pp 1-87.

Mansingh, A., and A. Wilson (1995). Insecticide contamination of Jamaican environment. Baseline studies on the status of insecticidal pollution of Kingston Harbour. *Mar. pollut. Bull.* 30: pp 640-645

Marten, R. (1976). Degradation of (8. 9-14) Endosulfan by soil microorganisms. *Applied Environmental microbiology* 31,pp 853-859.

Miles, J.R.W., and P. Moy (1997). Degradation of endosulfan and its metabolites by a mixed culture of soil microorganisms. *Bull. Environ. Contam. Toxicol.* 23: pp 13-19.

Pual, V., and E. Balasubramanaim. (1997). Effectof single and repeated administration of endosulfan on behavior and its interaction with centrally acting drugs in experimental animals: *Amini review. Environ. Toxicol. Pharmcol.* 3: pp 151-157

Shivaramaiah H. M. and Kennedy I. R. (2006). Biodegradation of Endosulfan by soil bacteria. J. *Enviorn. Sci. Health* B. 41: pp 895-905

Singh, S. K., and R. S. Pandey**.** (1990). Effect of subchronic endosulfan on plasma gonadotropins, testosterone, testicular testosterone and enzymes of androgen biosynthesis. Indian J. Exp. Biol. 28**:**pp 953-956

Sinha, N., R. Naarayan and D. K. Saxena. (1997). Effect of endosulfan on testis of growing rats. *Bull. Environ. Contam. Toxicol*. 58: pp 79-86

Sunderam, R. I. M., D. M. H. Cheng, and G. B. Thompson. (1992). Toxicity of endosulfan to native and introduced fish in Australia. *Environ. Toxicol. Chem.* 11: pp 1469-1476

Tariq S,B. Benedict C., Okeke, M.A. and William, T. F (2000). Van Woerden, H. F**.** (1963). Organic sulfites. Chem. Rev. 63: pp 557-571

Enrichmend and Isolation of Endosulfan-Degrading Microorganisms. *Journal of Environmental Quality* 32: pp 47-54.

Turner, K. O., M. Syvanen, and S. Meizel**.** (1997). The human acrosome reaction is highly sensitive to inhibition by cyclodiene insecticides. J. Androl. 18**:** pp 571-575