Full Length Research Paper

Biodegradation and dissipation of ^{14}C-carbofuran in clay soil from Loukkos perimeter, Northwestern Morocco

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Mineralization and dissipation of ^{14}C-ring labeled carbofuran (2, 3-dihydro-2, 2-dimethylbenzofuran-7-yl-methylcarbamate), one of the highly toxic N-methyl carbamate pesticides, in an untreated Vertisol Moroccan soil used for sugar beet cultivation was studied under laboratory conditions over a period of 63 days. The experiment was conducted in flooded and non-flooded conditions. Carbofuran mineralization was determined by monitoring the $^{14}CO_2$ production from soil amended with U-ring[14]C-carbofuran. Under both conditions, higher mineralization rates of ^{14}C-carbofuran were observed. The extent of mineralization being more in moist (19.2%) than in flooded conditions (12.4%). In both conditions, the soil extractable pesticide residues decreased with time, while the bound residues gradually increased. The extractable ^{14}C-activity disappearance rate was much higher in flooded than in non-flooded conditions. At the end of the experiment, 15.8% and 31% of applied dose were recovered as extractable residues under flooded and moist conditions respectively. Soil bound residues were formed to the extent of 33.3% of the applied dose in flooded soil, while 29.1% was formed in non-flooded soil. The amount of the organic volatiles was much higher in flooded condition (18.2%), as compared to 5.6% in non-flooded soil. The main degradation product formed was carbofuran phenol.

Key words: Carbofuran, mineralization, degradation, volatilization, ^{14}C, biodegradation.

INTRODUCTION

Development and productivity of sugar beet system are tightly linked to pesticide use in Morocco. However, increased sugar beet production is generally associated to environmental problems due to the use of a wide variety of pesticides in the culture; about 93 commercial products (Benicha, 2005; Ezzahiri et al., 2011).

In recent years, there is an increasing public concern over the presence of pesticide residues in food products because of the known toxicity of pesticides (Charlier and Plomteux, 1997), so many substances are already banned in many countries since 2008 (Seghier, 2008). Studying the fate of these agrochemicals in soil is therefore, of interest with the aim of promoting the health of consumers and the environment.

Microorganisms are considered to be one of the primary factors that determine the fate of these agrochemicals in soil (Alexander, 1999). They influence directly and indirectly the persistence of pesticides in soil (Farenhorst et al., 2000). Numerous studies have documented the ability of soil microorganisms to utilize pesticides as a carbon and energy source for their growth, either by the isolation of pure or mixed cultures through enrichment techniques, or by the demonstration of mineralization in soil (release of $^{14}CO_2$ from labelled pesticides) (Chaudhry and Ali, 1988; Feng et al., 1997; Fournier et al., 1997). Through mineralization, the

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persistence of pesticides is reduced and, as a result, reduces the environmental risk (Fournier et al., 1997).

The insecticide carbamates have been developed as an alternative to recalcitrant organochlorine pesticide; however, many of these carbamates are highly toxic and inhibit acetyl cholinesterase, an enzyme vital for the functioning of central nervous system (Fisher et al., 1999). Among them, carbofuran (2,3-dihydro-2,2-dimethyl-7-benzo-furanyl methylcarbamate), a versatile broad spectrum systemic insecticide, nematicide and acaricide, registered for use worldwide to control many of insect pests (that is, the green leafhoppers, rootworms, brown plant hoppers, stemborers, whorl maggots and Diptera Cecidomyiidae) on a variety of crops such as sugar beet, sugar cane, rice, corn, peanuts, cotton, strawberries, potatoes and wheat (Venkateswarlu et al., 1977; Lhaloui et al., 1993; Fournier et al., 1997; Jaramillo et al., 2001). Consequently, carbofuran has attracted much attention as a potential alternative to the persistent and toxic chlorinated hydrocarbon insecticides. Due to its widespread use and high water solubility, carbofuran has been detected in groundwater, surface and rain water, in soils, air, food and wildlife (Bouchway et al., 1992; Arraez-Roman et al., 2004; Caldas et al., 2010).

The use of carbofuran raises environmental concerns because of its high mammalian toxicity having an oral LD_{50} = 2 mg/Kg for rats (Chaudhry and Ali, 1988) and LC_{50} = 0.2 to 0.8 mg/Kg for fish (Jinhe et al., 1989); and to its potential endocrine disrupting activity (Lau et al., 2007). Its use was banned by USEPA since 1994 and in Europe since 2008. In Morocco, as in many other parts of the world, however, carbofuran is readily available and still being used by farmers on horticulture crops (Ezzahiri et al., 2011), wheat and barley (Lhaloui et al., 1993) and mainly on sugar beet cultivation as nematicide at a rate of 20 kg/ha, as granular formulation (Furadan 5G) (Ezzahiri et al., 2011).

Carbofuran is a relatively persistent insecticide with a half-life in soil ranging from 3 weeks to more than 50 weeks depending of the pH of the soil (Getzin, 1973). A field study by Caro et al. (1973) showed that half-life of carbofuran in soil ranged from about 6 to 17 weeks. Persistence of carbofuran in soil has also been reported by Szeto and Price (1991) who found 78 µg/g of carbofuran near a year after application. The major degradation products of carbofuran in soil include carbofuran phenol, 3-hydroxycarbofuran, 3-ketocarbofuran and 3-ketocarbofuranphenol.

A number of bacteria strains capable of using carbofuran as source of C and N for their growth, have been isolated from soil and characterized (Slaoui et al., 2007; Krishna and Ligy, 2008, Chanika et al., 2011). These bacteria are capable of mineralizing both carbonyl group and the aromatic ring of carbofuran and residues (Feng et al. 1997; Trabue et al., 2001) leading to CO_2 and H_2O.

The vast and intensive agricultural use of pesticides in some Moroccan zones such as Loukkos perimeter (North-western) has important implications for the contamination of the environment. With increasing population, efforts are being made to intensify sugar beet production to reach national self-sufficiency in sugar production. In addition, with the implementation of the recommendations of the Green Moroccan Plan, new Government Agricultural Development Strategy, which provides increase of agricultural areas it is expected to further increase use of pesticides including carbofuran, especially in areas with intensive agricultural activities (MAPM, 2008).

No studies on carbofuran behaviour have been carried out in the perimeter. Hence, the present work aimed at studying the rate of mineralization and degradation of carbofuran, under laboratory conditions, in sugar beet soil of Loukkos perimeter under flooded and non-flooded conditions using radio-tracer technique, with the aim of improving human health and protecting the environment.

MATERIALS AND METHODS

Soil samples

Soil samples were taken randomly to a depth of 0 to 30 cm from a sugar beet field of Loukkos perimeter (North-western of Morocco) and mixed thoroughly. The Vertisol soil, which has no history of carbofuran treatment, was air dried ground and screened through a 2-mm sieve before treatment.

Physical and chemical soil properties

Soil properties including organic matter, total nitrogen, and pH and soil texture were analyzed at the Regional Soil Laboratory of INRA of Tangier. Soil pH was measured in water: soil (1: 2.5), organic matter was determined according to the Walkley and Black method (Walkley and Black, 1934), total nitrogen was carried out using Kjeldhal method, while texture was determined according to the Robinson Pipette method.

Chemicals

[U-phenyl-^{14}C] carbofuran (specific activity 1.12 GBq/mmol) was purchased from the Institute of Isotopes of the Hungarian Academy of Sciences via the International Atomic Energy Agency (IAEA). The chemical was determined to be more than 98% radiochemically pure by thin layer chromatography before use. (U-phenyl-^{14}C) carbofuran was diluted with unlabelled carbofuran (to specific activity: 4.08 MBq/mmol) before application. Carbofuran (98.8%), 3-OH-carbofuran (>98%) and 3-keto-carbofuran (>98%) and carbofuran phenol (>97%) analytical standards where purchased from Sigma Aldrich /Riedel de Haën. All the reagents used in the study were of analytical or scintillation grade. All the solvents used were residues, HPLC, or analytical grades.

Mineralization and degradation study

50 g of soil samples were transferred into standard 250 ml Bellco biometer flasks placed in the laboratory. The experiments were carried out under moist and flooded conditions referring respect-tively to irrigation and intensive rainfall systems Characteristics of the perimeter. The moist conditions were maintained by adding

water equivalent to 60 % field capacity (FC) of the soil and kept at this level throughout the course of the experiment by weighing the flasks. For the experiments under flooded conditions, the water level was maintained at 2 cm above the soil surface. 1.0 mg (20 ppm) carbofuran equivalent to 0.50 µCi of ^{14}C-carbofuran was added to each flask and stirred to obtain a homogenous sample. In the side arm of each flask was added 20 ml of ethanolamine (scintillation grade) to trap the $^{14}CO_2$ produced while the organic volatiles were trapped by polyurethane plugs placed at the passage between the soil sample and ethanolamine trap. Flasks were incubated at about 25°C in the darkness for 63 days. Each flask was tightly closed with a rubber stopper. At weekly intervals, the rubbers stoppers were removed for few minutes to ensure aerobic conditions.

Sampling of flasks

3 flasks were taken off immediately, after application of carbofuran, for analysis to determine the initial applied dose (100%). The other flasks were sampled in triplicate at 15, 31, 45 and 63 days after treatment. The soil samples were air-dried under a ventilated fume hood, ground and homogenized then stored in plastic bags at -21°C until analysis.

Extraction, clean-up and analysis

The trapped $^{14}CO_2$ in the ethanolamine traps was quantified by Liquid Scintillation Counting (LSC), while the organic volatiles were extracted with methanol and aliquot (1ml) of the extracts were counted with LSC. Total and bound residues were determined by combustion of soil samples in a Harvey-Biological Oxidizer (Harvey OX-600) followed by counting in the LSC. Extractable residues were determined by extracting 10 g (dry weight basis) soil samples, in triplicate in a soxhlet extraction apparatus with methanol for 6 h (5 to 6 cycles/h) followed by counting in the LSC.

Radioassay method

Radioactivity was measured on a Packard Tricarb1100 Scintillation Counter after mixing the samples with toluene-based-scintillator cocktail. The internal standardization technique was used to correct for quenching effect.

HPLC analysis

To determine the nature of the ^{14}C-residus in methanol extracts, solution was gently concentrated to 1 ml with Rotavapor (Buchï, Switzerland) at 30°C and cleaned-up on Bond Elut C_{18} SPE cartridges, which were previously conditioned with 3 ml HPLC water followed by 3 ml HPLC methanol, then analysed with HPLC Shimadzu (LC-10ADvp) , fitted with a Nucleosil C_{18} stainless-steel column (5 µm, 4.6mm × 250mm), equipped with a Nucleosil C_{18} guard column, injection volume was 20 µl. Mobile phase used was acetonitrile : water (50 : 50 v/v) with a flow rate of 0.7 ml/min; ultraviolet absorption at 230 nm (SPD-10Avp UV detector) was measured and the peak area used for quantification. The identification was done by comparison the pick samples with the authentic ones of standards.

RESULTS AND DISCUSSION

The soil was classified as clay and had the charac-teristics cited in Table 1.

In the present investigations, monitoring of liberated $^{14}CO_2$ from the degradation of ring labelled, carbofuran in Moroccan clay soil showed that considerable amounts of this substance are mineralized to $^{14}CO_2$ during 63 days under both moist and flooded conditions. Figure 1 illustrates the cumulative evolution of $^{14}CO_2$ from ^{14}C-U-ring carbofuran in clay soil under flooded and non flooded conditions. The evolution of $^{14}CO_2$ from carbofuran labelled at the phenyl ring indicates the biological attack on the phenyl ring. The percentage of mineralization showed a consistent increase with time. The rate of mineralization was found to be more rapid in non-flooded than in flooded conditions.
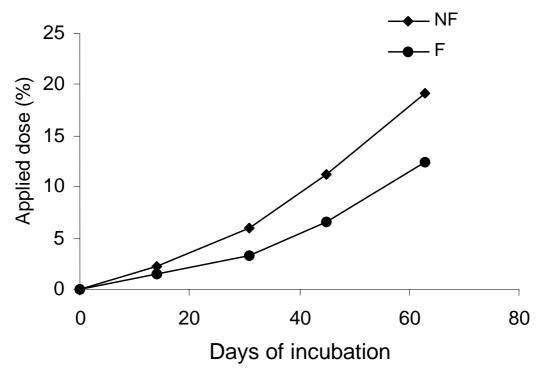
In the microbial breakdown of pesticide molecules, it has been suggested, that there is an initial lag phase, during which no significant breakdown of the pesticide molecule occurs (Fournier et al., 1997). This is the adaptation period of soil microorganisms to the new substance. This may be the fact that no significant increase amount of mineralization has occurred during the first 14 days (only 2.2 and 1.4% of applied dose was mineralized respectively under moist and flooded conditions). Afterward, a rapid increase in the rate of mineralization was observed; at day 45, the $^{14}CO_2$ activity amounted to 11.8 and 7.4% respectively under moist and flooded conditions. The amount of mineralization increased to reach more than 19% for moist soil and more than 12% for flooded condition at the end of the experiment. This is in accordance with the pattern of microbial breakdown of a pesticide, that is, a period of rapid degradation follows the initial lag phase (Fournier et al., 1997).

Similar high mineralization levels of carbofuran have been reported in the literature. Hussain et al. (1986) found that 35.6% of applied dose was lost as $^{14}CO_2$ after 20 days incubation in a clay loam soil. Lalah et al. (1996) reported mineralization of 13 and 11% of applied dose, from U-ring-^{14}C-carbofuran after 33 days of incubation respectively under flooded and moist conditions. In experiments using a continuous flow system, Kale et al. (2001) found near 30% of carbofuran mineralization under moist condition after 30 days of incubation in a Vertisol soil. High mineralization rate was also reported by Trabue et al. (1997); the mineralization of the aromatic ring of carbofuran exceeded 58% during 28 days of incubation. Kale and Raghu (1996) reported also extensive carbofuran mineralization in clay soil; after 30 days, 29.9 and 33.7% of applied dose was mineralized under moist and flooded conditions respectively. Zayed et al. (2001) reported mineralization of 13.9% of applied activity of ^{14}C-carbofuran during 90 days of incubation in soil under moist condition. Liyanage et al. (2007) found carbofuran mineralization ranging from about 20 to near 23% in Sri Lankan soils.

The relative low mineralization obtained in flooded conditions as compared to that in moist conditions can be explained by the following: in flooded soil, which could give rise to anaerobic conditions, carbofuran is subjected

Table 1. Physical and chemical characteristics of the soil used.

pH (water)	pH (KCl)	Total nitrogen (%)	Organic matter (%)	Clay (%) (<2µm)	Silt (%) (2-50µm)	Sand (%) (50-200 µm)	Texture
7.8	7.2	0.22	2.14	50.2	35.6	14.2	Clay

Figure 1. Cumulative evolution of $^{14}CO_2$ from ring labelled carbofuran in soil samples (average of triplicate values. Applied radioactivity = 100%). (F: Flooded condition, NF: non flooded condition).

to hydrolysis to give carbofuran phenol as main metabolite that undergoes immediately strong adsorption reaction with soil constituents and hence it is slowly metabolized by microorganisms (Getzin, 1973). Accordingly, bound residues were high in flooded compared to moist conditions (Table 3). Inversely, in aerobic conditions, carbofuran phenol is instable (Venkateswarlu and Sethunathan, 1978) and tends to be degraded further through aromatic ring cleavage yielding CO_2 and H_2O (Ou et al., 1982; Trabue et al., 1997). Therefore, the same microorganisms that mineralized the aromatic ring of carbofuran were likely to be responsible for carrying out mineralization of the aromatic ring of Carbofuran phenol (Feng et al., 1997). Ramanand et al. (1988) suggested that the microbial mediated hydrolysis of the carbamate linkage was the first step in complete mineralization of carbofuran. Turco and Konupka (1990) concluded that the biodegradation of carbofuran in the soil is a two-step process: hydrolysis of the carbonyl side-chain followed by the adsorption of the primary meta-bolite, causing a decrease in ring breakdown. Thus, we

assumed that there is a competition between mineralization of carbofuran phenol leading to $^{14}CO_2$ in aerobic conditions and its adsorption to soil components and bound residue formation. Venkateswarlu and Sethunathan (1978) and Ramanand et al. (1988) reported that the first suggestion is more likely to be under aerobic condition. Binding of carbofuran phenol to soil components is therefore more likely to occur in flooded conditions than to its microbial breakdown, which may explain the difference observed in the amount of bound residues formation in the two conditions.

Table 2 shows the extent of organic volatiles under flooded and moist conditions. The results indicated that carbofuran underwent considerable volatilization in flooded than in non flooded conditions. At the end of the experiment, more than 18 % was lost by volatilization in flooded soil, as compared to only 5.6 % in non flooded conditions. This can be explained by the fact that carbofuran is very water-soluble (700 mg/L), therefore it is lost rapidly by co-evaporation with the water on the surface of the soil. In a similar study, Lalah et al. (1996)

Table 2. ^{14}C-organic volatiles trapped from the soil samples.

Incubation period (days)	Non-flooded soil		Flooded soil	
	µg carbofuran equivalent	Percent	µg carbofuran equivalent	Percent
14	1.30	0.29 (±0.04)	24.50	3.45 (±0.44)
31	3.60	1.37 (±0.42)	82.70	7.07 (1±.09)
45	13.80	3.08 (±0.62)	121.30	11.13 (±1.88)
63	26.70	5.67 (±0.95)	198.20	18.25 (±2.84)

100% corresponds to 1 mg=1.000µg

Table 3. Masse balance of ^{14}C-carbofuran dissipation in flooded and non-flooded clay soil as percent of applied activity (values are mean of triplicate, applied activity= 100%).

Incubation Period (days)	Extracts (%)		Bound (%)		[14]CO$_2$ (%)		Volatilization (%)		Recovery (%)	
	F	NF	F	NF	F	NF	F	NF	F	NF
14	65.88	76.9	18.1	14.22	1.43	2.28	3.45	0.29	88.86	93.69
31	43.37	60.67	28.3	22.76	3.22	5.92	7.07	1.37	81.96	90.75
45	27.21	46.01	32.6	27.4	7.44	11.87	11.3	3.08	78.55	88.36
63	15.78	31.05	33.3	29.11	12.41	19.23	18.25	5.67	79.74	85.06

F: flooded; NF: non-flooded

found that 50 and 13% of carbofuran initial applied dose was recovered as volatile products respectively under flooded and moist soils 33 days after incubation. Caro et al. (1976) reported that 36.2% of the applied carbofuran volatilized from a sandy soil after 60 days of incubation. Venkateswarlu et al. (1977) reported that carbofuran under flooded conditions, is subjected to hydrolysis, rapid adsorption and volatilization.

Table 3 represents the dissipation of carbofuran in soil. The result showed that the substance underwent extensive degradation under both conditions. The degradation was more rapid in flooded than in moist condition. With increasing time, the extractable residues in general decreased. Within the first two weeks, they decreased rapidly and reached about 66% and 77% of applied dose under flooded and moist soil respectively. The amount of extractable residues showed a continuous decrease with time to reach at the end of the experiment 15.8 and 31% respectively under flooded and moist soil. Consequently, the disappearance rate of the extractable residues was more rapid in flooded than in non flooded conditions.

Bound residues in both conditions were high, the extent being more in flooded soil (33% of applied activity) than in moist soil (29% of applied dose). In both conditions, the binding process occurred very rapidly within the first 2 weeks after application and reach more than 18 and 14% of applied activity respectively under flooded and moist soil. With increasing time, they increased more: at 31 days, they amounted to 28.3 and 22.7% respectively under flooded and moist soil. After day 31, binding activity was slower, reaching 33 and 29% in the flooded

and moist soil treatments, respectively. The amount of binding activity of carbofuran to the Vertisol was relatively high in both treatments.

A similar trend was obtained by many researchers; Venkateswarlu et al. (1977) and Venkateswarlu and Sethunathan (1978) reported that carbofuran degraded more rapidly in flooded soils (anaerobic conditions) than in non-flooded soils (aerobic conditions). Kale and Raghu (1996) found, in a clay soil, 20 and 31% of applied dose as extractable residues and formation of 48% and 23% as bound residues, respectively, under moist and flooded conditions. Kale et al. (2001) obtained in a Vertisol soil 8.5 and 10% of applied dose as extractable and 55 and 42% as bound residues under moist and flooded conditions respectively after 60 days of incubation of carbofuran. Lalah et al. (1996) reported formation of 40% of bound residues in flooded soils after 40 days of incubation. Ou et al. (1982) reported formation of bound residues of ^{14}C-carbofuran from 5.1 to 94.5% depending on soil type. Greater amounts of bound residues were formed in soils with high clay and soil organic matter content. Soil adsorption of carbofuran and its ^{14}C-residues was suggested to cause the increase of unextractable carbofuran with time in the soil (Isensee and Tayaputch, 1986; Wilems et al., 1996). These authors suggested that formation of carbofuran phenol likely plays a key role in the incorporation of carbofuran residues into organic matter.

Literature showed variable results on carbofuran metabolite recovery from soils ranging from no metabolite (Isensee and Tayaputch, 1986) or only carbofuran

Table 4. Percent metabolites identified in flooded and non-flooded soil sample extracts at 63 days after application.

Compound	Retention time (min)	Metabolites (%)	
		Moist soil	Flooded soil
3-ketocarbofuran	5.45	4.08	6.24
Carbofuran	6.42	77.24	67.31
3-ketocarbofuranphenol	6.61	3.46	4.38
Carbofuran-7-phenol	8.30	8.22	18.37

phenol up to 46% (Brahmaprakash and Senthunathan, 1985) to the presence of the three metabolites carbofuran phenol, 3-hydroxy- and 3-keto carbofuran (Lallah et al., 1996).

A good balance sheet was obtained for non-flooded condition and the percentage recovery was generally between 85 to 93%. This suggests that volatilization probably does not represent a significant percentage in the dissipation of carbofuran from soil under this soil condition. While in flooded soil the balance sheet obtained was lower than in moist conditions suggesting that there was some loss during water evaporation (co-evaporation). This was explained by the high percentage (18.25%) obtained for ^{14}C-organic volatile products compared to only 5.67% under non-flooded conditions.

HPLC analyses of the extractable residues after 63 days of incubation showed that 78.24 and 67.31% was in the form of carbofuran respectively under moist and flooded soils. In both conditions, carbofuranphenol was the major significant metabolite observed with 18.3 and 8.2% of applied activity under respectively flooded and moist soil (Table 4). Similar results were obtained by Venkateswarlu and Sethunathan (1978) and Lalah et al. (1996). They reported substantial formation of carbofuran phenol as the major degradation product.

Carbofuran was reported to be subject to hydrolysis, mainly in alkaline conditions, and its hydrolysis occurs at the carbamate linkage yielding carbofuran phenol (Ou et al., 1982). This is the fact that high amount of carbofuran phenol was observed in flooded conditions. The slight alkaline nature of soil (pH = 7.8) may be responsible for the significance presence of carbofuran phenol. 3-hydroxycarbofuran metabolite however was not detected. This may be due probably to its rapid conversion to 3-keto carbofuran metabolite as reported by Camper et al. (1987).

Conclusion

The results confirm that carbofuran is more rapidly mineralized in soil under moist than flooded conditions; likewise it is important to note the large difference in degradation rate of the substance in moist and flooded soils. Under moist condition, higher mineralization rates (amounts) occurred reaching 19.2%, compared to those in flooded soil levelling 12.4%. Whereas, soil bound residues were formed at high amounts. The extent was more in flooded (33.3%) than in moist soil (29.1%) indicating high potential for soil binding. Considerable dissipation occurred through volatilization in flooded soil. Consequently chemical hydrolysis and volatilization are important dissipation ways in flooded condition.

ACKNOWLEDGEMENTS

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