POTENTIAL OF BIOAUGMENTATION FOR REMEDIATION OF POLLUTED ENVIRONMENTS

***Getenga*, Z.M., *Ngige*, A., *Kimosop*, K., *Mutua*, G., *Orata*, F., *Kowino*, I., *Were*, H. and *Onunga*, D.**

Chuka University, P. O. Box 109-60400, Chuka, Email: zgetenga@yahoo.com, Tel.: 0729171505
Multi-Media University of Kenya, P. O. Box 30305, Nairobi
Masinde Muliro University of Science and technology, P.O. Box 190-50100, Kakamega

library@chuka.ac.ke; www.chuka.ac.ke

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ABSTRACT
After repeated applications and long use of persistent pesticides in soils, a phenomenon known as enhanced (accelerated) degradation of some pesticides has been observed in soils. This has led to isolation of key microbes known to degrade these persistent pesticides in soils. The isolated microbes which are characterized and identified have been used to enhance the degradation of pesticides in contaminated soils and hence the term, bioaugmentation. In this paper we discuss various pesticide compounds which for so long were known to be recalcitrant, but later could be subject to accelerated degradation. Key degraders were isolated and characterized and are potential candidates for bioaugmentation for remediation of contaminated sites. We report the successes registered in studies of atrazine, terbuthylazine, hexazinone, diuron, carbofuran and chlorpyrifos and metribuzin.

Keywords: Adaptation; Bioaugmentation; Remediation; Pesticides; Pollution

INTRODUCTION
Xenobiotic compounds are chemicals which are foreign to the biosphere. They are chemically synthesized compounds that do not occur in nature and thus are foreign to the biosphere. They have unnatural structural features to which microorganisms have not been exposed to during evolution. They may resist biodegradation, or they may undergo incomplete biodegradation or just biotransformation[1]. Degradation of chemicals can involve biotic and abiotic processes, where microbially facilitated biodegradation is especially interesting, as it is a major process in the complete mineralization of aromatic compounds to

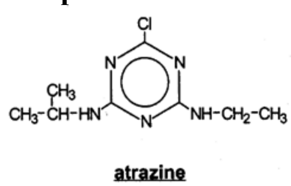
harmless inorganic products[2]. However, the halogen, methylthioether, and N-alkyl substituents on the s-triazine ring of the herbicides impede facile microbial metabolism[3]. This has also been observed in some of the halogenated phenylurea herbicides such as methabenzthiazuron, diuron, metobromuron and monuron[4]. As a result some of the compounds most frequently used such as atrazine and diuron have been detected in surface and ground waters[5,6,7].

However, enhanced degradation has been observed for s-triazine and phenylurea compounds in soils where they have been applied repeatedly and used for a long time with subsequent isolation of the bacterial strains which metabolize the pesticides to get C, N and energy for growth[8,9]. Enhanced degradation is a phenomenon whereby, a soil-applied pesticide is rapidly biodegraded by a population of microorganisms that has developed the ability to use the compound as a C, energy and or nutrient because of the previous exposure to it or its analogue[10].

In Kenya both s-triazine and phenylurea herbicides have been used in various sugarcane fields to control weeds for more than 20 years. One of the s-triazine herbicides that have been used for a long time in the sugarcane fields is atrazine while diuron is a model compound for the phenylurea herbicides. Both compounds have been investigated for enhanced biodegradation in the soils that have had long exposure to both chemicals. After detection of enhanced biodegradation of the chemicals in the soils, the microbial community which is responsible is enriched in liquid media with subsequent isolation and characterization of bacterial strains responsible for the biodegradation[11,12,13].

In order to minimize dispersion of the same chemicals outside the agricultural environments, laboratory studies have been undertaken to increase the degradation (biostimulation) of the same by the indigenous soil bacteria by applying appropriate limiting nutrient amendments to the soils without adapted microflora[11,14,15,16]. We report in this paper various methods that have been used in the course of our studies in determining the utilization of the selected chemicals (pesticides) by the adapted microbes as a source of C and N for growth and energy. We also present some of the compounds we have worked with and the successes registered in isolating key degraders of the respective pesticides and the extent the locally generated organic materials are able to enhance the degradation of the respective chemicals in soil.

Compounds which have been investigated and their chemical structures



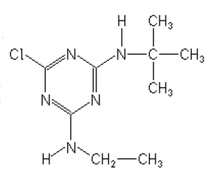
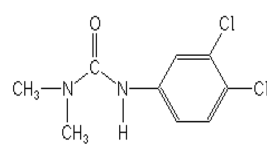



Figure i: Atrazine Figure ii: Terbuthylazine Figure iii: Diuron

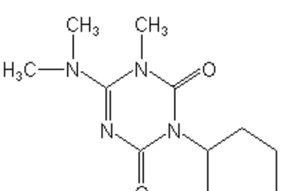
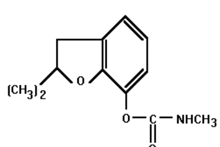



Figure iv: Hexazinone Figure v: Carbofuran

METHODOLOGY

Screening the soils for enhanced biodegradation of the chemicals

Soil treatment

Soil samples were collected from sugarcane fields where the chemicals are intensively used for pest's control (weeds or insects). The soils were analyzed for their physico-chemical characteristics (Table 1). Aliquots were taken, homogenized and sieved through a 2 mm sieve. Water retention curves for the various soils were determined to determine the optimum water content at the soil water tension of -15kPa at the compacted soil density of 1.3 gcm^{-3}.

Chemical application to soil

Radio-isotopically ^{14}C-labelled compounds were used as it is fast to screen the soils for enhanced degradation. The mineralization of the radioablled chemicals released $^{14}CO_2$ which was trapped and radio-assayed by a liquid scintillation counter (LSC). Part of the chemicals which gets bound to the soil is obtained after combustion of the soil releasing also $^{14}CO_2$ which was also radio-assayed. The portion of the chemical which was not mineralized was extracted from the soil and the extract was analyzed for ^{14}C labeled chemical. Whenever ^{14}C-labelled compound was not available, a non-labeled one was used. The residual compound at different time intervals in the incubation systems was determined by appropriate chromatographic techniques (Gas chromatography, UV spectrophotometer or high pressure liquid chromatography). Other transformation products were also determined.

At the end of the experiment a mass balance for the chemical initially applied to the soil was determined. From the data generated, it was possible to determine the kinetics of biodegradation of the chemical and the extent of mineralization. It was then possible to determine that the soils harboured soil microbes which had adapted to the chemical and hence, could be cultured, isolated and characterized using the current molecular techniques. Experiments included soil samples which had not been exposed to the chemical as well. Controls in which sterile soils were used were included to determine the extent of the influence of chemical degradation.

Physico-chemical properties of the selected soils

Table 4.1a: Physico-chemical characteristics for soils from Chemelil, Nzoia and KESREF

Sample fields No.	pH	%N	%C	P mg/kg	S mg/kg
F_1	6.25	0.16	1.22	3.50	98.21
F_6	5.76	0.14	1.10	2.77	82.14
F_9	6.16	0.14	0.92	3.79	94.64
F_{10}	5.93	0.11	1.28	3.35	60.71
24D	6.07	0.28	1.96	2.48	47.32
D_8	5.43	0.17	1.66	2.19	320.54
F_{10C}	5.56	0.22	1.66	2.19	333.93
F_{26}	6.02	0.33	3.36	63.44	141.07
124	5.10	0.12	1.44	8.23	446.67
312	6.17	0.16	2.14	12.71	47.50
314	5.37	0.11	1.76	19.06	75.83
1120	5.01	0.15	1.68	3.90	236.67
7011	4.80	0.14	1.58	14.30	64.17
7013	4.60	0.15	1.50	2.89	19.17
8100	4.67	0.13	1.72	6.35	30.83
9090	5.08	0.05	0.80	8.23	78.33
Chemelil	6.08	0.19	2.07	80	-
Nzoia	4.9	0.16	3.06	12.3	-

Bio-stimulation of the soil microorganisms to degrade the chemicals

Microbes in soil require both macro- and micro- nutrients for optimal proliferation. Absence of any of the nutrients can retard growth. The affected nutrients can be supplemented by the addition of chemical fertilizers. However, the inorganic fertilizers are also sources of pollution in the environment. Alternative source of the nutrients are organic materials that are added to soil as soil organic amendments. In our studies organic materials from garbage, by-products (filter mud) of sugarcane processing and *Tithonia diversifolia* (TD), a plant grown extensively along hedges of the sugarcane fields, were used to bio-stimulate soil microorganisms to degrade the chemicals in soil. The organic materials were added to soil at different concentrations and their impact on the biodegradation of the chemicals was observed. The physico-chemical data for the organic materials used in our studies are provided (Table 2).

Table 2: The physico-chemical characterisitcs of organic amendments

Organic Amendment	pH	Organic Carbon (%)	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Fe (%)	Mn (%)
Filter mud	6.3	12.9	3.0	0.07	0.10	0.12	0.001	0.50	0.001
Tithonia diversifolia	-	24.84	2.94	0.09	0.08	0.11	0.004	0.30	0.003
Compost from garbage	-		1.14	0-72	2.57	1.28	0.36	0.13	0.212

Physicochemical characteristics of organic amendments

Liquid culture enrichment studies

Mineral salt medium (MSM) for the respective pesticides (diuron, atrazine, terbuthylazine and carbofuran) for liqud culture experiments were prepared. Appropriate amounts of the chemicals were added as the sole sources of either C or N. An aliquot of 5 g of soil was added as inoculants to the medium and incubated on orbital shakers at 100 rpm in the dark at 20^oC. At various time intervals, aliquots of 1 ml were taken to 24 ml of fresh growth medium to give a total volume of 25 ml in the biometer flasks; thereafter, liquid medium cultures were transferred weekly to fresh media. Detailed procedures for liquid culture enrichments for the individual pesticides are provideded[12,13,17]. Bacterial growth in the liquid culture was followed by measurement of; $^{14}CO_2$ when the pesticide was ^{14}C-labelled, concentration levels of residues of the pesticide in the culture, growth of microbes in the culture by monitoring turbidity at the optical density (OD_{600}).

The 10th enrichment culture was used for the isolation of bacteria strains; aliquots were taken and serial dilutions were spread on BSM agar plates. Detailed procedures are described[17]. Single colonies were transferred using sterile inoculation loop onto BSM agar plates to get pure cultures. Obtained strains were re-transferred to liquid cultures and mineralizing capacities for the respective pesticides were tested in mineralization experiments.

DNA extraction, PCR amplification and sequencing of 16S-rDNA coding genes

Total DNA samples were extracted from isolates for the various pesticides according to procedures described by Sharma et al[18]. The detailed procedures for each pesticide is described[12,13,17]. The obtained sequences were further analyzed phylogenetically using the software package ARB (http://www.arb-home.de)[19]. Sequences were added to an existing database of well aligned small-subunit rRNA gene sequences (SILVA_95)[20] by using the fast alignment tool implemented in the ARB software package.

RESULTS AND DISCUSSION

Biostimulation of the soil microorganisms by organic amendments

Atrazine degradation in soil amended with compost from municipal garbage

Soil amended with different concentrations of the compost made from municipal garbage strongly increased atrazine degradation in soil from Chemelil sugarcane fields incubated in the laboratory (Fig. 1).
The figure below shows clearly that the mineralization of atrazine to $^{14}CO_2$ increased from 31% in soil without compost to 55% in soil with the highest concentration of 5000 ppm of compost. The bound residue

of atrazine was highest (57%) in sterile soil while the lowest bound residue (25%) was in soil with 5000 ppm of compost. The addition of compost added more nutrients to soil thus favouring the proliferation of soil microorganisms which degraded atrazine. It is evident that the atrazine degradation was microbially driven as the sterile soil registered nil atrazine mineralization[11].

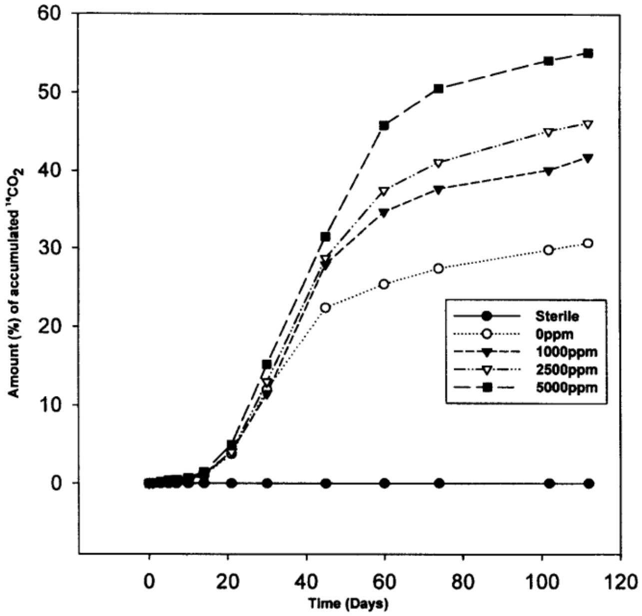


Figure 1. Mineralization of ^{14}C-Atrazine at different compost concentrations in soil.

Diuron degradation in soil amended by filter mud and T*ithonia diversifolia*

The results below show that the dissipation of diuron was significantly enhanced (p <0.05) from soils amended with the two organic amendments *Tithonia diversofolia* and filter mud compost as shown in figure 2. However, there was no significant difference between the soils amended with filter mud compost and *Tithonia diversifolia*.

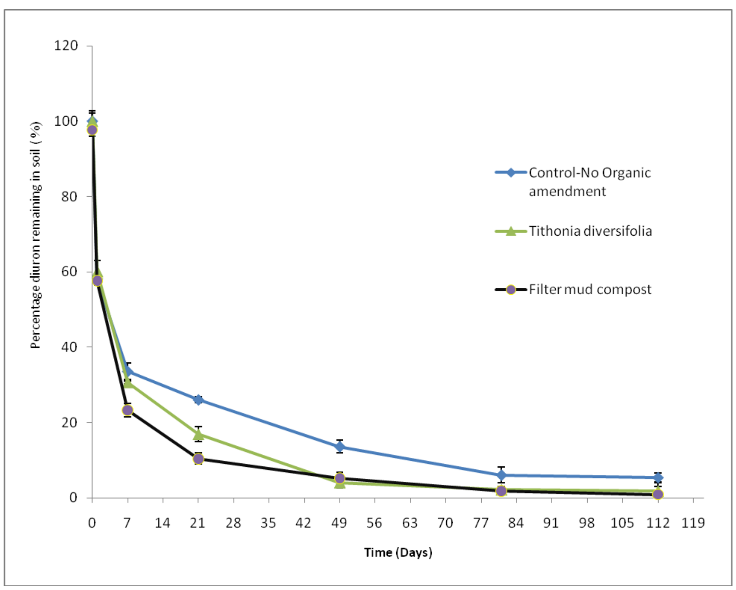


Figure 2: Dissipation behaviour of diuron in soils amended with organic materials

Enhanced degradation studies of pesticides in soils with long exposure to the pesticides

In laboratory degradation study, ^{14}C-uniformly ring labeled atrazine was rapidly degraded (mineralized) in soil from KESREF where atrazine had been used for over 20 years. Atrazine was mineralized by 90% after 100 days of incubation of soil in the laboratory. The soil which had not been exposed to atrazine could not mineralize atrazine, with only 0.16% of atrazine having been mineralized after 163 days of incubation in the laboratory. When compost from municipal garbage was added to the soil with enhanced atrazine degradation, there was a negative effect. Addition of compost at all levels (1000 to 10,000 mg/kg) caused a lag phase of 8 days in the atrazine mineralization. The detailed results are discussed[14].

Diuron degradation in soil with and without exposure to carbofuran

Figure 3 below shows the dissipation rates of diuron in two different soils, one with prior exposure to diuron and another without prior exposure.

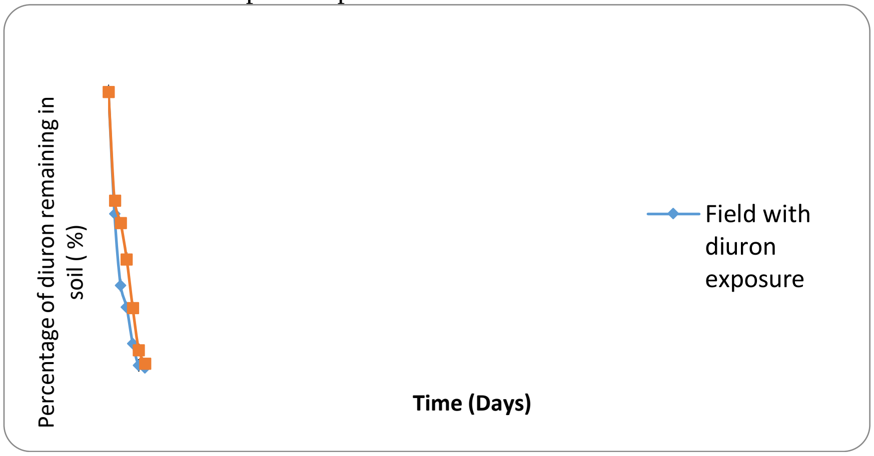


Figure 3: dissipation behavior of diuron in soils with and without prior exposure

The results showed that the dissipation of diuron with prior exposure to the pesticide was faster than the dissipation from soil with no previous history of diuron application. However, the difference was not significant using the t-test ($P>0.05$).

In the study of carbofuran dissipation from soils with and without prior exposure to carbofuran (figure 4), there was significant difference in the dissipation rates in the two soils ($p<0.05$) with dissipation half-lives of 19 days from soil with no prior exposure to the pesticide and 8 days in soils that had been exposed to carbofuran before.

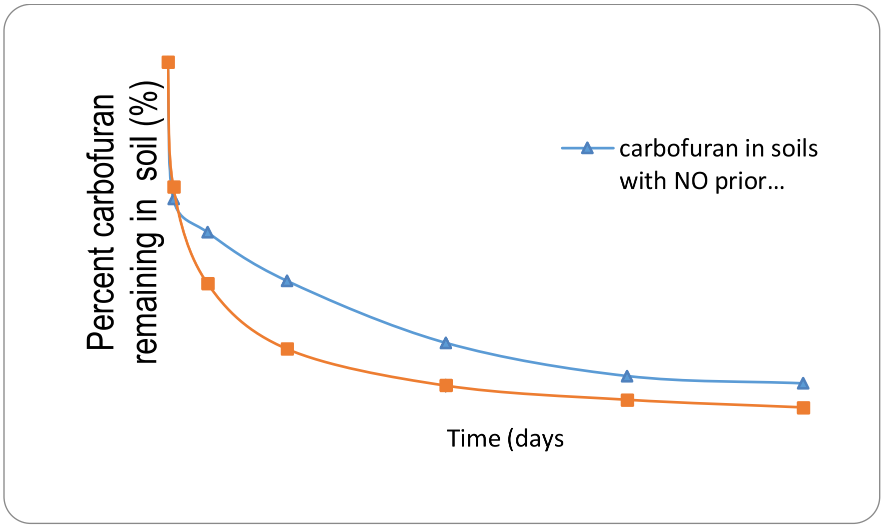


Figure 4: Dissipation behavior of carbofuran from soil in rice paddy fields

Isolation and characterization of pesticide-metabolizing bacterial strains from soil

Soils which showed enhanced degradation of the respective pesticides were used as inoculants with the pesticides as sole source of either C or N in liquid cultures. Through a series of liquid culture enrichments, communities of the bacterial strains adapted to the pesticides were enriched from which pure bacterial strains were isolated and finally characterized.

Arthrobacter sp. strain GZK-1 isolated from soil in KESREF sugarcane field

This is the soil which mineralized ^{14}C-ring labeled atrazine up to 90% after 100 days. The isolated *Arthrobacter* sp. strain GZK-1 was characterized by comparative sequence analysis of the 16S-rRNA coding genes as a member of the genus *Arthrobacter* strain. *Arthrobacter* sp. GZK-1 (accession number of the 16S-rRNA sequence FJ766438) shows the highest similarity of 99.2% to a sequence derived from an uncultured *Actinobacterium* (accession number AY622266) and exhibited 99.0% similarity to the next related cultured bacterium, *Arthrobacter* sp. SMCC G964 (accession number AF197029).

Several *Arthrobacter* sp. had been isolated from agricultural soils in France, Canada, USA, China, New Zealand, and India which were able to start the degradation process but could not totally degrade ^{14}C-ring labelled atrazine to $^{14}CO_2$ and NH_3. The species isolated in those countries carry the s-triazine-degrading genes atzA, atzB, atzC or atzABC which enable them to degrade atrazine to cyanuric acid and to yield carbon dioxide and ammonia. The *Arthrobacter* sp. GZK-1 isolated in this work was able to solely mineralize atrazine completely, but the enzymatic arsenal of this strain cannot be described, since up to now the degrading genes have not been identified[17]. Moreover, the isolated *Arthrobacter* sp. GZK-1 could also mineralize terbuthylazine. Previous studies[21,22,23] found terbuthylazine to be poorly mineralized even in soils which had been exposed to the herbicide.

In other related studies with soils in other fields within KESREF, two bacterial strains coded ISL 8 and ISL 15 isolated from two different fields were shown to have 94 and 96% 16S- rRNA gene sequence similarity to *Burkholderia cepacia*, respectively. Another bacterial sp., ISL 14 was closely related to *Enterobacter cloacae* with a 96% 16S-rRNA gene sequence similarity[12]. *Burkholderia* sp. was isolated from the same field (F_6) in KESREF where *Arthrobacter* sp. strain GZK-1 had previously been isolated and from another field (1120) in Nzoia sugarcane fields. *Enterobacter cloacae* sp. was isolated from field 8100 in the Nzoia sugarcane fields where atrazine had been discontinued 10 years before. However, Velpar 75DF (hexazinone) had been applied to the field in 2007 at the rate of 10 kg ha^{-1}. In a separate liquid culture enrichment experiment with hexazinone as a sole N-source, *Enterobacter cloacae* sp.was also isolated from soil in field 7013 where hexazinone was being used[12].

Diuron degrading strains isolated from soil in sugarcane fields

Different bacterial species, *Bacillus cereus, Vagococcus fluvialis*, *Burkholderia ambifaria* and *Bacillus spp1* were isolated from fields in the Nzoia sugarcane company where diuron is being used to control weeds. The combination of *V. fluvialis* and *B. ambifaria* showed enhanced degradation of diuron up to 30% from their individual degradation levels of 25% and 22% for *V. fluvialis* and *B. ambifaria*, respectively[13]. From the extent of degradation of diuron by the individual bacterial species, it is clear no one bacterial species could completely degrade diuron and therefore, complete degradation of diuron could be realized by a consortium of these bacterial strains, unlike with atrazine which was completely mineralized by the *Arthrobacter sp*. strain GZK-1.

Carbofuran degrading bacterial strains isolated from Bunyala rice paddy fields

The latest isolated bacterial strains are *Bacillus cereus* and *Bacillus thuringiensis* from rice paddy fields in Bunyala rice paddy fields within the Nzoia River drainage basin, Kenya. *Bacillus cereus* had been isolated also from Nzoia sugarcane fields, where diuron was being used. In addition to the bacterial strains, two fungi have tentatively been isolated and partially characterized and are currently under intense investigation for full characterization. The fungi are *Fusarium merismoides* and *Nectria sp.* In an earlier study by Omolo et al[24], partial 16S rDNA sequence analysis indicated that the carbofuran-degrading strains isolated from soils collected from horticultural farms in Naivasha, Gilgil (R.Valley) and Thika were closely related to members of the genus *Pseudomonas* and *Alcaligenes*.

CONCLUSION

Biostimulation by locally generated organic materials significantly enhanced the degradation of the xenobiotics introduced into the soils through agricultural activities. This strategy is likely to not only reduce pollution but will also improve soil fertility and crop yields as has been established independently.

We have also established that much of the agricultural soils exposed to various organic chemicals have adapted and developed the ability to degrade the chemicals and hence, helped reduce the contamination of both the aquatic and terrestrial environments. This promises a future strategy for environmental restoration through bio-augmentation. The rich biodiversity in our soils need to be mapped, characterized and documented for industrial use in line with vision 2030.

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