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Biodegradation of carbamate pesticides by natural river biofilms in different seasons and their effects on biofilm community structure

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ABSTRACT

This study investigated the ability of natural river biofilms from different seasons to degrade the carbamate pesticides methomyl, carbaryl and carbofuran in single and multiple pesticide systems, and the effects of these pesticides on algal and bacterial communities within biofilms. Spring biofilms had the lowest biomass of algae and bacteria but showed the highest methomyl degradation (>99%) and dissipation rates, suggesting that they might contain microorganisms with high methomyl degradation abilities. Degradation of carbofuran (54.1–59.5%) by biofilms in four seasons was similar, but low degradation of carbaryl (0–27.5%) was observed. The coexistence of other pesticides was found to cause certain effects on pesticide degradation and primarily resulted in lower diversity of diatoms and bacteria than when using a single pesticide. The tolerant diatoms and bacteria potentially having the ability to degrade test pesticides were identified. River biofilms could be suitable biomaterials or used to isolate degraders for bioremediating pesticide-contaminated water.

1. Introduction

The intensification of agriculture has resulted in an increased loading of soil, surface and ground water with pesticides, greatly impacting ecosystems by changing the bacterial diversity in soil and threatening aquatic organisms (Khan and Law, 2005; Zhang et al., 2010). Carbamate pesticides have seen large-scale use in the past 50 years as insecticides, fungicides, herbicides or nematocides. The toxicity of carbamates is caused by the inhibition of acetylcholinesterase. Methomyl, carbaryl and carbofuran were commonly used carbamates, and they have been detected in the aquatic environment (Wilsont and Foos, 2006). The half-lives of 1 mg L^{-1} Methomyl in water were 20.8, 27.9 and 15.8 days at pH 4.00, 7.00 and 9.20, respectively (Aktar et al., 2010). Carbaryl was degraded rapidly, with half lives of 2–3 weeks at 10 °C and of 1–5 days at 25 °C (Starner et al., 1999). Carbofuran was degraded in water by hydrolysis, with a half-life of 5.00, 6.60 and 8.60 days in unfiltered and filtered river water exposed to sunlight and filtered river water kept in the dark, respectively (Farahani et al., 2012). It has been found that microbial degradation is a major degradation route of these three pesticides in soils (Trabue et al., 2001; Naqvi et al., 2011; Van Scoy et al., 2013). In the aquatic environment, methomyl, carbofuran and carbaryl were rapidly biodegraded by bacterial strains isolated from activated sludge, drinking water and the standing water of carbofuran-retreated *Azolla* plot (Singh et al., 1993; Xu et al., 2009; El-Fakharany et al., 2011).

River biofilms consist of mainly bacteria and algae that are embedded in an organic polymer matrix, which traps nutrients from the water column and provides protection for microorganisms within the biofilms (Das et al., 2012). The biofilms are found in nearly submerged surfaces and present a highly reactive surface area for the sorption and metabolism of contaminants (Lawrence et al., 2001; Araya et al., 2003). It has been shown that river biofilms are important in biogeochemical cycling and the biodegradation of pollutants within riverine systems and contribute significantly to the self-purification of the river (Lawrence et al., 2001; Chenier et al., 2003). Biofilm-based reactors have been used to degrade herbicides and pesticides such as 2,4-dichlorophenoxyacetic acid, 2-(2-methyl-4-chlorophenoxy) propionic acid, diclofop, carbendazim, diazinon, chloropropham (Oh and Tuovinen, 1994; Wolfaardt et al., 1995; Pattanasupong et al., 2004; Tien et al., 2011; Verhagen et al., 2011). However, degradation of methomyl, carbofuran and carbaryl by river biofilms in the aquatic environment has not been investigated.

Environmental stresses such as nutrients, metals and organic pollutants have been shown to have a significant impact on the

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diversity of the microbial community and on the function of river biofilms (Lee et al., 1995; Araya et al., 2003; Lawrence et al., 2004). Accordingly, river biofilms have been suggested as good indicators for heavy metal pollution (Fuchs et al., 1996) and water quality changes (Tien et al., 2009). Araya et al. (2003) showed that the bacterial community in river biofilms was disturbed by the addition of aniline-derived compounds, and one dominant bacterium appeared during the degradation of these compounds. Therefore, understanding the variation in the microbial community structure of river biofilms during the biodegradation processes may be helpful for their future use in bioremediation processes.

The co-occurrence of pesticides is normally found in aquatic environments. Thus, the objectives of this study were to investigate the ability of river biofilms in different seasons to remove methomyl, carbaryl and carbofuran in single-, two- and three-pesticide systems and to determine the effects of these pesticides on the biomass and community structures of the algae and bacteria within river biofilms. The DGGE technique was used to determine the molecular profile of the bacteria in the river biofilms and to identify the bacterial species that potentially contributed to the degradation of carbamate pesticides.

2. Materials and methods

2.1. Colonization of natural river biofilms

Unglazed ceramic discs (diameter: 4.5 cm, thickness: 0.7 cm) were used to simulate natural stones (Araya et al., 2003) for biofilm colonization to obtain a fixed surface area for the following tests. One hundred sixty unglazed ceramic discs were cleaned using phosphate free detergent and hung on two paint-covered steel racks with plastic strings. Each rack was placed vertically in the river at a 1 m depth parallel to the current flow for 21 days in each season (April, June and October 2008 and January 2009) to colonize the biofilms. The racks were placed at the Se-Kung Bridge (23°6′26″N, 120°12′18″E), which is downstream of the Tseng-Wen River and has intense agricultural activities with frequently pesticide spraying in the watershed area. It has been found that river biofilms grown in this site showed high ability to degrade the organophosphorus pesticide, diazinon (Tien et al., 2011). Accordingly, it is anticipated that river biofilms from this site may be able to degrade carbamate pesticides.

2.2. Degradation tests

The natural river biofilms that formed on the unglazed ceramic discs in different seasons were collected, placed in a 60 L polypropylene container containing 30 L of river water, and transported to the laboratory within 2 h. Twenty liters of river water were collected and sterilized at 121 °C for 15 min to remove any suspended microorganisms. In the single pesticide system, the experimental groups consisted of 17 ceramic discs covered with river biofilms plus 1 L of sterilized river water and 10 mg L^{-1} of a single pesticide (i.e., methomyl, carbaryl or carbofuran) in order to determine the pesticide degradation kinetics and select for pesticide-degrading microorganisms (El-Fakharany et al., 2011; Verhagen et al., 2011). To examine the interactive effects of a mixture of two or three pesticides on the microbial community structures and the pesticide removal by biofilms, the two-pesticide (i.e., methomyl + carbaryl, methomyl + carbofuran, or carbaryl + carbofuran) and three-pesticide (i.e., methomyl + carbaryl + carbofuran) systems were tested in each experimental group instead of a single pesticide. The control groups consisted of 1 L of sterilized river water and 10 mg L^{-1} of a single pesticide or multiple pesticides with no biofilm addition. The blank groups consisted of river biofilms and 1 L of sterilized river water (no pesticide addition) to determine the effects of the incubation condition on microbial communities within biofilms during degradation tests. Each system was supplied with air bubbles by pumps to provide sufficient dissolved oxygen. All systems were run in duplicates and in an ambient-control laboratory at 25 °C under illumination (500 lux) with a 12:12 h light:dark period for 10 days.

After adding the pesticides, 50 mL of sterilized river water were collected from each group on Days 0, 1, 2, 3, 5, 7, and 10 to determine the concentrations of pesticides. One ceramic disc with river biofilms was collected on Days 0, 3, 7, 10 to determine the concentrations of pesticides in biofilms. Five ceramic discs with biofilms were collected to investigate changes in the biomass and the community structure of the algae and bacteria within the biofilms on Days 0, 3, and 10. Before adding pesticides, concentrations of pesticides in sterilized river water were measured.

2.3. Determining the concentrations of carbamate pesticides in sterilized river water

Ten mL of sterilized river water collected from each group was filtered with nylon filter membranes (25 mm diameter, 0.22 μm pore size). The filtrate was added to an equal amount of acetonitrile and injected into a high performance liquid chromatography (HPLC, Agilent 1100, Palo Alto, CA, USA) for pesticide analysis. The ceramic disc with biofilms was placed in 10 mL acetonitrile and sonicated for 30 min to extract carbamate pesticides in biofilms. The extract was then filtered with nylon filter membranes, and the filtrate was added to an equal amount of deionized water and injected into the HPLC. An Ascentis C_{18} (25 cm long × 4.6 mm diameter, 5 μm particle size, Supelco, Bellefonte, PA, USA) was used in the HPLC analysis. The mobile phase utilized acetonitrile and double deionized water at 50:50, with a flow rate of 1 mL min^{-1}. A UV detector was used at a detection wavelength of 254 nm for methomyl and 280 nm for carbaryl and carbofuran.

Data quality was managed by spiking known amounts (5 mg L^{-1}) of pesticides in a blank of sterilized river water (20 mL) and biofilms, and then carrying out the procedure as described above. Procedural blanks ($n = 2$) and quality control check samples ($n = 3$) were included with every batch of samples (10 samples per batch). The recoveries of the pesticides were calculated by dividing the concentrations of the spiked samples (after subtracting the concentration of an unspiked sample) with the original spiked concentrations. The recoveries of methomyl, carbaryl and carbofuran in blank sterilized river water ranged from 89.1 to 109.3%, 82.2–99.5% and 70.6–96.5%, respectively. The recoveries of methomyl, carbaryl and carbofuran in blank biofilms ranged from 94.3 to 98.6%, 90.8–92.9% and 86.2–88.1%, respectively. The precision of the 5 mg L^{-1} pesticide standards was 3.0%–5.6%, 3.0%–5.1% and 2.9%–5.0% for methomyl, carbaryl and carbofuran, respectively. The method detection limit for methomyl, carbaryl and carbofuran was 8.0, 11.0 and 11.0 μg L^{-1}, respectively.

The percentage of pesticide removal in the sterilized river water during the tests was calculated by dividing the mean pesticide removal of the duplicated water samples collected at a particular time by the amount of original pesticide. The Student's *T* Test (with significance of $p < 0.05$) was used to determine the differences between different treatments using STATISTICA software (release 6.0, StatSoft, Inc. USA). The multiple regression method with the least-square method was used to fit the experimental data with the first-order model (Schimdt et al., 1985; Tien et al., 2011) to determine the pesticide removal kinetics. The goodness of fit for the regression line was evaluated by the ANOVA (analysis of variance) *F*-test (with significance of $p < 0.05$).

2.4. Analysis of algal biomass and diatom diversity

Algal biomass was determined by measuring the concentration of chlorophyll a. The ceramic discs (in two replicates) covered with biofilms after different exposure times were soaked in 10 mL of 90% ethanol to extract chlorophyll a by using a 60 °C water bath for 30 min (mixing every 10 min). The extract was measured by the ISO standard method (ISO10260, 1992).

The river biofilms containing diatoms were removed from the ceramic discs using mild sonication and a scraper in 10 mL of a phosphate buffer solution (pH 7.4). The diatom cells were treated and counted according to the method of Tien et al. (2009). The Shannon-Weaver diversity index (H′ for diatoms) of the diatoms was calculated using the equation $H' = -\Sigma(Pi) \times \ln Pi$, where Pi is the proportion of each species in the sample.

2.5. Total bacterial counts

One ceramic disc covered with biofilm was placed in 20 mL of a 5% glutaraldehyde solution, and the biofilm was removed with a scraper and further sonicated for 10 min to obtain biofilm suspensions. The two replicate samples were performed. The suspensions were stained with 4′6-diamidino-2-phenylindol (Sigma, St. Louis, MO, USA) and the number of bacteria was counted on three different slides from a single disc according to the method of Porter and Feig (1980).

2.6. Molecular technique to determine bacterial diversity

2.6.1. DNA extraction and polymerase chain reaction (PCR) amplification of 16S rDNA fragments

The river biofilms were removed from the duplicate ceramic discs using a scraper. The DNA of the removed biofilms was extracted using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The variable V6–V8 region of the 16S rDNA was enzymatically amplified in a total volume of 50 μL PCR reaction mixture (US patents owned by Hoffman-La Roche, Pleasanton, CA, USA) in a thermal cycler (MyCycler, Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. To increase the sensitivity and specificity to facilitate the DGGE, a nested PCR technique was applied. In the first PCR round, the primers 11F and 1512R (Amann et al., 1995) were used. The primers 968F-gc (Heuer et al., 1997) and 1392R (Ferris et al., 1996) were used for the second PCR round. The GC-rich sequence (Ferris et al., 1996) was attached to the 5′ end of primer 968F to avoid completely melting the PCR products during the separation on the DGGE.