CHAPTER III

RESEARCH METHODOLOGY

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals and reagents

Carbofuran (98% purity) and carbofuran phenol (99% purity) were purchased from Sigma-Aldrich, USA, and 3-keto carbofuran (98.5% purity) was purchased from Ehrenstorfer Quality, Germany. Methanol (HPLC grade) was purchased from BDH, England. Iodonitrotetrazolium violet (INT) and iodonitrotetrazolium formazan (INTF) was purchased from Sigma-Aldrich, USA.

3.1.2 Soil samples

Soil samples were collected from the rice field of Ban Non-muang, A. Muang, Khon Kaen, Thailand, at a depth of 0-15 cm, air dried and passed through a 2-mm sieve (pore size). Soil samples were kept in plastic bag and stored at 4 °C prior the usage. Analysis of carbofuran residue, total nitrogen, organic carbon, pH and soil texture were conducted by HPLC, Kjeldahl method (Mckenzie et al., 1954), Walkey-Black method (Nelson et al., 1996), pH meter, and Hydrometer method (Mocek et al., 1997), respectively.

3.1.3 Carbofuran aged soil sample

Aged soil was prepared by spiking soil with carbofuran to achieve a final concentration of 50 mg/kg soil and incubated for 4 months in the dark condition at the room temperature then carbofuran residue in soil was analyzed by HPLC.

3.1.4 Organic and inorganic amendment

Organic amendments: rice straw (RS), cassava pulp (CS), corn cob (CC), cattle manure (CM), compost (CP), glucose (GL), molass (ML). Each of these amendments except CP, CM, GL, and ML were blended into small pieces, then passed through a 2-mm sieve (pore size) and analyzed for carbon and nitrogen contents using Wakley Black method (Wakley Black, 1934) and Kjeldahl method (AOAC, 2000), respectively.

Inorganic solution contained (in g/L): 1.060 $CaSO_4$, 1.412 K_2HPO_4, and 7.140 NH_4NO_3 (Moorman et al., 2001). The inorganic solution was sterilized by filtrating through 0.2 μm cellulose acetate membrane filter and kept at 4 °C prior the usage.

3.1.5 Culture media

Basal Salt Medium (BSM) contained (in g/L): 5.57 Na_2HPO, 2.44 KP, 2.00 NH_4Cl, 0.20 $MgCl_2.6H_2O$, 0.0004 $MnCl_2.4O$, 0.001 $FeCl_3.6O$ and 0.001 $CaCl_2$. In order to enumerate the carbofuran degraders possessing the ability to use carbofuran as its sole C-source or sole N-source or a sole C and N-sources, three types of BSM were used. For C-limited BSM (C-BSM), 50 mg/L of carbofuran was used as a sole C-source instead of glucose. For N-limited BSM (N-BSM), 50 mg/L of carbofuran was used as a sole N-source instead of NH_4Cl and 10 g/L of glucose was used as C-source. For C and N-limited BSM (C,N-BSM), 50 mg/L of carbofuran was used as sole C and N sources. pH of the media was adjusted to 7 before autoclaved at 121 °C for 15 min. Carbofuran solution in methanol was added after the media was sterilized. For the BSM agar, 1.5% of bactoagarose was added to BSM before sterilization and coated with 50 mg/l of carbofuran solution (in H_2O) after sterilization.

3.2 Methods

3.2.1 Biostimulation experiment

3.2.1.1 Biostimulation by organic amendment

Forty grams dry weight of soil was mixed with carbofuran at an average concentration of 50 mg/kg dry soil in a 280 mL glass bottle. Each of organic amendments was added at 0.5%, 1%, 1.5% and 5% (w/w) (Moorman et al., 2001) to soil samples. The initial moisture content of soil microcosm was adjusted to 15-18%. The bottle was capped with a plastid lid and incubated at room temperature. Controls were autoclaved soil with the amendment, soil without amendment, soil with autoclaved amendments, and soil without carbofuran and amendment. All treatments were conducted in triplicate. Every 7 days, a glass bottle was opened for aeration and moisture adjustment. Soil samples were collected at 0, 7, 14, 21, 35, 70, 100 days and analyzed for concentrations of carbofuran and its metabolites i.e., carbofuran phenol and 3-ketocarbofuran using HPLC. In addition, number of carbofuran degraders, soil

respiration and microbial activity in soils were determined at each sampling date by spread plate technique, an accumulation of CO_2 by titration method and intracellular dehydrogenase enzyme by bioassay method, respectively.

3.2.1.2 Biostimulation by inorganic amendment

Forty grams dry weight of soil was mixed with carbofuran at an average concentration of 50 mg/kg dry soil in a 280 mL glass bottle. The inorganic solution was then added at two different levels of volume i.e., 1 mL and 2 mL (Moorman et al., 2001) to soil samples. The initial soil moisture content was adjusted to 15-18%. The bottle was capped with a plastid lid and incubated at room temperature. Controls were autoclaved soil with the amendment, soil without amendment, and soil without carbofuran and amendment. All treatments were conducted in triplicate. Every 7 days, a glass bottle was opened for aeration and moisture adjustment. Soil samples were collected at 0, 7, 14, 21, 35, 70, 100 days and analyzed for concentrations of carbofuran and its metabolites i.e., carbofuran phenol and 3-ketocarbofuran using HPLC. In addition, number of carbofuran degraders, soil respiration and microbial activity in soils were determined by spread plate technique, an accumulation of CO_2 by titration method and intracellular dehydrogenase enzyme by bioassay method, respectively.

3.2.1.3 Biostimulation by organic and inorganic amendments in carbofuan aged soil

Forty grams dry weight of carbofuran aged soil was added into a 280 mL glass bottle. RS and inorganic solution at optimal load, previously determined, of 1.5% and 1 mL, respectively, were added to soil sample. The initial soil moisture content was adjusted to 15-18%. The bottle was capped with a plastid lid and incubated at room temperature. Controls were aged soil without any amendments. All treatments were conducted in triplicate. Every 7 days, a glass bottle was opened for aeration and moisture adjustment. Soil samples were collected at 0, 7, 14, 21, 35, 70, 100 days and analyzed for concentrations of carbofuran and its metabolites i.e., carbofuran phenol and 3-ketocarbofuran using HPLC. In addition, number of carbofuran degraders, soil respiration and microbial activity in soil were determined by spread plate technique, an accumulation of CO_2 by titration method and intracellular dehydrogenase enzyme by bioassay method, respectively.

3.2.2 Soil respiration

Soil respiration was determined by quantifying the total CO_2 generation from soil using titration technique. Forty grams of dry soil were weighted into a 280 mL glass bottle, then mixed with each of organic amendment i.e., RS, CS, CC, CM, CP, GL, ML at the varying concentrations of 0.5%, 1%, 1.5% and 5% (w/w). For total CO_2 generation in soil amended with inorganic solution, the experiment was conducted in a similar manner but the amendment was changed from organic to be inorganic solution with a varying volume of 1 and 2 mL. Carbofuran at a concentration of 50 mg/kg soil was spiked into soil. Controls were soil without carbofuran and amendments, soil mixed with 50 mg/kg soil carbofuran without any amendments, soil mixed with each of organic amendments without carbofuran, and soil mixed with two levels of inorganic solution without carbofuran. All of treatments were conducted in triplicate. A 20 mL plastic box contained 15 ml of 1N NaOH solution was inserted into the soil sample bottles and hung above soil surface. Then the bottle was capped with plastic lid. All treatments were conducted in triplicate. At days 0, 7, 14, 21, 35, 70, 100 NaOH in plastic box was emptied and then replaced with 15 ml of freshly prepared 1N NaOH. A total CO_2 generation in soil was quantified by titrating the excess NaOH by 1N HCl and phenolphthalein was used as the indicator. The end point was observed when the color changed from pink to white. The amount of total CO_2 (mg) was calculated by a following equation (Crossno et al., 1996).

$$CO_2 = (B\text{-}V)\,NE \qquad (1)$$

Where B = amount of HCl that used to titrate NaOH of the control,
V= amount of HCl that used to titrate NaOH of each sample, N= concentration of HCl (1 N), E = equivalent weight of CO_2

3.2.3 Kinetic characterization of carbofuran degrader

Kinetic of carbofuran degraders responsible for a degradation of carbofuran in successful biostimulated soil microcosms were characterized. In order to obtain the carbofuran degraders, the enrichment technique was conducted by adding 5 grams of soil sample from the microcosm that showed the shortest half-life of carbofuran from the biostimulation experiment into 100 ml C-BSM, N-BSM, C,N-BSM contained 5 mg/L carbofuran then incubated at room temperature and shaken at 150 rpm. Every 7 days, the media was transfered to a fresh C-BSM, N-BSM, C,N-BSM media contained 5 mg/L of carbofuran, incubated at room temperature and shaken at 150 rpm. Microorganisms growing in these media were considered as carbofuran degrader capable of using carbofuran as C, N, or C,N sources, respectively. This step was repeated until soil free enrichment was obtained. This soil free enrichment was used as inoculums in the kinetic characterization experiment. Ten percent (v/v) of soil free enrichment was added into 100 ml C-BSM, N-BSM, and C,N-BSM contained 5, 50, 100 and 150 mg/L carbofuran, incubated at room temperature and shaken at 150 rpm. All of treatments were conducted in triplicate. Samples were collected at day 0, 2, 4, 6, 8, 10, 14, 18, 24, and 30. Carbofuran and its metabolites in BSM i.e., 3-ketocarbofuran and carbofuran phenol were extracted by liquid-liquid partitioning method. Concentration of carbofuran and its metabolites was determined by HPLC. The dissolved oxygen (DO) in the bottle was measured by digital oxygen meter model 5510 to ensure that the experiment was under aerobic condition. Number of carbofuran degraders was measured by plate count method on BSM agar coated with 5, 50, 100, 150 mg/L carbofuran, respectively. The cell growth yield of the carbofuran degraders was determined by measuring cell dry weight (Montie et al., 2006).

Growth of carbofuran degraders in each BSM with each initial carbofuran concentration was described by fitting to the first order growth kinetic model as follow (Bernard and Ferda, 1991):

$$dX/dt = \mu X \qquad (2)$$

Where X = number of carbofuran degraders (CFU/mL)

t = times (days)

μ = specific growth rate (1/day)

A specific growth rate, μ of mixed carbofuran degraders in the BSM was obtained from plotting log X_t versus t using a linear regression method (Figure 3.1). μ is equal to slope multiplied by 2.303.

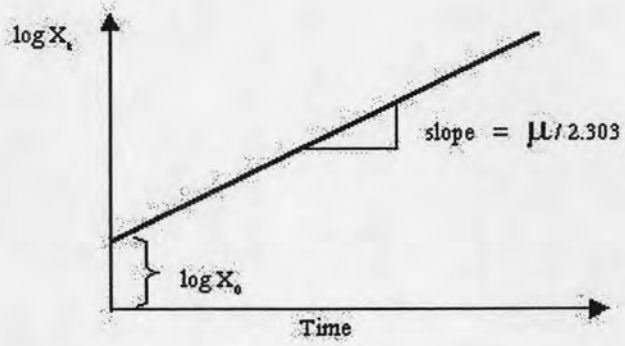

Figure 3.1 Growth of microorganisms fitting to the first order kinetic model

Kinetic analysis for bacteria growth was evaluated by fitting the data to the bacterial growth model using the non-linear least squares technique from MATLAB program (Harmonic software Inc, 2003). Two types of bacteria growth model for carbofuran degraders were used in this study. First is a non-substrate inhibition model described as the addition of excess nutrients when μ equal to μ_{max} did not affect microbial growth. Monod's equation (Equation 3) was used to describe these phenomena as described in Figure 3.2. Second is a substrate inhibition model described as the addition of excess nutrient when μ equal to μ_{max} reduced the growth of microorganisms described by Haldane equation (Equation 4) as described in Figure 3.2.

$$\mu = \frac{\mu_{max} \times S}{K_S + S} \qquad (3)$$

$$\mu = \frac{\mu_{max} \times S}{K_s + S + \left(S^2 + K_i\right)} \qquad (4)$$

Where: μ is the specific growth rate (1/day), K_s is the half-saturation constant (mg/L), μ_{max} is the maximum specific growth rate (1/day), K_i is substrate inhibition coefficient (mg/L), and S is carbofuran concentration (mg/L).

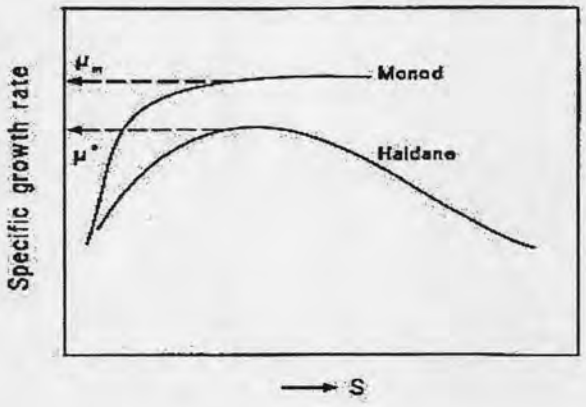

Figure 3.2 Growth models of microorganisms (Metcalf and Eddy, 2003)

K_i and K_s values obtained from fitting the data to Haldane equation were further used to calculate the lowest carbofuran concentration which can cause the death of carbofuran degraders (S*), in which $S^* = (K_s \times K_i)^{1/2}$ (Metcalf and Eddy, 2003)

3.3 Analytical methods

3.3.1 Extraction of carbofuran and its metabolites from soil samples

Carbofuran and its metabolites were extracted from soil samples by using an ASE 100 Accelerated Solvent Extractor (Dionex, Austria) equipped with 11-mL stainless-steel extraction cell. The extraction cell was prepared as follows: the bottom of the extraction cell was covered with a cellulose acetate membrane (16.2 mm I.D., Dionex, Austria) as a filter agent to prevent a frit blockage and fine soil breakthrough into the collection bottle. Afterwards, 13 g of dried soil sample was transferred into the extraction cell and finally the top of the extraction cell was covered with a cellulose acetate membrane as filter agent before closed. The samples were extracted under the conditions which are 100 °C extraction temperature, 5 min static extraction time, 60% methanol (AR grade) with the volume of 11 mL/cycle as the extraction solvent and two extraction cycles. The ASE parameters were used according to the default settings, i.e., the maximum extraction pressure was set not to exceed 1,500 psi, the flush volume was 60% of the extraction cell volume, and the N_2-purge time was set to 1 min. After static extraction, the raw extracts were collected in a special 200-mL glass bottle, adjusted by 60% methanol (HPLC grade) to the volume of 25 mL in volumetric flask and passed through 0.45 μm nylon membrane syringe filter prior analyzing by HPLC (Plangklang, 2004). Percent recovery of this method was 98%.

3.3.2 Extraction of carbofuran residues and its metabolites from culture media by the liquid-liquid partitioning method

Two-mL of each culture media was added with 2 mL of methanol and then sonicated for 10 min for two times. Carbofuran and its metabolites from the sonicated culture media was extracted three times with 4, 2 and 2 mL of dichloromethane in a separation funnel. The organic fraction of the samples was evaporated to dryness in the fume hood and then redissolved in 4 mL of 60% methanol and passed through a 0.45 μm nylon membrane syringe filter before analyzing by HPLC (Plangklang, 2004). Percent recovery of this method was 98%.

3.3.3 Analysis of carbofuran and its metabolite concentrations in soil samples by HPLC

Concentrations of carbofuran, carbofuran phenol and 3-keto carbofuran in extracted solvent was analyzed by Shimadzu 10-A HPLC equipped with 4.6x150 mm-Lunar 0.5 μm C-18 columns (Phenomenex, USA), a UV detector operating at 220 nm and a 20 μL injector loop. Column temperature was ambient temperature and the mobile phase was methanol-water (60:40) at a flow rate of 1 mL/min. External standard linear calibration curves of carbofuran, carbofuran phenol and 3-keto carbofuran were used to quantify their concentrations in the aqueous phase. The observed concentration was characterized by its peak areas (Plangklang, 2004). The detection limits of the HPLC for analyzing carbofuran, carbofuran phenol and 3-keto carbofuran were 0.07, 0.01 and 0.01 mg/L, respectively.

3.3.4 Microbial activity

Dehydrogenase activity (DHA) was commonly used as an indicator of biological activity in soil (Burns, 1978). Dehydrogenase enzyme plays a significant role in the biological oxidation of soil organic matter by transferring protons and electrons from substrates to acceptors. These processes were being a part of respiration pathways of soil microorganisms. (Glinski and Stepniewski, 1985). The DHA was determined by reducing iodonitrotetrazolium violet (INT) to iodonitrotetrazolium formazan (INTF). Two and a half grams of soil dry weight was collected from each experiment and added into a 20-ml vial, mixed with 1.25 ml of DI water and 0.5 ml of INT solution (5 g/L). The vials were sealed with septum and

incubated for 22 h at 27° C in dark. After the 22 h incubation period, the metabolic product i.e., INTF was extracted by adding 12.5 ml methanol, mixed and filtrated the extract through a Whatman paper No 42 and measured the absorbance of the metabolic product at λ_{max}= 428 nm using a spectrophotometer. The INTF standard was prepared in methanol. DHA was expressed as mg INTF/kg dry soil/h (Dungan et al., 2006).

3.4 Diagram of this study