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ORIGINAL PAPER

Bioremediation of carbofuran contaminated soil under saturated condition: soil column study

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Abstract Disturbed soil columns, 5.8 cm in diameter and 25 cm in length, were used as a basic model to simulate the movement of carbofuran in rice field soil under saturated conditions. Bioaugmentation using a specific carbofuran degrader, *Burkholderia* sp. PCL3, in free and immobilized cell forms and biostimulation using rice straw as organic amendment were applied with the aim of enhancing the degradation of carbofuran in soil and to prevent the movement of carbofuran along with the flow through. In the abiotic control and the treatment with only indigenous microorganisms, the mass recovery percentage of carbofuran in the effluent was 52.1 and 22.5%, respectively. The application of bioaugmentation or biostimulation significantly enhanced carbofuran degradation in soil and reduced the movement of carbofuran as indicated by a low mass recovery percentage of carbofuran in the effluent of 14.6–15.5%. A low efficiency of carbofuran removal was obtained from the soil column with bioaugmentation together with biostimulation treatments in which the mass recovery percentage of carbofuran in the effluent was in the range of 22.1–22.6%. Sorption of carbofuran to soil, rice straw and corncob, formation of carbofuran metabolite and colony forming unit (CFU) and pH variation with the time were also investigated during column operation.

Keywords Soil column · Bioremediation · Carbofuran · Saturated zone

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Introduction

Carbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in agriculture, especially in rice fields. It is used to control insects and nematodes on contact or after ingestion. Carbofuran is of environmental concern because of its highly acute toxicity to mammals through cholinesterase inhibition, neurotoxicity and adverse reproductive effects (Gupta 1994). Continuous use of

carbofuran in agricultural areas may cause contamination risk to surface water and soil (Thapinta and Hudak 2000). Carbofuran is soluble in water and highly mobile in soil (Tariq et al. 2006), so the subsequent high potential for groundwater contamination should be of great concern. Understanding the movement of carbofuran in soil and the removal of carbofuran from contaminated areas is necessary to prevent the contamination of groundwater by carbofuran.

One of the most effective routes for pesticide removal is microbial degradation by a specific degrader and/or indigenous microorganisms. Previous studies reported the discovery of microorganisms capable of degrading carbofuran and other pesticides from contaminated natural matrices (Yan et al. 2007; Bano and Musarrat 2004). These degraders could use the pesticide as their energy source, i.e., C- or N-, or C and N-sources. The addition of microbial cultures capable of degrading pesticides or so-called bioaugmentation techniques, is reported to be an effective bioremediation approach for improving pesticide degradation in contaminated soils and water that lack any indigenous microbial activity (Parameswarappa et al. 2008; Marecik et al. 2008). Carbofuran degrading bacterium strain PCL3 affiliated with *Burkholderia cepacia* was isolated from carbofuran phytoremediated rhizosphere soil (Plangklang and Reungsang 2011). The PCL3 strain was used in our previous studies to degrade carbofuran in synthetic medium, soil, as well as soil slurry phase yielding carbofuran phenol as the metabolite. The half-lives of carbofuran in synthetic medium, soil and soil slurry inoculated with PCL3 were approximately 3, 12 and 2 days, respectively (Plangklang and Reungsang 2009, 2010). In this study, the ability of PCL3 to degrade carbofuran under saturated condition was investigated.

In addition to bioaugmentation, biostimulation is another bioremediation treatment to remove pesticide contamination in the environment. This treatment stimulates the activity of the indigenous microorganisms by adding organic and/or inorganic additives. The amendments added would be used by the indigenous microorganisms for cell growth resulting in an increase in cell numbers and an increase in their pesticide degradation activities. In addition, the amendments could act as the enzyme-inducers and/or the co-metabolic substrates in the pesticide degradation pathways (Robles-González et al. 2008; Tyagi et al. 2010; Önneby et al. 2010).

The use of soil columns to simulate the pesticide movement in soil is well documented (Abdullah et al. 2001; Farahani et al. 2008). However, the report on the prevention of the movement of pesticides to soil is limited. In the present study, the soil columns were used to simulate the movement of carbofuran in rice field soil under saturated conditions. The mobility of carbofuran in disturbed soil collected from a rice field was investigated to understand the possibility of carbofuran contamination in groundwater. The bioremediation treatments, i.e., bioaugmentation using *B. cepacia* PCL3 and biostimulation using rice straw as organic amendment were applied with the aim of enhancing the degradation of carbofuran in soil and to prevent the movement of carbofuran to groundwater, especially in an area where carbofuran has been extensively used.

Materials and methods

Chemicals

Carbofuran (98.0% purity) and carbofuran phenol (99.0% purity) were purchased from Sigma-Aldrich, USA, and 3-keto carbofuran (98.5% purity) was purchased from Ehrenstorfer Quality, Germany. The physicochemical properties of carbofuran, carbofuran phenol and 3-keto carbofuran are shown in Table 1.

Rice straw

Rice straw was used as organic amendment in biostimulation treatments. It was blended into small pieces using domestic blender (HR2068/20, Philips, Malaysia) and passed through a 2-mm sieve. Organic carbon content of the rice straw, analyzed by Wakley Black method (1934), was 41.5% (dry weight basis) and total nitrogen content, analyzed by Kjeldahl method (AOAC 2000), was 0.4%.

Microorganism

B. cepacia PCL3 (GenBank accession no. EF990634) (Plangklang and Reungsang 2011) was used as the carbofuran degrader. It was grown in 100 ml nutrient broth (NB) containing 5 mg l^{-1} of carbofuran at 30°C and shook at 150 rpm for 36 h before being harvested by centrifugation at 5,000 rpm for 10 min at 4°C. The

Table 1 Selected physicochemical properties of carbofuran and its metabolites, i.e., carbofuran phenol and 3-ketocarbofuran

Property	Carbofuran	Carbofuran phenol	3-Keto carbofuran
Chemical formula	$C_{12}H_{15}NO_3$	$C_{10}H_{12}O_2$	$C_{12}H_{13}NO_4$
Molecular weight	221.25	164.20	235.24
Solubility in water (mg l^{-1}, 25°C)	351	1,096	4,464
Kow (25°C)	208.9	120.2	8.7
Koc	70.85	890.4	10
Vapor pressure (mm Hg, 25°C)	1.1×10^{-3}	5.3×10^{-3}	1.5×10^{-4}

Data was obtained from EPISuite™ (US Environmental Protection Agency, USA)

cell pellets were washed and re-suspended in basal salt medium (BSM) and were used as seed inoculums. The immobilization of *B. cepacia* PCL3 on corncob was conducted following the procedures previously described by Plangklang and Reungsang (2009). Corncob (0.7 × 0.7 × 0.7 cm cubes) was boiled in 1.0% NaOH (1 g corncob:10 ml NaOH solution) for 3 h to remove lignin which might react with the cells (Bardi and Koutinas 1994). The alkaline-boiled corncob was washed three times and soaked in distilled water overnight and then sterilized at 121°C for 15 min. The organic carbon and total nitrogen contents of the sterile corncob were 38.8 and 0.8% (dry weight basis), respectively. Cell immobilization was conducted by adding 75 g of sterile corncob into the sterile 250 ml NB containing 5 mg l^{-1} of carbofuran in 500 ml-Erlenmeyer flask before inoculated with the isolate PCL3 (10^6 CFU ml^{-1}). The flask was incubated at room temperature and shaken at 150 rpm for 48 h. After incubation, cells immobilized on corncob were transferred to a fresh NB containing 5 mg l^{-1} of carbofuran and incubated as previously described before collection by filtration through Buchner filter funnel and washed twice with 0.85% NaCl using aseptic technique. Immobilized cells were kept at 4°C until the usage. The internal cell density on corncob was $4.64 \times 10^7 \pm 1.36 \times 10^6$ CFU g^{-1} dry material.

Synthetic surface water

A modified BSM (pH 6.8) was used as synthetic surface water. It consisted of (in g l^{-1}) 5.57, Na_2HPO_4; 2.44, KH_2PO_4; 2.00, NH_4Cl; 0.20, $MgCl_2 \cdot 6H_2O$; 0.0004, $MnCl_2 \cdot 4H_2O$; 0.001, $FeCl_3 \cdot 6H_2O$ and 0.001, $CaCl_2$ (Mo et al. 1997).

Soil sample

The soil sample was collected from a rice field at Ban Nonmuang, A. Muang, Khon Kaen in May, 2010. Furandan 3G, granular formulation of carbofuran with 3.0% active ingredient (a.i.) was applied annually to this rice field at the rate of approximately 7 kg a.i. ha^{-1} over a 6-year period (2003–2008). The soil was passed through a 2 mm sieve and kept in a plastic bag at 4°C until it was used. Soil pH was 6.84. Soil texture analysis was conducted by hydrometer method (Mocek et al. 1997) including the treatment with hydrogen peroxide to remove of organic matters (Whitehead 1973). The soil sample was classified as sandy loam soil with percentages of sand, silt, and clay of 57.4, 31.1, and 10.1, respectively. The remainder was made up of 1.4% of organic matters.

Soil column experiment

The soil columns were made of glass with an inner diameter of 5.8 cm and a height of 30 cm with the inlet and outlet ports situated at the bottom and 5 cm from the top of the column, respectively (Fig. 1). The columns were first packed with 0.5 g of glass wool followed by 2 g of gravel and 3 g of sand at the bottom of the column to prevent soil loss and to provide a homogenous distribution for flow-through. Eight different column treatments (Table 2) were conducted

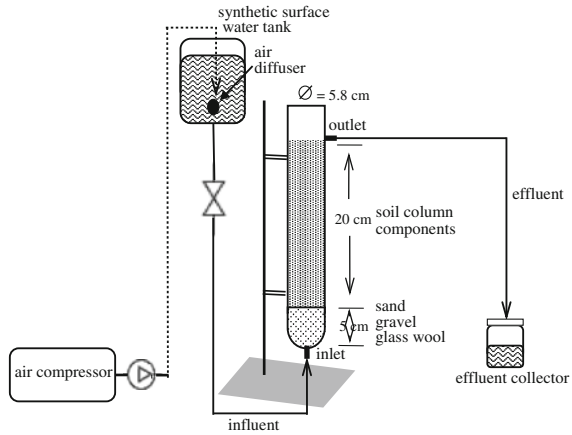

Fig. 1 Soil column schematic (not to scale)

Table 2 Experimental setup

Treatment	Soil (g)	Carbofuran (mg)	Inoculum (10^6 CFU g^{-1} soil)	Autoclaved corncob (g)	Rice straw (g)	Objective
A	830	10.43 ± 0.77	–	–	–	To study the biodegradation of carbofuran by indigenous microorganisms present in soil
B	830	9.61 ± 2.08	–	–	–	Abiotic control: to study the degradation of carbofuran without biological activity
C	830	9.26 ± 1.12	–	–	–	To study the effect of free cells of PCL3 on carbofuran degradation efficiency (bioaugmentation)
D	750	7.21 ± 1.13	Free cells 15 ml	–		To study the effect of immobilized PCL3 on carbofuran degradation efficiency (bioaugmentation)
E	810	9.75 ± 0.26	Immobilized cells 45 g	–	12.50	To study the effect of rice straw as organic amendment on carbofuran degradation efficiency (biostimulation)
F	810	8.69 ± 0.94	–	–	12.50	To study the effect of biostimulation together with bioaugmentation using free cells of PCL3 on the efficiency of carbofuran degradation
G	730	7.21 ± 1.42	Immobilized cells 45 g	–	10.95	To study the effect of biostimulation together with bioaugmentation using the immobilized PCL3 on the efficiency of carbofuran degradation
H	750	7.97 ± 1.55	–	45.00	–	Control of immobilized cell treatment: to study the effect of corncob on carbofuran degradation efficiency

The amounts of soil, immobilized cells, autoclaved corncob and rice straw are pressed in dry basis

The moisture contents of soil, immobilized cells, autoclaved corncob and rice straw are 10.1, 53.2, 50.13 and 1.0%, respectively

The amount of carbofuran added to each column was calculated by multiplied the amount of soil (g) by the concentration of carbofuran in soil (mg g^{-1}) which was analyzed as described in Sect. 2

with tree replicate columns per treatment. The soil was well mixed with carbofuran by hand stirring to achieve a final concentration of approximately 10 mg kg^{-1} dry soil before being packed into the columns. This carbofuran concentration was chosen base upon the residual occurrence in Thailand (0.05–8.42 mg kg^{-1} soil) (Thapinta and Hudak 2000). In the bioaugmentation treatment, PCL3 in free or immobilized cell form were inoculated into the carbofuran contaminated soil at the final cell concentration of 10^6 CFU g^{-1} dry soil. In the biostimulation treatment, rice straw at 1.5% (w/w) was used as the biostimulant because it is an agricultural residue with high organic and nitrogen content and was freely available in the rice field. In addition, it is bulky and light in weight; this could increase soil porosity, thus improving air ventilation and oxygen diffusion and has been previously reported to enhance carbofuran degradation in soil (Sittijunda and Reungsang 2009). The pulverized rice straw (approximately 2 mm particle size) was mixed with the carbofuran contaminated soil before being packed into the columns. The synthetic surface water stored in the plastic bottle was fed into each column through a silicone tube at the inlet port at the bottom of the column with a flow rate of 50 ml day^{-1}. The flow rate was adjusted and controlled using the manual valve. This flow rate was the result from calculation with the assumption that the rice field receives the heavy rainfall of 50 mm day^{-1} and 30–45% of the rainfall is the downward water flow (Tsubo et al. 2007). The columns were set upright to allow vertical flow of the influent in the bottom-up direction. Wetting the soil from the bottom force pore gases upward and allowed more complete soil saturation than did surface infiltration (Boopathy 2004). The columns were operated under saturated condition to mimic the saturation soil during rice cultivation. Carbofuran contaminated soil in the column was saturated with synthetic surface water for 24 h before being continuously fed by synthetic surface water. The

overflow effluent was collected every 2 days for 45 days to determine the concentrations of carbofuran and its metabolites, pH and the number of carbofuran degraders.

At the end of the column operation, each 5-cm depth of the soil was sectioned. Each section was passed through a 1 mm sieve to separate the soil from the rice straw and/or corncob. The soil and amendments from each section were air-dried at room temperature, weighed and analyzed for carbofuran and its metabolite concentrations, and the number of carbofuran degraders as described below.

Equilibrium sorption experiment

Adsorption isotherms of carbofuran onto the soil, corncob and rice straw were determined by conducting batch equilibrium experiment at carbofuran concentrations of 0.1, 1.0, 5.0, 10.0, and 20.0 mg l^{-1}. All solutions were prepared in 0.01 M $CaCl_2$. For each batch experiment, blank samples (carbofuran solutions without solid) were prepared and monitored. The significant carbofuran degradation or sorptive losses on the glassware during the course of the experiment was not detected. All experiments were conducted in triplicates.

For adsorption of carbofuran onto soil, a total of 3 g of air-dried soil was put into 15-ml glass tubes containing 9 ml of carbofuran solution. The tubes were shaken on a horizontal shaker for 48 h (time to reach sorption equilibrium) at 90 cycles per minute at an average room temperature of 29 ± 3°C. After centrifugation at 5000 rpm, the supernatant was filtered through a 0.45 µm nylon membrane filter and analyzed for carbofuran concentration using HPLC. The data was fitted to the Freundlich equation (Eq. 1) (Sposito 1980) to describe the kinetics of carbofuran sorption to the soil.

$$C_s = K_f C_{\mathrm{eq}}^{1/n} \quad (1)$$

where C_s is the carbofuran concentration in the soil (mg kg^{-1} soil), C_{eq} is the equilibrium solution concentration of carbofuran (mg l^{-1}), K_f is an index of adsorption capacity (l kg^{-1}) and $1/n$ is an empirical constant. Corncob and rice straw were air-dried overnight and ground into small pieces using a blender then passed through a 2 mm sieve. The air-dried corncob or rice straw, 0.25 g, were put into 250-ml conical flasks and mixed with 50 ml of carbofuran solution. The flasks were horizontally shaken at a constant speed of 90 cycles per minute for 48 h at an average room temperature of 29 ± 3°C. After 48 h, the solution was passed through Whatmann filter paper No. 1 and the filtrate was extracted using the liquid–liquid partitioning method and quantified for carbofuran concentration by HPLC. The data was fitted to the Freundlich equation (Eq. 1) (Sposito 1980) to describe the kinetics of carbofuran sorption to corncob and rice straw.

The high solid to liquid ratio of 3 g:9 ml of carbofuran solution for soil was used according to the previous experimental results which suggested that the solid to liquid ratio of soil suspension should be taken as large as possible to minimize the experimental error of sorption coefficient unless the equilibrium concentration becomes too low to be measured accurately. If a solid to liquid ratio of less than 0.2 g ml^{-1} is used, the error in the sorption coefficient will usually be unacceptable for sorption coefficients of less than 1 l kg^{-1} (Delle Site 2001). However, the high solid to liquid ratio could not be used for corncob and rice straw because they were swollen after soaking in the carbofuran solution, hence, the solid to liquid ratio of 0.25 g:50 ml was used.

Extraction and analytical methods

Carbofuran and its metabolites were extracted from the soil samples by an Accelerated Solvent Extractor ASE 100 (Dionex, USA) and from the synthetic surface water by liquid–liquid partitioning method. These operations were carried out prior to analysis by HPLC following the conditions described by Plangklang and Reungsang (2008). The recovery percentage of the extraction methods were 97.1 and 98.5 for soil and synthetic surface water, respectively.

Enumeration of carbofuran degraders in effluent and soil and on support material

The number of carbofuran degraders in the column effluent and soil was determined by the drop-plate technique. Briefly, the serial-diluted aliquots of the effluent or soil sample, 20 µl, were plated onto BSM agar coated with 5 mg l^{-1} of carbofuran and incubated at 30°C until colony appeared. BSM agar was prepared by adding 15 g l^{-1} of agar to the modified BSM before autoclaving at 121°C for 15 min.

To determine the number of PCL3 on corncob, 10 g of the immobilized cells (wet weight) was washed with sterile 0.85% NaCl solution three times. The washed immobilized cells were blended to small particles using a blender and then added to 50 ml sterile 0.85% NaCl solution and shaken at 250 rpm for 5 min in order to dislodge cells from the corncob. The number of PCL3 in the liquid phase was then determined by drop plate technique.

Data analysis

Data were analyzed by SPSS program Version 10.0 (SPSS Inc., Chicago, IL). The significance of treatments was set at a *P*-value of less than or equal to 0.05 by the one-way ANOVA test.

Results and discussion

Sorption of carbofuran to soil, corncob, and rice straw

The sorption isotherm of carbofuran onto soil, corncob, and rice straw was investigated. The data was fairly fitted to the Freundlich isotherm with the regression coefficient between 0.82 and 0.99 (Table 3). The K_f value was calculated from the intercept of the linear plot and the value of $1/n$ was computed from the slope of the linear plot. Results indicated that the sorption isotherms were not linear ($1/n \neq 1$). The $1/n$ value was less than 1.0, which indicates less effect of concentration change on the adsorptive capacity of the matrices (Faust and Aly 1987). The K_f value was used to describe the extent of sorption between carbofuran and soil. The K_f value of 0.899, 0.634 and 0.028 l kg^{-1} were obtained for rice straw, soil and corncob, respectively, which suggests that carbofuran might be adsorbed on rice straw more than on soil and corncob, respectively. However, the K_f values obtained were very low which implied that the sorption phenomena might not have a significant effect on the dissipation of carbofuran in a soil column system. Carbofuran might not be adsorbed well in the matrices added into soil column and might be dissolved easily into the water phase.

Table 3 Sorption isotherms of carbofuran to corncob, rice straw, and soil

Material	K_f (l kg^{-1})	$1/n$	r^2
Corncob	0.028 ± 0.004	0.440 ± 0.013	0.99
Rice straw	0.899 ± 0.095	0.855 ± 0.080	0.95
Soil	0.634 ± 0.104	0.759 ± 0.091	0.82

Distribution of carbofuran in column effluent and soil

The carbofuran mass in column effluent during column operation is shown in Fig. 2. The percentage recovery of carbofuran mass in the effluent and carbofuran residues in the soil were examined at the end of column operation (Table 4). The assumed amounts of carbofuran degraded (Table 4) were calculated by subtracting the average carbofuran mass detected in the soil and in the column effluent from the average carbofuran mass that was added to the soil.

A relatively high desorption of carbofuran from soil into the effluent could be found at the early state, i.e., 5–9 days of column operation in all treatments. The hydrophilic nature of carbofuran, i.e., a high water solubility of 351 mg l^{-1} at 25°C (Evert 2004) and low soil adsorption coefficient of 0.63 l kg^{-1} (Table 3) might be responsible for this trend.

In the abiotic control, column B, carbofuran could be detected in the effluent until the end of column operation (45 days) (Fig. 2) with a mass recovery of carbofuran of 52.1% (Table 4). With the presence of only indigenous microorganisms (column A), the percentage recovery of carbofuran in the effluent was 22.5%, which was approximately 2 times lower than in the abiotic control treatment (column B) (Table 4). Carbofuran was undetectable in the effluent from column A after 39 days of column operation (Fig. 2). These results indicate that biological activity of indigenous carbofuran degraders could partially prevent the movement of carbofuran along with the flow through. In this study, the soil samples packed in the columns were taken from a rice field with a history of carbofuran application. It is therefore possible that the microorganisms in the soil have the ability to adapt themselves to use carbofuran as their energy source for growing in the soil during column operation.

Bioaugmentation of PCL3 in free and immobilized cell forms (columns C and D, respectively) significantly reduced the movement of carbofuran in

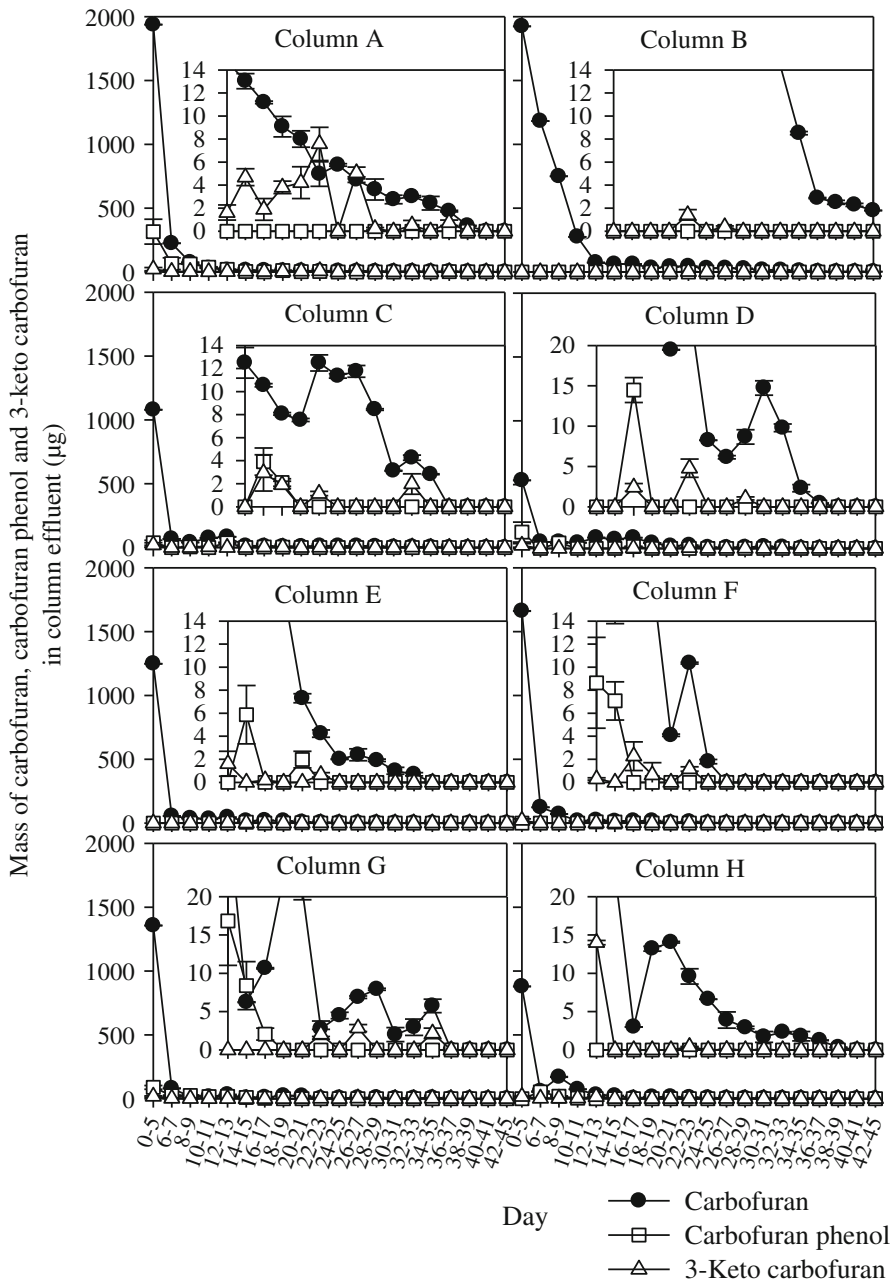

Fig. 2 Mass of carbofuran, carbofuran phenol and 3-keto carbofuran in column effluent (A: soil, B: abiotic control, C: soil + free cells of PCL3, D: soil + immobilized PCL3, E: soil + rice straw, F: soil + free cells of PCL3 and rice straw, G: soil + immobilized PCL3 and rice straw, H: soil + autoclaved corncob). The *y*-axis scales are magnified to facilitate resolution and presented in the small Figures

soil as indicated by the low percentage recovery of carbofuran mass in the effluent, 15.5 and 14.6%, respectively (Table 4). The detectable amounts of carbofuran in columns C and D were observed until 35 and 37 days of column operation (Fig. 2), respectively. Biostimulation using rice straw as organic amendment (column E) provided a similar result to the bioaugmentation treatments (columns C and D). The percentage of carbofuran recovery in the effluent from column E was 15.3% (Table 4) and the carbofuran residues in the effluent were undetectable after 33 days of column operation (Fig. 2). These results suggest an increase in the number of effective carbofuran degraders in the soil by bioaugmentation, i.e., the addition of PCL3 or biostimulation, i.e., stimulating the activity of indigenous carbofuran degraders in the soil by the addition of rice straw. These two techniques could improve the

Table 4 Percentage recovery of carbofuran from day 5 until day 45

Parameter[a]	Column							
	A	B	C	D	E	F	G	H
% Mass recovery of carbofuran in effluent[b]	22.5b ± 1.33	52.1c ± 2.15	15.5a ± 1.55	14.6a ± 2.3	15.3a ± 1.16	22.6b ± 1.02	22.1b ± 1.76	16.2a ± 2.54
% Mass of carbofuran residues in soil at day 45	nd	18.9 ± 7.18	nd	nd	nd	nd	nd	nd
% Mass of carbofuran assumably degraded	77.5	29.0	84.5	85.4	84.7	77.4	77.9	83.8

nd not detectable, *A* soil, *B* abiotic control, *C* soil + free cells of PCL3, *D* soil + immobilized PCL3, *E* soil + rice straw, *F* soil + free cells of PCL3 + rice straw, *G* soil + immobilized PCL3 + rice straw, *H* soil + autoclaved corncob

[a] Mass of carbofuran residues in corncob and rice straw was not detectable

[b] Comparison between treatments in rows are significantly different ($P < 0.05$) if marked with different *lowercase letters*

carbofuran degradation efficiency and prevent the movement of carbofuran along with the effluent.

The addition of PCL3 in free and immobilized cell forms together with rice straw (columns F and G, respectively) decreased the carbofuran degradation efficiency in the soil columns. The percentage recoveries of carbofuran mass in the effluent of 22.6 and 22.1% were obtained from columns F and G, respectively (Table 4), which were higher than that observed in the treatments with bioaugmentation or biostimulation alone (columns C, D, and E). This might be because PCL3 and/or indigenous carbofuran degraders might prefer to use by-products such as carbon, nitrogen, and phosphorus from the rice straw degradation (Fores et al. 1988), rather than carbofuran. These phenomena could result in a decrease in carbofuran degradation efficiency and increase the movement of carbofuran along with the effluent.

Upon completion of the column operation, carbofuran residues in the soil could not be detected in all treatments with biological activity, whereas, in the abiotic control, the percentage recovery of carbofuran mass in the soil was 18.9% (Table 4). The results confirm that biological treatments are an effective tool to enhance carbofuran degradation in soil and prevent the movement of carbofuran in soil along with the effluent. However, the abiotic control demonstrated a relatively high percentage of carbofuran degradation of 29.0%. This might have been as a result of abiotic hydrolysis under flooded conditions and oxidation processes due to the continuous aeration of water before feeding into the column. Although the abiotic degradation processes are not as important as microbial degradation, they contributed to dissipation processes which could be found in the abiotic control as reported in a previous study (Lalah et al. 1996; Plangklang and Reungsang 2009).

Carbofuran metabolites production during column operation

Carbofuran phenol and 3-keto carbofuran were observed as metabolites in effluent from the columns (Fig. 2) but no metabolite was detected in the soil section (data not shown). Carbofuran phenol was found in the effluent from columns with the presence of biological activity (columns A, C, D, E, F, G and H), but could not be found in effluent from the abiotic control (column B) (Fig. 2). The results suggest that carbofuran phenol is a metabolite from the biodegradation process. The concentration of carbofuran phenol tended to decrease over time and could not be detected at the end of column operation in all treatments. This implies that carbofuran phenol could

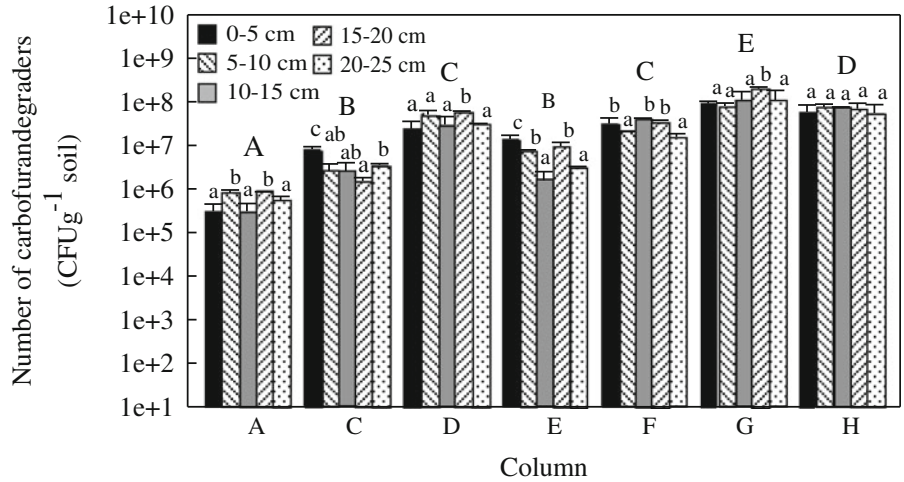

Fig. 3 The number of carbofuran degraders in each soil section (A: soil, C: soil + free cells of PCL3, D: soil + immobilized PCL3, E: soil + rice straw, F: soil + free cells of PCL3 and rice straw, G: soil + immobilized PCL3 and rice straw, H: soil + autoclaved corncob). Bar groups with different capital letters indicate a significant difference ($P < 0.05$) between numbers of carbofuran degraders in column treatment. Bars with different lowercase letters indicate a significant difference ($P < 0.05$) among the numbers of carbofuran degraders in five soil sections

be metabolized by PCL3 and/or indigenous microorganisms, which agreed with the previously published report (Park et al. 2006; Yan et al. 2007; Peng et al. 2008).

The 3-keto carbofuran was observed in the effluent from all column treatments (Fig. 2). It was detected as a key by-product of carbofuran degradation prevalence in water (Evert 2004) and in mostly flooded conditions (Kale et al. 2001). Since PCL3 degrades carbofuran to carbofuran phenol but not 3-keto carbofuran (Plangklang and Reungsang 2008, 2009), we suggested that 3-keto carbofuran was the metabolite from indigenous microorganism activity and/or abiotic degradation processes. Indigenous microorganisms might oxidize carbofuran into 3-keto carbofuran via oxidation reactions.

A smaller amount of carbofuran phenol and 3-keto carbofuran was observed in the effluent from the bioaugmented columns C and D and the biostimulated column E in comparison to column A, which contained only indigenous microorganisms (Fig. 2). This result suggests that applying the bioremediation technique, i.e., bioaugmentation (the addition of immobilized and free cells of PCL3) and biostimulation (rice straw supplementation) could improve the degradation of carbofuran metabolites produced during column operation.

CFU variation during column operation

Number of carbofuran degraders in soil

The initial number of carbofuran degraders in the soil without inoculation (column A) and soil augmented with the immobilized PCL3 (columns D and G) was $2.07 \times 10^5 \pm 0.57 \times 10^3$, $4.78 \times 10^5 \pm 1.41 \times 10^3$ and $7.59 \times 10^5 \pm 0.71 \times 10^3$ CFU g^{-1} soil, respectively. The initial number of carbofuran degraders in the soil inoculated with free cells of PCL3 (columns C and F) was $2.76 \times 10^6 \pm 5.67 \times 10^3$ and $3.65 \times 10^6 \pm 7.07 \times 10^3$ CFU g^{-1} soil.

The number of carbofuran degraders in soil sections at the end of column operation is shown in Fig. 3. In column A (without inoculation), significant greater numbers of carbofuran degraders were observed at 5–10 and 15–20 cm depths in comparison to the other soil sections. The greatest number of carbofuran degraders was found at 0–5 cm depth while the least was found at 15–20 cm depth of column C (inoculated with free cells). Column E (added with rice straw) had the greatest number of carbofuran degraders at 0–5 cm depth followed by 5–10 and 15–20 cm depths and 10–15 and 20–25 cm depths, respectively. Column F (added with free cells and rice straw) had the significant greater number of carbofuran degraders at

0–5 and 10–20 cm depths when compared to the other soil sections.

For the column added with corncob (column H), it was found that the number of carbofuran degraders was not markedly different among soil sections. For the columns added with the immobilized cells (columns D and G), only at the depth of 15–20 cm showed significantly greater number of carbofuran degraders. Our results implied that the addition corncob might increase the porosity hence facilitate the distribution of bacteria in soil.

The distribution of carbofuran degraders in soil varied among column treatments. In column A (with only indigenous microorganisms), the number of carbofuran degraders was relatively unchanged when compared to the initial value ($P = 0.083$). The addition of rice straw (column E) or corncob (column H) resulted in a significant increase in the number of carbofuran degraders ($P = 0.027$ and $P < 0.001$, respectively) (Fig. 3) to approximately 10^6–10^7 and 10^7–10^8 CFU g^{-1} soil, respectively, at the end of column operation. The results indicate that indigenous microorganisms could use rice straw or corncob as the energy sources for their growth, resulting in an increase in their population, which could improve carbofuran degradation in soil.

The addition of PCL3 (free cells) into soil resulted in a higher initial number of carbofuran degraders when compared to the other treatments ($P < 0.001$). This may be responsible for the enhanced degradation of carbofuran in soil in comparison to the treatment with no bioaugmentation. The number of carbofuran degraders in the soil bioaugmented with free cells of PCL3 (column C) at the end of column operation was not significant different from the initial number ($P = 0.523$) (Fig. 3). The results indicate that PCL3 in free cells form have the capability to survive but can not grow well in soil for long term column operation.

In the column inoculated with immobilized PCL3 (column D), the number of carbofuran degraders in the soil increased to 10^7 CFU g^{-1} soil at the end of column operation (Fig. 3). An increase in the number of carbofuran degraders in the soil might be the result of the stimulation effect of corncob on the indigenous microorganisms and/or PCL3. The number of carbofuran degraders in the soil augmented with immobilized cells of PCL3 (column D) was greater than that with free cells (column C). This indicates that the immobilization technique could improve the growth and survival of PCL3 in the soil.

The addition of PCL3 together with rice straw (column F and G) gave a high number of carbofuran degraders in the soil of approximately 10^8 CFU g^{-1} soil at the end of column operation. However, the carbofuran degradation efficiency had not improved in comparison to the non-treated soil (column A) (Table 4). This confirmed that the rice straw could stimulate the growth of PCL3 and indigenous microorganisms but do not stimulate their carbofuran degradation activity. In addition, the increasing adsorption of carbofuran on rice straw compared to the other matrices might reduce the availability of carbofuran to the carbofuran degraders, hence the chance for biodegradation of carbofuran by the carbofuran degrading bacteria was reduced.

The number of immobilized PCL3 on corncob (taken from columns D and G) at the end of column operation were $5.04 \times 10^7 \pm 7.73 \times 10^6$ and $4.53 \times 10^7 \pm 5.13 \times 10^6$ CFU g^{-1} dry material respectively, which had not decreased when compared to the initial value ($P = 0.431$ and 0.745, respectively). This implied that corncob could act as a shelter, protecting PCL3 from diverse environments such as, substrate concentrations, pH, temperature and shear forces during column operation.

Numbers of carbofuran degraders in the column effluent

The number of carbofuran degraders in the effluent collected from the columns is shown in Fig. 4. The results agreed with the number of carbofuran degraders in the soil. The number of carbofuran degraders in the effluent from column A (with only indigenous microorganisms) was stable at approximately 10^5 CFU ml^{-1}. The higher number of carbofuran degraders in soil bioaugmented with free cells of PCL3 (column C and F) agreed with the greater number of carbofuran degraders in the effluent (10^6–10^8 CFU ml^{-1}), when compared to the other treatments.

The number of carbofuran degraders in the effluent from column C (inoculated with free cells of PCL3) decreased over 15 days of column operation, which implies that PCL3 in free cells form could not survive during column operation. The results agreed with the concentration of carbofuran in the effluent, which

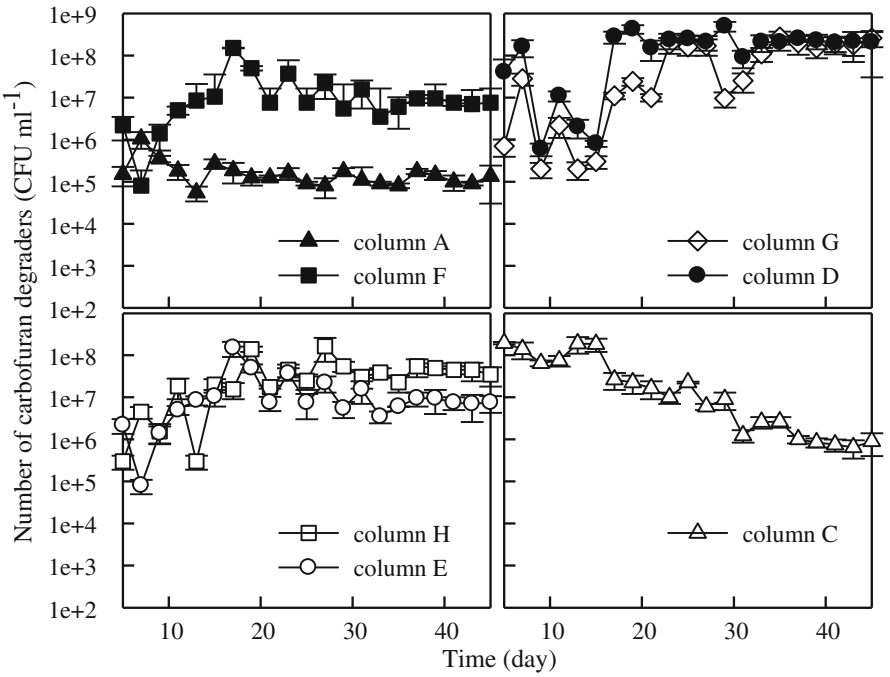

Fig. 4 The number of carbofuran degraders in column effluent (A: soil, C: soil + free cells of PCL3, D: soil + immobilized PCL3, E: soil + rice straw, F: soil + free cells of PCL3 and rice straw, G: soil + immobilized PCL3 and rice straw, H: soil + autoclaved corncob)

decreased rapidly from 1,079 mg l^{-1} at days 0–5 to 12.49 mg l^{-1} at days 14–15, decreasing very slowly thereafter (Fig. 2). These results indicate that the survival of augmented PCL3 is needed in order to effectively remove carbofuran from soil and prevent the movement of carbofuran from soil to groundwater.

The number of carbofuran degraders in the effluent from the column with the rice straw (column E) and corncob added (column H), slightly increased at the early stage of column operation and was stable at above 10^6 CFU ml^{-1}. These results indicate that rice straw or corncob might be used by indigenous microorganisms for their growth, resulting in an increase in the number of carbofuran degraders in the soil and effluent, hence enhancing carbofuran removal efficiency. However, the number of carbofuran degraders in the column with both PCL3 and rice straw added (columns F and G), remained above 10^7 CFU ml^{-1} throughout the experiment.

Number of carbofuran degraders in the column effluent of the treatments augmented with immobilized cells (columns D and G) tended to increase which was corresponded to the number of carbofuran degraders in soil. A similar result was noted by Plangklang and Reungsang (2009) who observed that cell leakage from corncob could increase the number of PCL3 in basal salt medium and soil. Kumar and Das (2001) also reported that *Enterobactor cloacae* II-BT 08 leaked from the supporting material to the culture media because of lack of space.

pH variation in column effluent

The pH of the effluent from the columns was monitored during column operation and the results are shown in Fig. 5. The pH of the effluent from the abiotic (column B) was observed to be stable at approximately 6.9 throughout the experiment, which might be due to the absence of microbial activity. The pH of the effluent with biological activity (columns A, C, D, E, F, G, and H) was more varied than the abiotic control (column B). In the bioaugmentation treatments (columns C, D, F, and G) the pH of the effluent tended to increase from day 0 to 19 of column operation and stable thereafter. With the presence of only indigenous microorganisms (column A, E and H), the pH value sharply increased until 9, 15 and 11 days, respectively and then decreased overtime. The pH of the effluent from column A and E tends to be stable after 19 days while the pH of the effluent from column H gradually decreased after 25 days till the end of column operation. These results suggested that the pH of the column effluent is considered to be an important criterion for determining the activity of different

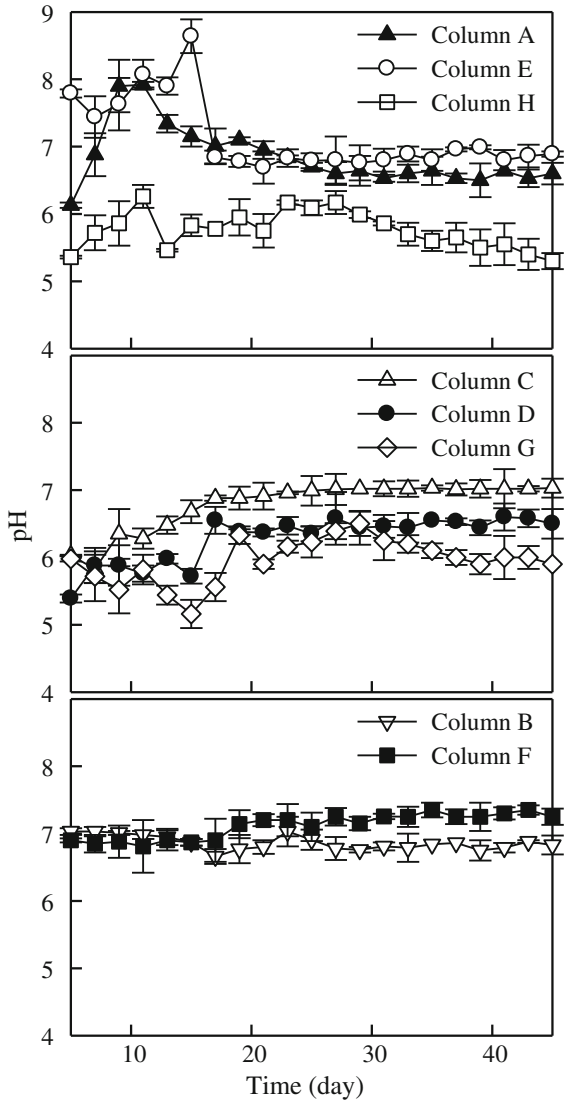

Fig. 5 The pH variation in columns effluent (A: soil, B: abiotic control, C: soil + free cells of PCL3, D: soil + immobilized cells of PCL3, E: soil + rice straw, F: soil + free cells of PCL3 and rice straw, G: soil + immobilized cells of PCL3 and rice straw, and H: soil + autoclaved corncob)

microorganisms present in the soil column. The variation in effluent pH could result from the by-products from biodegradation activity of different microorganisms in soil. The increase in effluent pH might be caused by the alkaline nature of by-products, i.e., 3-keto carbofuran and carbofuran phenol obtained during the metabolism of carbofuran and organic matter by microorganisms present in the soil (Prasanna et al. 2008). A decrease in effluent pH, however, might be due to the formation of CO_2 from the mineralization of carbofuran and organic matter in the soil (Venkata-Mohan et al. 2006).

Conclusions

The soil columns were used to simulate the movement of carbofuran in rice field soil under saturated conditions. The mass recovery percentages of carbofuran in the effluent from bioaugmentation or biostimulation column treatment were approximately 15.4%, which were less than that from abiotic control or natural attenuation (treatment with only indigenous microorganisms) (52.1 and 22.5%, respectively). Bioaugmentation together with biostimulation treatments gave a low efficiency of carbofuran removal in which the mass recovery percentage of carbofuran in the effluent was in the range of 22.1–22.6%. The results suggest that bioaugmentation and biostimulation techniques might be applicable to enhance carbofuran degradation in rice field soil in situ in order to prevent the movement of carbofuran to groundwater, especially in rice field areas where carbofuran has been applied.

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