IDENTIFICTION OF NITROFEN HERBICIDE METBOLITES UNDER DIFFERENT CONDITIONS

BY

Torki, M.A.; Khlil, A.R.A.; Nadia, Y. Attia and Megalla*, H.N.

*** Dept. of Soil and Biohcem., Fac. of Agric., Moshtohor, Zagazig Univ.**

ABSTRACT

The biochemical transformation of the herbicide i.e. nitrofen (from the diphenyl ethers group) was studied in soil suspensions and in pure bacterial cultures i.e. three organisms were isolated from soil suspension, Red, white and yellow. The organisms were capable of using nitrofen during incubation as a sole source of carbon. The red organisms was the most effective one. The addition of acetate, as an extra source of carbon, did not affect the adaptation of the three organisms towards nitrofen. Five intermediate fractions of nitrofen were detected from the culture extracts in HPLC chromatogram. Nitrofen was degraded in soil to more polar intermediate compounds and can be used successfuly as herbicide.

INTRODUCTION

Nitrofen has been used for many years as a pre-emergance herbicide toxic to a number of broad-leaved and grassy weeds. Its major application was as pre-emergance herbicide in cereals at 2 kg a.i. per hectar. The acute toxicity of the formulation to rats is 5.050 mg./kg., (Martin and Worthing, 1977).

Nitrated and or/halogenated diphenyl ether derivatives, have in general high biological activity as herbicides, at the same time being less toxic to mammals than the original dinitrophenol. Recently, it found a wide range of application in the field of agriculture.

Crosby and Nakagawa (1971), showed that photodecomposition of aqueous solutions of nitrofen in sunlight was characterized by rapid cleavage of the ether linkage to form P-nitrophenol and 2, 4-dichlorophenol.

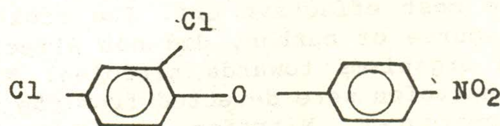
Kuwatsuka et al. (1976), indicated that diphenyl ether herbicides were rapidly degraded to form their amino derivatives in soils under reductive conditions but not under aerobic or oxidtive conditions.

Tawfik (1980), confirmed the cleavage of the ether bond of nitrofen by liberation of nitrite ions during the metabolism of this compound (dichloro phenyl-4-nitro phenyl ether) and chlorine was eliminated as inorganic chloride ions in the media.

MATERIAL AND METHODS

I- Sampling:

a- **Nitrofen:** Known commercially as "Tok", is: 2, 4-dichlorophenyl-4-nitrophenyl ether.



The pure compound forms yellow crystalls with M.P. 70-71°C. Nitrofen was recrystallized several times from the commercial product examined by HPLC. Nitrofen yields one peak with retention time (Rt), 14.2 min.

b- **Nitrofen metabolites:** Five different nitrofen metabolites, reference grade, were obtained from Aldrich Chemicals Company Inc., USA; i.e. 2, 4-dichlorophenol, p-chlorophenol, p-nitrophenol, p-aminophenol and quinol.

II- Incubation techniques: Basal medium was used according to Habib (1984): $(NH_4)_2SO_4$ 0.1%, K_2HPO_4 0.05%, $MgSO_4$-$7H_2O$ 0.05%, $Cacl_2.6H_2O$ 0.01%, NaCl 0.01% and $FeSO_4.7H_2O$ 0.005%. Nitrofen solely and nitrofen with organic substrate were added to all treatments with concentrations 0.02% (w/v), and the pH was adjusted to 7.5.

III- Soil leachate cultures: Fresh soil (clay loam soil from Agric. Res. Center, at Giza) (10 gm) and 100 ml. of phosphate buffer (0.05M) pH 7 were shaken in 500 ml. Conical flask on a rotatory shaker for one hour. After the soil settelement, its leachate was used as a source of microorganism. Each flask (1L).

Containing substrateinorganic medium, 300 ml (with or without the addition of other carbon source) was incubated at room temperature with 10 ml. of soil leachate. The addition carbon source used in the metabolism studies was soln. of Na-acetate at 1% (w/v). Samples were taken internals 3 to 7 days for the estimation of herbicide and microbial growth.

IV- The isolation of micro-organism adapted to the herbicides was accomplished as reported by Habib (1984).

V- Estimation of herbicide was carried out by mixing one ml. of the culture with four ml of methanol followed by centrifugation at 3000 r.p.m. to remove bacterial cells. The supernatants were poured in a selica cuvettes and scanning was performed against supernatants of control cultures without herbicide. The measurements were done in Lambda 3, U.V. visible double beam recording spectrophotometer (Perkin-Elmer Ltd.) at wavelength from 190-700 nm.

VI- **Extraction technique:** The natural culture was acidified (pH3) and extracted with ether, which was laterly dried under vacuum.

VII- High **performance liquid chromatography (HPLC):** HPLC analysis was carried out by Perkin-Elmer series two delivery system equiped with two pumps and attached to L-15 Perkin-Elmer U.V. fixed wavelength (254 nm) detector.

RESULTS AND DISCUSSION

1- Degradation of nitrofen by soil micro-organisms:

The data obtained is presented under three separate headings to facilitate the presentation of such tremendous data:

1- The degradation of nitrofen in soil culture.
2- Degradation of nitrofen by isolated bacteria in pure culture.
3- Studies on nitrofen metabolites:
   a- Chemical identification of nitrofen metabolites.
   b- HPLC analysis of culture extracts of different organisms at different periods.

The degradation of nitrofen in soil culture are shown in two figs. (1 and 2). Fig. (1) revealed that the disappearance of nitrofen was evidenced after three days of incubation, indicating that the soil micro-organisms did not

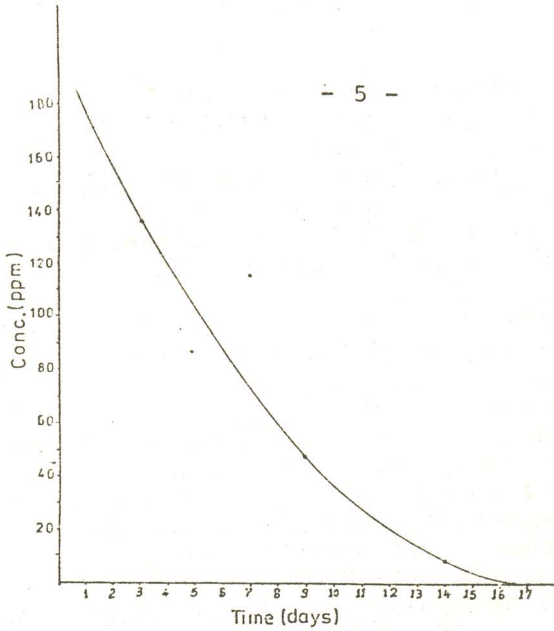
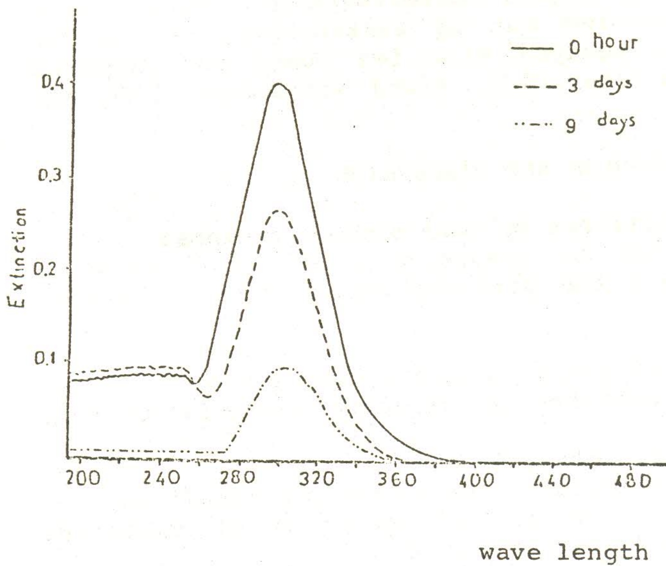


Fig. (1): Disappearance curve of nitrofen by soil microorganisms.



wave length (n.m.)

Fig.(2): Changes in the U.V. and visible spectra of nitrofen during degradation.

require any time for adaptation, if a lag phase existed, it was less than three days. The rate of degradation of nitrofen increased with the time of incubation, thus after nine days, over 75% of the herbicide was already consumed by soil microflora. However, after two weeks, the left over herbicide was less than 5% of the initial added dose. Such phenomenon confirms the well-known property of fast decomposition of this compound in the soil.

The resulted U.V. and visible spectrum of nitrofen during the degradation period (Fig. 2) showed a decrease in the absorption peak of nitrofen at 302 nm. After nine days of continuous incubation, a shoulder appearded at 315-320 nm, indicating the appearance of nitrofen intermediates.

2- Degradation of nitrofen by isolated bacteria in pure culture:

Nitrofen was incubated in soil shake culture, and after the consumption of the herbicide, an additional dose of nitrofen was added. When more than 50% of the new dose had disappeared, the bacteria existing in the culture were isolated in pure cultures.

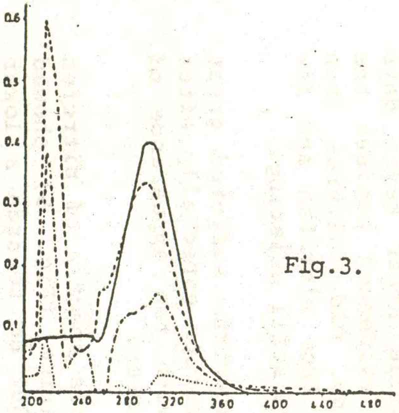
Three micro-organisms were isolated and designated as red (R), Yellow (Y) and white (w) according to the color of these organisms on the solid culture. From the floures-cence observed during its growth, it was pressumed that they were pseudomonads. The results obtained from each micro-organism were separately recorded.

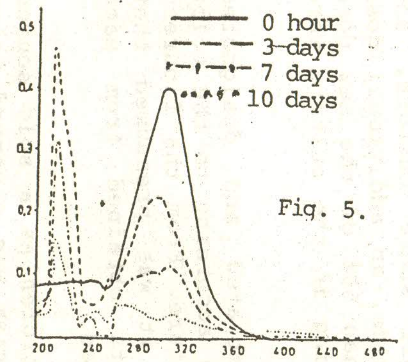
The resulted metabolites of nitrofen as a sole source of carbon by the action of red-organism was observed using U.V. as shown in Fig. (3). After three days of incubation, another peak appeared at 260 nm, while after seven days several peaks of intermediates were evidenced besides the original peak of nitrofen at 302 nm. Old cultures (ten days) showed a spectrum almost free from nitrofen and few peaks of some nitrofen metabolites were still existing.

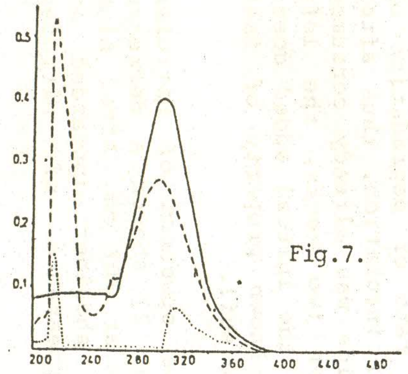
Fig. (4) showed that nitrofen spectrum suffered great changes during the metabolism with acetate especially after ten days of continous incubation, with the appearance of many unknown peaks of nitrofen metabolites.

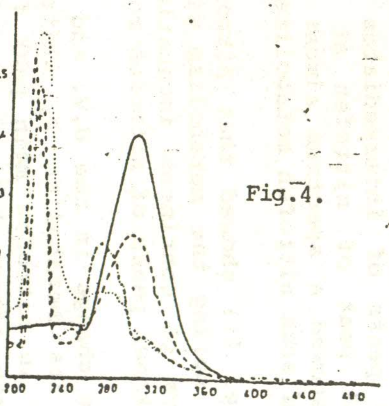
The change in the U.V. and visible spectra of nitrofen during its metabolism by white-organism (Fig. 5) showed a shift in nitrofen main peak from 302 nm towards a lower wavelength after three days. After seven and ten days of

Extinction

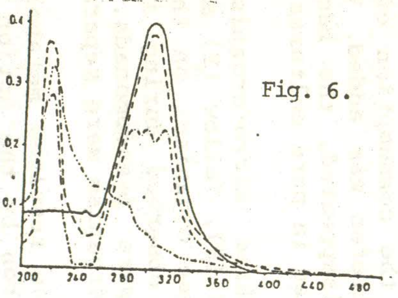






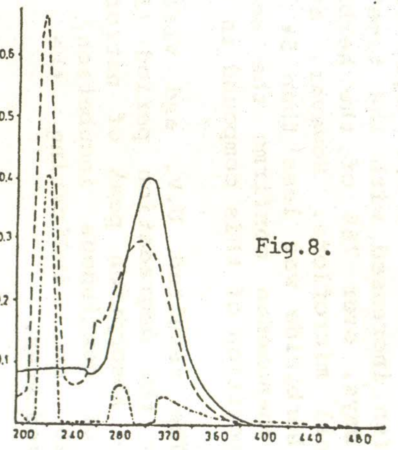


Changes in the U.V.and visible spectra of nitrofen Fig.(3),and nitrofen + Na-acetate,Fig.(4) with (R) microorganism.



WAVE - LENGTH (nm).

Changes in the U.V. and visible spectra of nitrofen Fig.(5),and nitrofen + Na-acetate,Fig.(6) with (W) microorganism.



Changes in the U.V. and visible spectra of nitrofen, Fig.(7); and nitrofen + Na-acetate Fig.(8) with (Y) microorganism.

incubation different spectra were detected with several peaks at lower and higher wavelength, indicating the accumulation of nitrofen metabolites in the cultures.

There was little change from the metabolism spectrum (Fig. 6). After three days incubation confirming that the micro-organism was mainly working on acetate during these period. However, after seven days there was a sharp decrease in the absortpion peak at 302 nm. with the appearance of other peaks at a lower wavelength corresponding to the accumulation of nitrofen intermediates.

The metabolism of nitrofen with the yellow-organism (Fig. 7) showed that the accumulation of nitrofen intermediates with this organism was much less than that observed in the previous two organisms. Thus after three days of incubation, only two peaks were observed at a lower wavelength.

The spectrum of nitrofen during the metabolism (Fig. 8) showed small accumulation of intermediates after three days indicating that the micro-organism was acting mainly on acetate at this stage of incubation.

From the previous results it may be concluded that the soil microflora can metabolize nitrofen without a lag phase or with an unnoticeable lag phase. This may explain, at least partly, the fact that nitrofen decomposes rapidly when incorporated in the soil, Tawfik (1966).

Addition of an organic compound beside nitrofen revealed that such addition did not improve the consumption of nitrofen by three isolated organisms. The delay in the metabolism of the herbicide through the addition of acetate varied from 3-7 days. Also it was clear that three different organisms are capable of degrading nitrofen since the structure of this herbicide is not difficult for the soil microflora to deal with. The efficiency in degrading this herbicide varied from one organism to another. The red-organism being the most effective one followed by white and yellow-organism.

3- Studies on nitrofen metabolites:

a- Chemical identification of nitrofen metabolites:

HPLC analysis of nitrofen and standard metabolites i.e. p-chlorophenol, p-aminophenol, p-nitrophenol, 2, 4-dichlorophenol and quinol were injected in a similar way to that used for the culture extracts.

Table (1) showed that every compound is identical in its retention time either singly or in mixture.

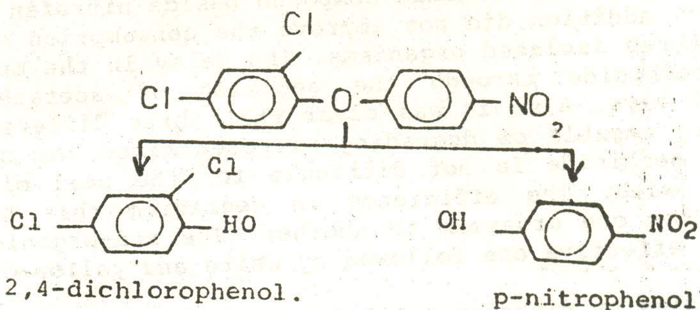
Table (1): Retention time (R_t) of nitrofen and its standard metabolites.

Compound	Retention time (Rt)
Nitrofen	14.2
p-aminophenol	3.6
quinol	6.2
p-nitrophenol	8.3
p-chlorophenol	10.4
2, 4-dichlorophenol	12.2

b- HPLC analysis of culture extracts of different organisms at different periods:

Cultures of red, white and yellow-organisms were grown either with nitrofen as the sole source of carbon, or in the presence of acetate were extracted after 15 and 30 days of incubation. The acid ether extracts of these cultures were analyzed using HPLC to examine the accumulation of the intermediates during the metabolism of this herbicide.

The data illustrated in Figs. (9, 10 and 11) indicated that the resulted metabolites of nitrofen revealed the existence of five intermediates i.e. 2, 4-dichlorophenol, p-chloro-phenol, p-nitrophenol, p-aminophenol and quinol. Hence, it could be stated that the metabolism of nitrofen in Egyptian soil proceeded through the initial hydrolysis of the ether linkage to give a major intermediates i.e., 2, 4-dichlorophenol and p-nitrophenol as follows:



p-nitrophenol is oxidized directly or via the amino derivative to quinol, which is further oxidized through ring fission as follows:

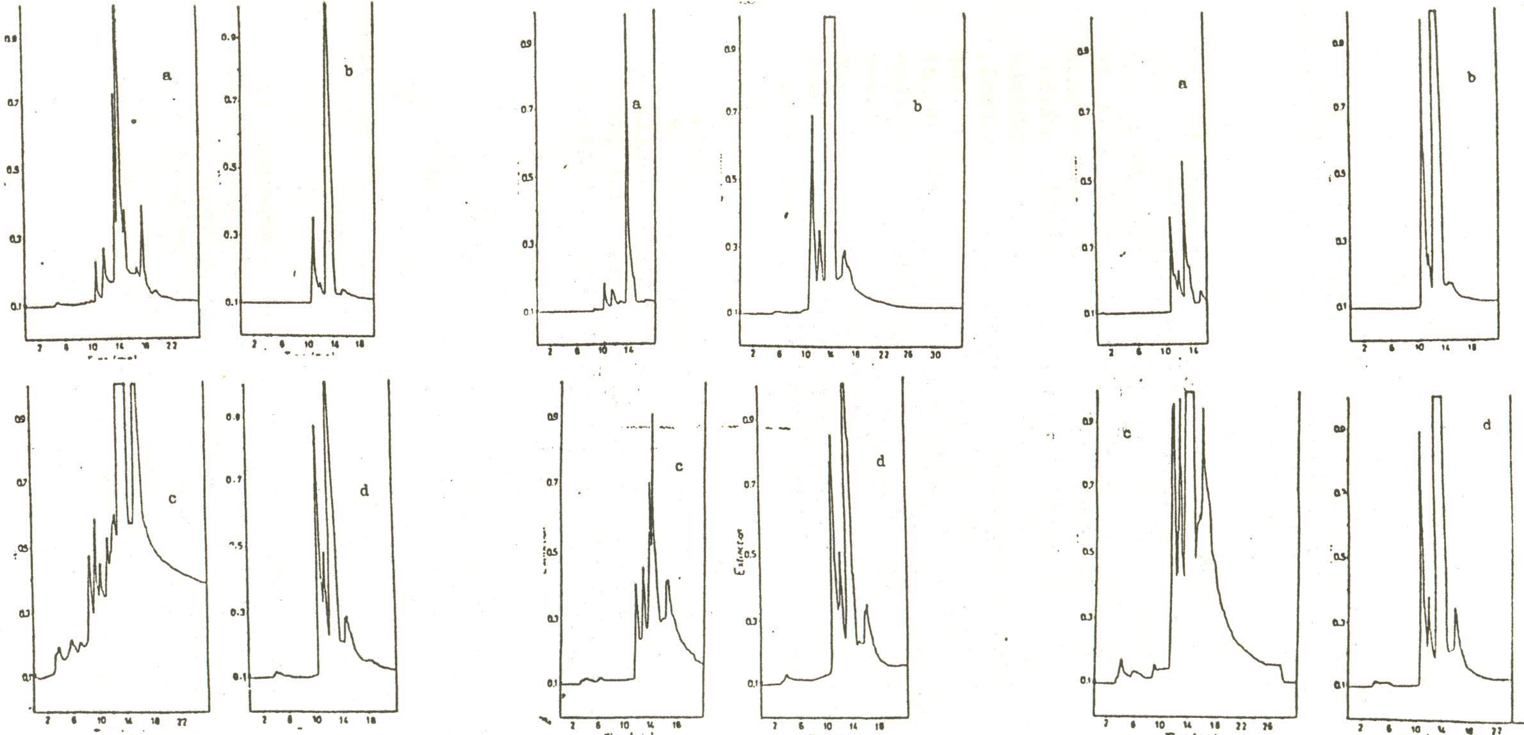
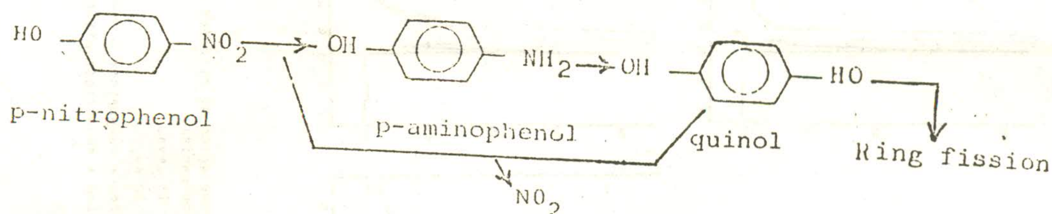


Fig. (9) : HPLC chromatogram of nitrofen degradation with (K) microorganism.
a. After 15 days.
b. After 30 days.
c. Nitrofen + Na-acetate after 15 days.
d. Nitrofen + Na-acetate after 30 days.

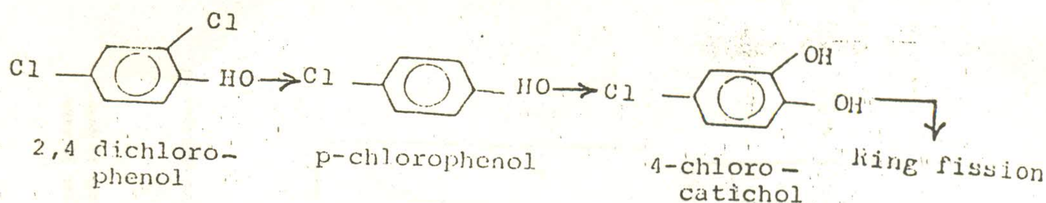
Fig. 10 : HPLC chromatogram of nitrofen degradation with (W) microorganism.
a. After 15 days.
b. After 30 days.
c. Nitrofen + Na-acetate after 15 days.
d. Nitrofen + Na-acetate after 30 days.

Fig. 11 : HPLC chromatogram of nitrofen degradation with (Y) microorganism.
a. After 15 days.
b. After 30 days.
c. Nitrofen + Na-acetate after 15 days.
d. Nitrofen + Na-acetate after 30 days.



Both p-aminophenol and quinol were identified in the different cultures. The resulted nitrite ions were also detected in the cultures of all organisms, therefore, it can be concluded that the present organisms were able to use the two metabolic pathways known for the metabolism of the aromatic nitro compounds (Tawfik and Hamdi, 1975). The first possibility is the reduction of p-nitrophenol to p-aminophenol before deamination to give rise to quinol, the other alternative is the direct elimination of the nitro group to give rise to quinol with the release of nitrite. It is clear that quinol will result from the metabolism of p-nitrophenol and then oxidized rapidly through ring fission to give simple aliphatic compound which are used in the terminal oxidation processes for the maintenance and the growth of the micro-organisms (Larway and Evans, 1965).

P-chlorophenol was identified in most cultures, it is probably produced by aromatic dehalogenation of 2, 4-dichlorophenol. Such mechanism was previously mentioned by several workers (Williams, 1950; Evans, 1963 and 1969). P-Chlorophenol is known to proceed through 4-chlorocatichol before ring fission (Evans, 1969) as follows:



The obtained data indicates that this compound was degraded to more polar chemical fragments by soil microflora which are capable of using this herbicide as a sole source of carbon for growth. Such conclusion lead to believe that nitrofen is quite safe from the point of view for long-term use in agriculture, since the accumulation of its degraded products in the soil is not harmful.

REFERENCES

Crosby, D.G. and Nakagawa, M. (1971): Photodecomposition of 2, 4-dichloro-4-nitrodiphenyl ether. Abs. Paps, 162nd Nut. Meet. Amer. Chem. Soci., Washington. (C.F. Weed Abs., 21(2), 1121, 1972).

Evans, W.C. (1963): The microbiological degradation of aromatic compound. J. Gen. Microbiol, 32: 177.

Evans, W.C. (1969): Microbial transformation of aromatic compounds. Fermentation Advances, p. 469. Academic Press, New York.

Habib, M.W. (1984): Biochemical transformation of certain herbicide in Egyptian soil. Ph.D. Thesis, Fac. of Agric., Cairo Univ.

Kuwatsuka, S.; Niki, Y.; Oyamado, M.+ Shimotori, H. and Ohyama, H. (1976): Fate of diphenylether herbicides in soil and plants. In Proc. 5th Asian-Pacific Weed Sci. Soci. Conf., Tokyo, Japan, 223-C.F. Weed Abs., 26(7): 2167, 1977.

Larway, P. and Evans, W.C. (1965): Metabolism of quinol and resorcinol by soil Pseudomonads. Biochem. J., 95, 52 pp.

Martin, H. and Worthing, C.R. (1977): Pesticide Manual 5th ed. British Crop Protection COuncil, Worcest, England.

Tawfik, M.S. (1966): The metabolism of certain aromatic nitro herbicides by soil micro-organisms. Ph.D. Thesis, University of Wales, Bangor.

Tawfik, M.S. (1980): The microbial metabolism and cometabolism of nitrofen. Absts. Paps. 1st. Conf. of Plant Prot. Res. Inst., p. 92.

Tawfik, M.S. and Hamdi, Y.A. (1975): Metabolism of fluorodifen by soil micro-organisms. Soil Boil. Biochem., 7(2): 79.

Williams, R.I. (1950): Detoxication metabolism of benzene. a- detection of benzene b- elimination of unchanged benzene by rabbits. Sym. Biochem. Soc., 5.

التعرف على نواتج تكسير النتروفين تحت ظروف مختلفة

أ.د/ منير عبدالعظيم تركى — د. عبدالخالق ربيع عبدالمجيد

د. نادية يحيى احمد عطية — هدى نجيب مجلع

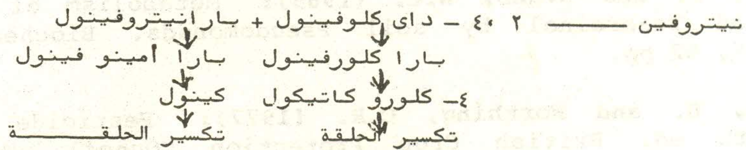
أمكن دراسة التغييرات الحيوية لاحد موبيدات الحشائش وهو مركب النيتروفين (من مجموعه الداى فينيل أثير) تحت ظروف بيئية مختلفة فى معلق التربه وفى بيئة بكتيرية نقيه معزولة من التربة.

أظهرت النتائج بدء هدم المبيد فى معلق التربه بعد ثلاثه ايام وهذا يشير الى ان ميكروبات التربه لها القدرة على تمثيل هذا المركب بعد فترة تحضير قصيرة .

أمكن عزل ثلاثه انواع من الميكروبات من معلق تربه بها نيتروفين بعد فترة تحضين صنفت حسب لونها الى ابيض - أحمر واصفر . واظهرت النتائج ان الميكروب الاحمر هو أكثرها فاعلية فى تمثيل النيتروفين كمصدر وحيد للكربون .

باضافة ملح الخلات الى بيئه بكتيريه نقيه معزوله من التربة وجد ان تمثيل النيتروفين قد تاخر من ٤ - ٧ أيام فى الانواع الثلاثة من الميكروبات الى ان تأقلم هذة الميكروبات على استعمال النيتروفين لم يتأثر .

استعمل جهاز الفصل الكروماتوجرافى للسوائل لدراسة السلوك الكيميائى لتكسير النيتروفين بواسطة الميكروبات الثلاثه وقد أمكن التعرف على خمسة مركبات وسطية . من النتائج المتحصل عليها يقترح أن يكون التمثيل الكيمائى للنيتروفين بواسطة الميكروبات فى التربه المصرية بالترتيب التالى :-



يتبين من النتائج المتحصل عليها ان مركب النيتروفين يتم تكسيره فى التربة المصرية بواسطة الكائنات الدقيقة الى مركبات اكثر قطبية مما يشجع استخدامه فى الزراعة دون ضرر على الصحة العامة.