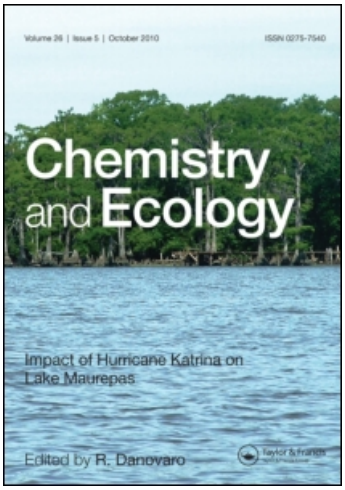

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MICROBIAL DEGRADATION OF TWO CARBAMATE INSECTICIDES AND THEIR MAIN METABOLITES IN SOIL

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Degradation studies in soil of the insecticides aldicarb and carbofuran and their metabolites (aldicarb sulfoxide, aldicarb sulfone; 3-ketocarbofuran and 3-hydroxycarbofuran) were carried out using laboratory systems under controlled conditions (temperature, water content, light). The insecticides were added to soil samples and subsamples of the soil were analyzed at different times to assess both the bacterial abundance and the concentration of the different chemicals. The epifluorescence direct count method was applied to the subsamples to estimate microorganism numbers (N/g soil). Untreated samples of soil were used as controls for evaluating the effects of the application of the insecticides on microbial abundance. Subsamples treated with the pesticides were analyzed using HPLC and the DT_{50}s of the different compounds studied were calculated.

The DT_{50} values show that neither the parent compounds nor the transformation products have a high persistence in soil and there is a general increase in the concentration of microorganisms as the pesticides diminish.

Keywords: Aldicarb; Carbofuran; Metabolites; Soil; Biodegradation

INTRODUCTION

Modern agricultural production systems are dependent on the application of plant protection products to soil. These products may cause undesirable side effects, *e.g.* leaching to groundwater or to surface water, uptake by plants and entry into the foodchains. While the toxicity inherent to organisms is important, the way in which undesirable effects are brought about largely depends on a compound's behaviour in the soil system. In this respect the persistence in soil, expressed as disappearance time of 50% (DT_{50}) of the applied dose of a parent compound, indicates its degradability. The degradation rates are dependent on biotic (microbial activity) and abiotic (temperature, water availability, hydrolytic stability, organic matter content) factors in a soil ecosystem.

Microbial metabolism is recognized as the primary force in pesticide transformation and mineralization. The chemical may be used as a substrate for growth, or in the case

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of co-metabolism it is transformed by metabolic reactions but does not serve as an energy source for microorganisms (Bollag and Liu, 1990; Skipper *et al.*, 1996; Zipper *et al.*, 1998).

Aldicarb and carbofuran are widely used carbamate insecticides that have been developed as a biodegradable and short-lived alternative to highly stable organochlorine. They are widely used in Italy (Sesia, 2000) but they are neverthless toxic and able to form metabolites retaining significant toxicological properties (Nelson *et al.*, 1981; Foran *et al.*, 1985; Baron and Merriam, 1988; Moye and Miles, 1988; Gupta, 1994; Canna-Michaelidou and Nicolau, 1996; Alvarez-Rodriguez *et al.*, 1997). Moreover, carbamates show a high mobility in soil (Barra Caracciolo *et al.*, 1999; Fava *et al.*, 2001) and have been detected in significant concentrations in groundwater (Williams *et al.*, 1988; USEPA 1984; IHP, 1998; Walker and Porter, 1990; Jones and Estes, 1995; Funari *et al.*, 1995). Since they have significant biological properties (Gupta, 1994), they can represent a human health hazard, especially if the water is used for drinking.

Aldicarb [2-methyl-2-(methylthio)propionaldehyde) *O*-(methylcarbamoyl)-oxime] is mainly oxidized by microorganisms to aldicarb sulfoxide [2-methyl-2-(methylsulfininyl) propionaldehyde*O*-(methylcarbamoyl)oxime], then a small proportion of it is oxidized to aldicarb sulfone [2-methyl-2-(methylsulfonyl)-propionaldehyde*O*-(methylcarbamoyl)oxime]. Under aerobic conditions microbial oxidation appears to be the major route for aldicarb degradation in surface soil. In anaerobic conditions aldicarb sulfone and sulfoxide may revert to the parent compound (Ou *et al.*, 1988; Baron and Merriam, 1988). Abiotic transformation of the latter in aldicarb sulfone and sulfoxide was also observed in a soil not previously treated with the insecticide, but at a degradation rate significantly lower than in the treated one (Trabue, 1997).

Carbofuran (2,3-dihydro-2, 2-dimethyl-7-benzofuranylmethylcarbamate) is degraded through both biotic and abiotic processes. The formation of 3-hydroxycarbofuran may be both chemical and biological, that of 3-ketocarbofuran is reported to be mainly a biotic oxidization (Yen *et al.*, 1997).

The degradation of these parent compounds and of their main metabolites has been studied in extensively (Kale *et al.*, 2001; Kazumi and Capone, 1995) and several soil microorganisms have been reported to transform aldicarb and carbofuran (Chaudry and Ali, 1988; Trabue *et al.*, 1997; Sahoo *et al.*, 1998; Salama, 1998) to their main metabolites; however, the biochemical pathways regarding the complete mineralization of the transformation products are still not well known.

The results reported here come from laboratory studies on aldicarb, aldicarb sulfoxide, aldicarb sulfone, carbofuran, 3-ketocarbofuran and 3-hydroxycarbofuran, in which soil DT_{50}s was assessed and the abundance of the microbial community were measured.

MATERIAL AND METHODS

Collection of the Soil Samples

Samples of soil were collected from the surface layer (5–15 cm depth) of an agricultural field located in Treviglio (province of Bergamo), Northern Italy. The criteria used for the selection of the site were the presence of intensive agriculture with a prior history of carbamate exposure and a high aquifer vulnerability. The site is representative of the geopedological conditions prevailing in a large area of the River Po Plain where the underground profile in terms of permeability features is favorable to the migration of pesticides towards the groundwater body and the water table is relatively shallow.

TABLE I Main Abiotic and Biotic Features of the Treviglio Site Samples.

Pedological horizon	A
Sample depth (cm)	5–15
Soil temperature at time of collection (°C)	5.2
Organic carbon (%)	1.4
pH (H_2O)	8
CEC (meq/100 g)	14.95
WHCmax (%)	34%
Coarse sand (%)	26.4
Fine sand (%)	15.1
Silt (%)	47.0
Clay (%)	11.5
Moisture content (w/w%)	22%
N bacteria/g soil	4×10^7

The soil was classified as a loam soil (USDA, 1994) with an organic carbon content of 1.4% (Table I). After sampling, the soil was sent to the laboratory in a portable refrigerated bag and stored at 4 °C for 1 week until use.

Chemicals

All substances were purchased from the Dr. Ehrenstorfer Laboratories, Augsburg, Germany. The purity degree was >98%. Distilled water for High Performance Liquid Chromatography (HPLC) was further purified through a Norganic cartridge (Millipore, Bedford, MA). All the organic solvents used were of HPLC grade (acetone – Baker, Holland; acetonitrile Merck, Germany; methanol – Sigma Aldrich – Germany).

Soil Degradation Experiments

The soil degradation experiments were conducted according to SETAC guideliness (SETAC, 1995), and procedures previously developed (Donati *et al.*, 1994; Bottoni *et al.*, 1996; Barra Caracciolo*et al.*, 1999).

The fresh soil was dried at room temperature and sieved with a 2 mm-mesh sieve. A set of sterilized beakers (2 replicates for each chemical plus 2 controls) was prepared, each containing the same quantity of soil (about 200 g). Six distilled water solutions, each containing separately aldicarb, carbofuran and the metabolites (respectively aldicarb sulfoxide, aldicarb sulfone, 3-ketocarbofuran, 3-hydroxycarbofuran), were applied to the soils at a dose of 5 mg a.i./kg^{-1} for every compound except for carbofuran (2.5 mg a.i. kg), which corresponds to the agricultural dose of the parent compounds. The control soils were prepared for each chemical applied with only distilled sterile water in order to obtain the same final moisture content of the treated soils (22% w/w, corresponding to 65–70% of soil maximum water holding capacity, WHC). All soils were thoroughly stirred with a spatula to distribute homogeneously the pesticides (treated soils) and the sterile water (control ones). The beakers were closed with a sterilized cotton plug wrapped in a gauze to allow air exchange; the soil moisture was kept constant during the entire period of the experiments by weighing the soil batches periodically and replacing any losses by adding sterile water according to SETAC (SETAC, 1995). All the experimental sets were incubated at 21 °C (±0.5) in the dark.

Chemical Analysis

The residue concentrations of aldicarb and carbofuran and their metabolites were measured immediately after the treatment and at fixed intervals until a reduction of 90% was reached for each compound.

Two grams of soil were collected in duplicate at each interval of sampling and extracted with 4 ml of water for aldicarb and with 8 ml of acetone for the other compounds. The resulting suspensions were shaken for 10 min with a wrist-action shaker and then centrifuged for 10 min (2420 g). Supernatants were filtered (0.45 μm membrane) and then directly analyzed by HPLC (aldicarb) or reduced under nitrogen flow to 1 ml (determined by weight), and then and analyzed (other compounds). All compounds were extracted from soils with initial recoveries of 71%, 95%, 98%, 66%, 94% and 98% for alicarb, aldicarb sulfone, aldicarb sulfoxide, carbofuran, 3-hydroxycarbofuran and 3-ketocarbofuran respectively. Concentrations were calculated through the external standard method (Barra Caracciolo *et al.*, 1999).

HPLC Apparatus

The equipment used in the experiments was a Model 9010 liquid chromatograph (Varian, Walnut Creek, CA, USA), a Varian 9050 UV detector (for aldicarb analysis), a Spectra System UV6000 LP Thermoquest diode array detector (for carbofuran analysis) and a Varian 9100 autosampler. The chromatographic raw data were elaborated with the Star Integrator 4.1 Varian Software.

For DT_{50} determinations, a Supelcosil LC-18 ABZ plus 25 cm × 4.6 mm i.d. column (Supelco Inc., Bellofonte, PA) was used. Compounds were eluted with gradient programs using a mixture of acetonitrile and water as follows: from 0 to 5 min acetonitrile was 20%; from 5 to 20 min the proportion of acetonitrile linearly increased from 20 to 80%; from 20 to 25 min the proportion of acetonitrile remained at 80%; then linearly decreased to 20% from 25 to 30 min, and remained at this value for a further 5 min.

The mobile phase flow was 1 ml^{-1}; the column was kept at 30 °C and the detectors set at 210 nm wavelength. The qualitative and quantitative determinations were obtained with the external standard methods. Each analytical datum represents the mean value of three HPLC determinations.

Microbial Analysis

Sampling

The microbial abundances were measured immediately after the treatment and at given intervals corresponding to chemical sampling. For each sampling, 1 g of soil was collected in triplicate both for control and for treated soils. Microbial analysis was performed by modifying (Di Corcia *et al.*, 1999; Barra Caracciolo *et al.*, 2001) a previously used procedure (Porter and Feig, 1980) of direct counting. This procedure involves using a fluorescent dye, DAPI, to distinguish bacteria (that appear with a luminescent blue colour) from non-living bacterium sized particles (that show up as yellow).

Soil Sample Treatment

A gram of soil was placed in a sterile 10 ml test tube with 9 ml of a filter-sterilized solution consisting of 4% formaldehyde solution and 0.5% of Tween 20. Then the test tube was shaken for 5 minutes (400 rpm) to facilitate the action of Tween 20 in detaching the bacteria

from the soil particles. After shaking, the suspension was left for 24 hours so that the larger soil particles could settle out.

An aliquot of supernatant (100 µl) was pipetted into a sterile tube containing 2 ml of sterilized physiological solution. Then 200 µl of DAPI (4′,6-diamidino-2-phenylindole) were added.

The DAPI was in contact with the supernatant for 20–30 minutes in the dark at 4 °C. The solution was then filtered through a 0.2 µm black membrane that was subsequently mounted on a glass microscope and the bacteria were counted by epifluorescence microscopy. A Leica microscope equipped with the appropriate filter blocks was used for the epifluorescence counts.

All bacterial cells in the grid field (100 µm × 100 µm) were counted with a 100 × oil immersion fluorescence objective. Twenty fields per slide were counted and at least 600–1000 cells were counted for each sample.

All the solutions and instruments utilized were sterilized and all the steps were performed in a sterile cabinet.

RESULTS

The degradation patterns of all examined compounds fit first-order kinetics.

DT_{50} values were calculated, for each compound, from the regression curve:

$$C_t = C_0 e^{-kt}$$

C_0 = theoretical initial concentration
k = exponential coefficient
t = time
fitting the residue concentration data (C_t) at the sampling times (t), when $C_t = \frac{1}{2} C_0$:

$$DT_{50} = \frac{\ln 2}{k}$$

Aldicarb and Metabolites

The declines in the concentrations in soil of aldicarb, aldicarb sulfoxide and sulfone are shown in Figure 1.

The DT_{50} value of the parent compound was less than 1 day, that of the metabolites respectively 7 days for aldicarb sulfoxide and 12 days for aldicarb sulfone. Among all the studied compounds only the formation of aldicarb sulfone from the degradation of aldicarb sulfoxide was detected (Fig. 1).

The number of bacteria (N/g) in the treated batches was generally greater than in the untreated ones as shown in Figure 2 A, B, C. Moreover a significant difference was found in bacterial abundance between the treated and untreated systems (t test, $p < 0.01$) for all the three compounds studied.

Carbofuran and the Metabolites

The declines in the concentrations in soil of carbofuran, 3-ketocarbofuran, 3-hydroxycarbofuran are shown in Figure 3. The DT_{50}s were respectively 12 days for carbofuran, 5 days for 3-ketocarbofuran, and less than one day for 3-hydroxycarbofuran.

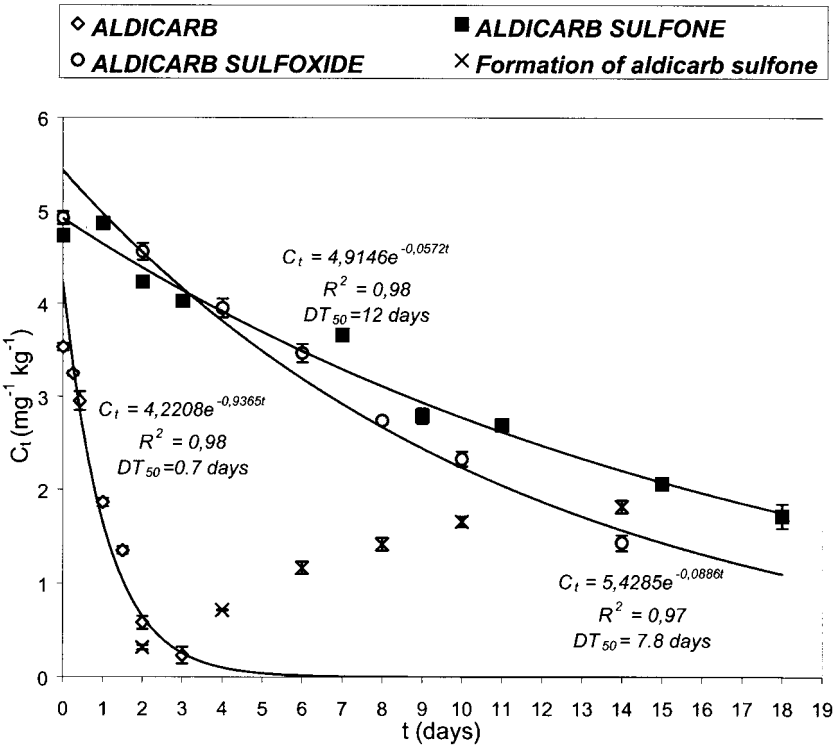

FIGURE 1 Decline in the concentrations (mg/kg) of aldicarb, aldicarb sulfone, aldicarb sulfoxide (and formation of aldicarb sulfone) in soil degradation experiments. The vertical bars represent the standard errors. The cross symbols represent the data on formation of aldicarb sulfone from aldicarb sulfoxide.

The number of bacteria (N/g) in the treated batches was generally greater than in the untreated ones (Fig. 4 A, B, C), although a significant difference in abundance between the treated and untreated batches (*t* test, $p < 0.01$) was found only for carbofuran.

DISCUSSION AND CONCLUSION

The soil DT_{50}s of aldicarb and carbofuran and their metabolites calculated in this work are in accordance with other values reported in literature (Montgomery, 1993; Greenhalgh and Balanger 1981; Ramanand *et al.*,1988; Salama, 1998; IHP, 1998), although other works have shown values with a relatively high variability (Getzin, 1973; Jury *et al.*, 1987; Tomlin, 1995). The variability of DT_{50} values may be due to the different conditions used in the experiment (laboratory or field experiments and different biotic/abiotic factors present, such as soil moisture, temperature and microbial activity, etc.), which significantly influence the degradation rates. In any case the carbamates studied do not show a high persistence; since they are reported to be very mobile in soil (Barra Caracciolo *et al*, 1999; Fava *et al.*, 2001), it is very important that the environment contains a microbial community which has adapted to them (Moorman, 1990) and is capable of degrading them in a short time to avoid their transport to groundwater. The results of the bacterial counts reported above show not only that the studied compounds at the applied dose do not inhibit microbial

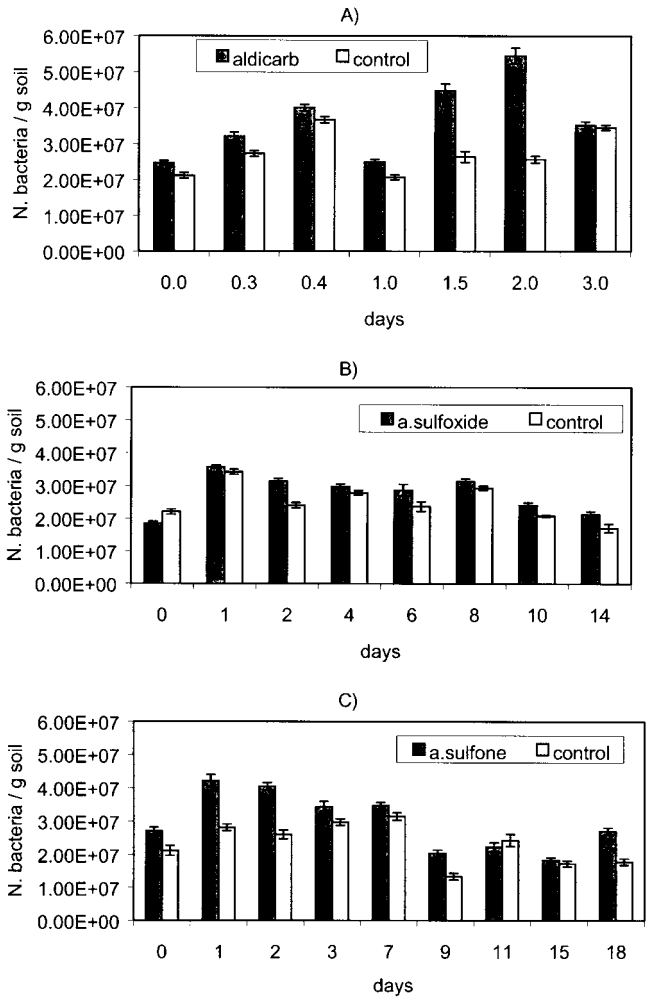

FIGURE 2 Bacterial numbers (N/g soil) in aldicarb (A), aldicarb sulfoxide, (a. sulfoxide) (B), aldicarb sulfone, (a. sulfone) (C) in the degradation experiments. The vertical bars represent the standard errors.

growth, but that also, in the case of aldicarb, sulfone, sulfoxide and carbofuran, the microbial component has a significant role in the degradation of these compounds. Many authors, in fact, report the capability of soil microrganisms to use the carbamate insecticides (aldicarb and carbofuran and some metabolites) as a source of carbon and nitrogen for growth (Ou *et al.*, 1988; Baron and Merriam, 1988; Rasul Chaudry and Ali, 1988; Sahoo *et al.*, 1998; Salama, 1998). Since in the case of hydroxycarbofuran and ketocarbofuran the differences in bacterial numbers between treated soils and controls are not significant, the degradation might be due to both chemical and co-metabolic processes. Co-metabolism is a biological process in which pesticides are transformed by metabolic reactions, but do not serve as an energy source for micro-organism growth. In any case knowledge about the degradative pathways of the metabolites is in general still scarce and further studies are necessary to assess the specific role of the micro-organisms in the different phases of degradation.

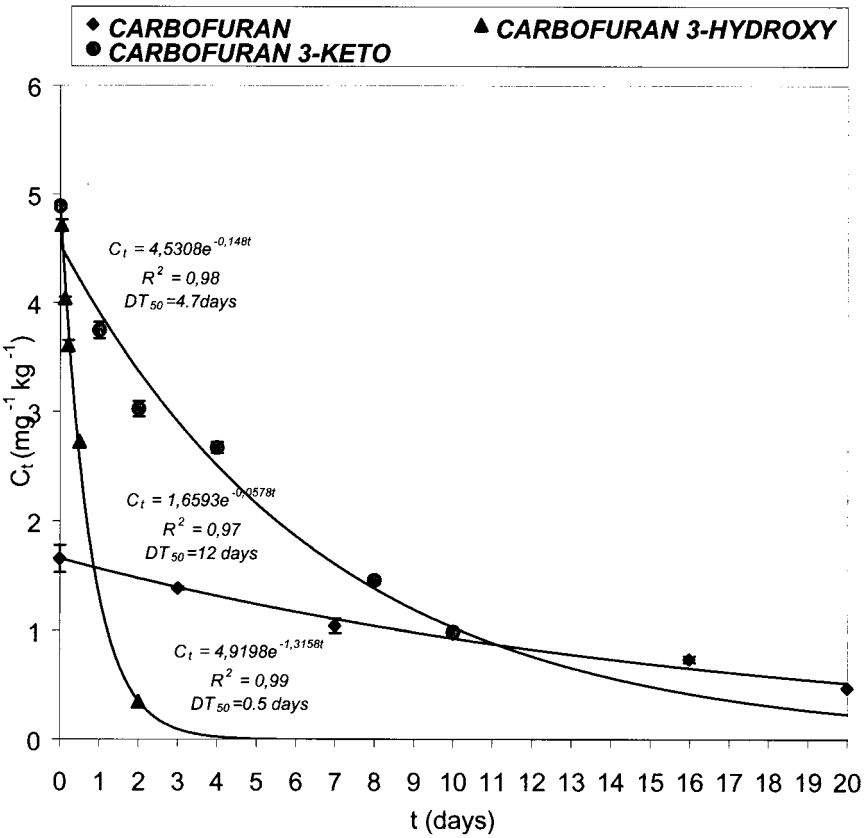

FIGURE 3 Decline in the concentrations (mg/kg) of carbofuran, 3-ketocarbofuran (carbofuran 3-keto), 3-hydroxycarbofuran (carbofuran 3-hydroxy) in soil degradation experiments. The vertical bars represent the standard errors. The vertical bars represent the standard errors.

The present study was performed at a relatively high temperature (21 °C) and with a soil water content of 22% w/w, (corresponding to 60–70% of soil maximum water holding capacity), both being optimal conditions for soil microorganism activity (Atlas and Bartha, 1997) so that the soil degradation rates may be over-estimated. Using a lower temperature (Barra Caracciolo *et al.*, 2001) and a different soil-water concentration, simulating different environmental conditions, could allow the measurement of the degradation of the same chemicals in less favourable conditions. Experimental studies of this kind are in progress.

Laboratory studies, allowing the measurement and/or the control of the different environmental factors, are very useful to compare both different chemicals with the same conditions and the same compound with different conditions, in order to increase our knowledge of pesticide behaviour.

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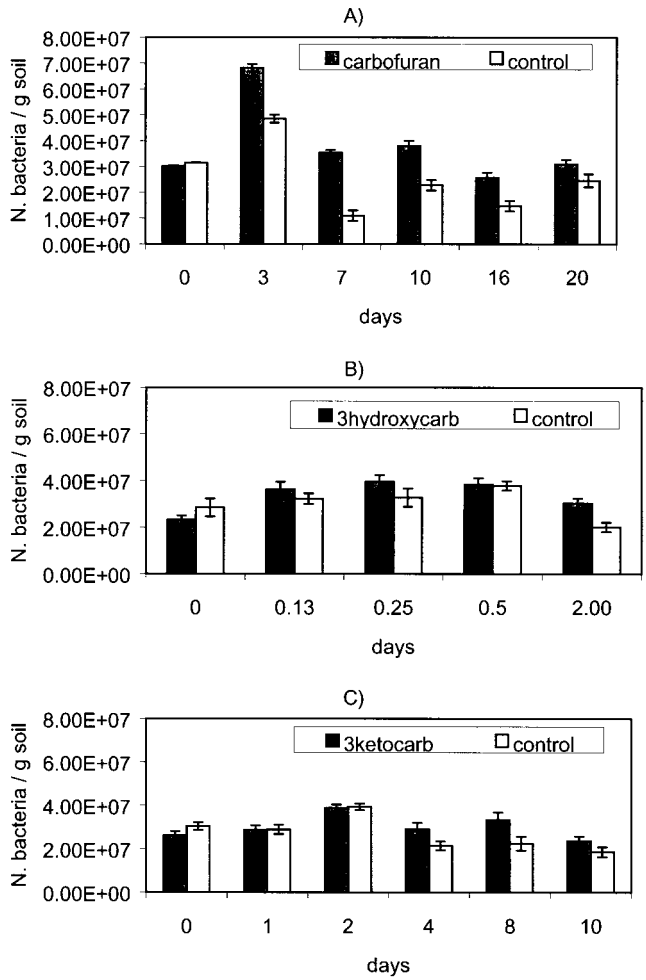

FIGURE 4 Bacterial numbers (N/g soil) of carbofuran (A), 3-hydroxy carbofuran, (3hydroxycarb) (B), 3-ketocarbofuran, (3ketocarb) (C) in the degradation experiments. The vertical bars represent the standard errors.

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