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Note

Evaluation of river model biofilm for assessing pesticide effects: a case study with atrazine

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The dose response of the biological parameters of the model river biofilm, consisting of two diatom strains and three bacterial strains on the glass surface, was examined with atrazine. Good correlations (r^2=0.90–0.93) between the atrazine log concentration and the relative increase rates of microbial cell numbers and the amount of chlorophyll *a* in the model biofilm formation were shown, and similar EC_{50} values for the biological parameters including esterase activity were tentatively obtained as an endpoint. The results suggested that this model biofilm could be used for testing the potential effects of pesticides on natural river biofilms at a community level with high reproducibility. © Pesticide Science Society of Japan

Keywords: river model biofilm, assessment of pesticide effects, atrazine.

Introduction

The surfaces of rocks and pebbles in a river bed are covered by biofilms, which consist of algae, bacteria, and protozoa as producers, decomposers, and consumers, respectively,[1,2] and play important roles in river ecosystems.[2–4] Because the epilithic microbial communities in the river biofilm are the first organisms in the river to be exposed to pesticide runoff from agricultural fields,[5,6] it is important to evaluate the adverse effects and to assess the ecological risk of pesticides on the biofilms. There have been reports on the pesticide effects on a river biofilm formed on stones or artificial substances set in a river or in a laboratory experimental system[7–9]; however, it would be difficult to evaluate the effects of pesticides on natural biofilms due to the poor reproducibility of biofilm settlement in which biological parameters such as biomass, activity and community structure vary among season and sites,[10,11] and are affected by environmental conditions.[12] Additionally, it has been reported that environmental conditions influence the effects of pesticides on river biofilm[13–17]; therefore, a test system with high reproducibility and reliable endpoints should be established to determine the toxicity of pesticides on natural river biofilms.

In our previous study,[18] we constructed a model biofilm consisting of known microorganisms (two diatom and three bacterial strains) on the inside surface of a glass test tube continuously rotated under a light–dark regime under controlled conditions to assess the pesticide effects on biofilm formation. Preliminary experiments using atrazine at an excessive concentration of 1000 ppb was conducted to examine if the adverse effects of the herbicide could be experimentally detected, and the results demonstrated the potential of the model biofilm in which good reproducibility of the following biological parameters was obtained: bacterial and diatom cell numbers, microbial community structure, esterase activity, and amount of chlorophyll *a*.[18] In this study, we conducted a toxicological test with a series of concentrations of atrazine, and examined the dose response of the biological parameters with atrazine. Then, we evaluated the model biofilm if EC_{50} values of the parameters can be obtained as endpoints for assessing the pesticide effects on biofilm formation.

Materials and Methods

Two diatom strains, *Achnanthes minutissima* N71 and *Nitzschia palea* N489, and three bacterial strains, *Pedobacter* sp. 7–11, *Stenotrophomonas* sp. 3–7 and *Aquaspirillum* sp. T-5, were selected to construct the model biofilm based on our previous report.[18] Each diatom and bacterial strain was precultured in Csi medium[18] at 20°C under a 12-hr light–dark cycle at 5000 lux (natural white), and in 1/10 PTYG medium[19] with shaking at 25°C in the dark, respectively. Initial cell densities were set at 4.0×10^4 and 4.0×10^6 cells/ml for each diatom and bacterial strain, respectively, in fresh Csi medium determined by the direct counting method.[20] Atrazine (98% purity; Wako Pure Chemical Industries, Osaka, Japan) was dissolved in acetone at 1000 ppm, and test media were prepared at final concentrations of 0, 200, 400 and 800 ppb. The same amount of acetone (100 μl/l) was added at 0 ppb. The model biofilm was constructed using 8 ml culture on the inside surface of a glass test tube (flat bottom 25 mm inner diameter×125 mm without a rim; IWAKI, Tokyo, Japan) continuously rotated (1 rpm at an angle of 15°) at 20°C under a 12-hr light–dark regime at 5000 lux,[18] and the following biological parameters in plankton and biofilm were measured at 0, 1, 2, 3 and 4 weeks after inoculation according to the methods described previously:[18] diatom and bacterial cell numbers by the direct counting method, microbial community structures by PCR-DGGE of bacterial and diatom chloroplast 16S rRNA genes, chlorophyll *a* content by absorbance measurement, and esterase (fluorescein diacetate degrading) activity by a colorimetrical method. The concentration of atrazine in the culture was measured by HPLC. An aliquot of the culture was taken at 0, 1, 2, 3

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and 4 weeks, and directly analyzed after centrifugation at 21,880×*g* for 10 min according to the procedure described previously[21] except for the detection at 264 nm. The experiment was conducted in triplicate. The toxicity of atrazine on biofilm formation was expressed as the EC_{50} value for each biological parameter. The EC_{50} values were determined manually using increase rate inhibition %-log atrazine concentration plots. The increase rates of each biological parameter of the model biofilm were calculated using the data by which the linearity of the increase was observed.

Results and Discussion

In this study, we focused on the atrazine effects on biofilm formation, not on the microorganisms in plankton, but showed changes in the biological parameters in both plankton and biofilm as a reference (Figs. 1 and 2). Although no biofilm was present at 0 week, immediate adhesion of microorganisms to the glass surface was observed. All biological parameters suggested that biofilm formation was completely inhibited by atrazine at 800 ppb; thus, the results at 800 ppb were considered to be attributed to microorganisms that physically adhered to the glass surface, and their values at 1 week were used for the calculation of EC_{50} as the value of each biological parameter of the biofilm at 0 week.

During incubation of the control (0 ppb of atrazine), the diatom number in biofilm increased to 8.0×10^6 cells/tube at 1 week and remained almost constant. Most diatoms were present in biofilm rather than in plankton. The increase of the diatom population in biofilm accompanied the increase of the amount of chlorophyll *a* in the biofilm. The planktonic bacterial number increased concomitantly with the increase of the diatom population in biofilm, and then the bacterial number in biofilm gradually increased to 1.1×10^8 cells/tube during 4 weeks of incubation. The percentage of bacteria in the biofilm was about 10% of the total bacteria in the culture at 4 weeks. The increase of the bacterial population in biofilm accompanied the increase of esterase activity in the biofilm. The PCR-DGGE profiles of the 16S rRNA gene indicated that all of the inoculated bacterial and diatom strains proliferated in the biofilm throughout the incubation period (Fig. 2). Immediately after inoculation, the intensity of the bacterial bands was almost the same among them, while in the diatom strains, the intensity of the bands was not similar despite almost the same cell numbers (data not shown), perhaps due to PCR amplification bias. Apparent adhesion of diatoms without bacteria as well as the growth of bacteria without diatoms was not observed under the study conditions[18]; therefore, the model biofilm found in this study was considered to be formed as a result of mutual interaction between the bacterial and diatom strains.

Atrazine was stable in the cultures (105–133% of nominal concentrations) throughout the incubation period. Atrazine inhibited the growth of diatoms and bacteria in biofilm depending on the concentration of atrazine except for the similar inhibition of bacterial growth at 200 and 400 ppb (Fig. 1). The diatom growth rates in biofilm at 0, 200, 400 and 800 ppb were determined based on the data during 0–1, 0–2, 0–3 and 0–4 weeks, respectively, considering the linearity of diatom growth (Fig. 1), and the EC_{50} value was determined to be 213 ppb (Fig. 3). In a separate experiment under the same conditions without the bacterial strains, the cell number of individual diatom strains N71 and N489 increased constantly in plankton during 1 and 2 weeks, respectively, and the EC_{50} values of strains N71 and N489 were determined to be 255 and 207 ppb, respectively (data not shown). The EC_{50} values for diatom growth were not significantly different between their individual cultures and in the model biofilm. Bacterial growth rates in the biofilm were determined using the data during 4 weeks of incubation for all atrazine concentrations, and the EC_{50} values were calculated to be 215 ppb (Fig. 3). Atrazine did not affect the growth of each individual bacterial strain even at 1000 ppb, and was not degraded by the bacterial strains in an organic medium at the concentrations used in this study (data not shown).

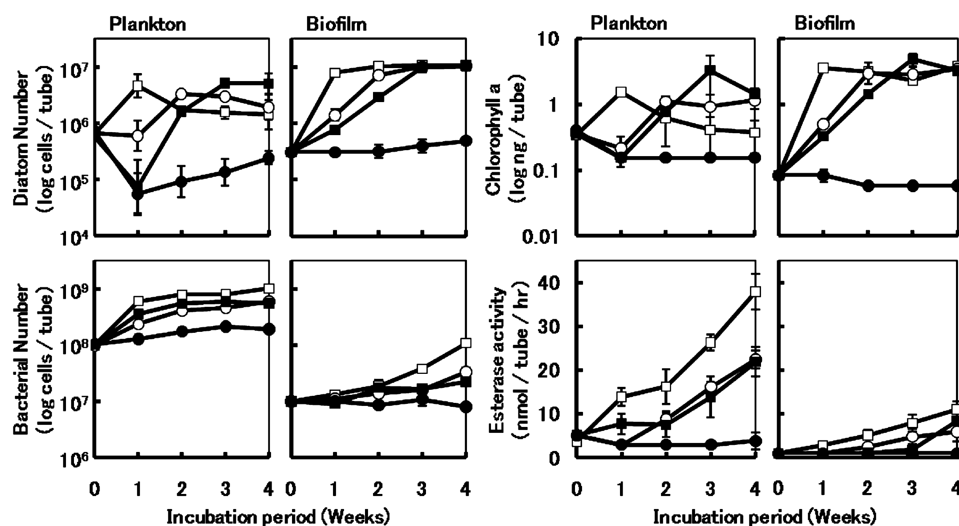

Fig. 1. Effect of atrazine on biological parameters of plankton and biofilm in the formation of model biofilm. Open square, open circle, closed square and closed circle indicate control, atrazine at 200 ppb, 400 ppb and 800 ppb, respectively. Bars indicate standard deviation ($n=3$).

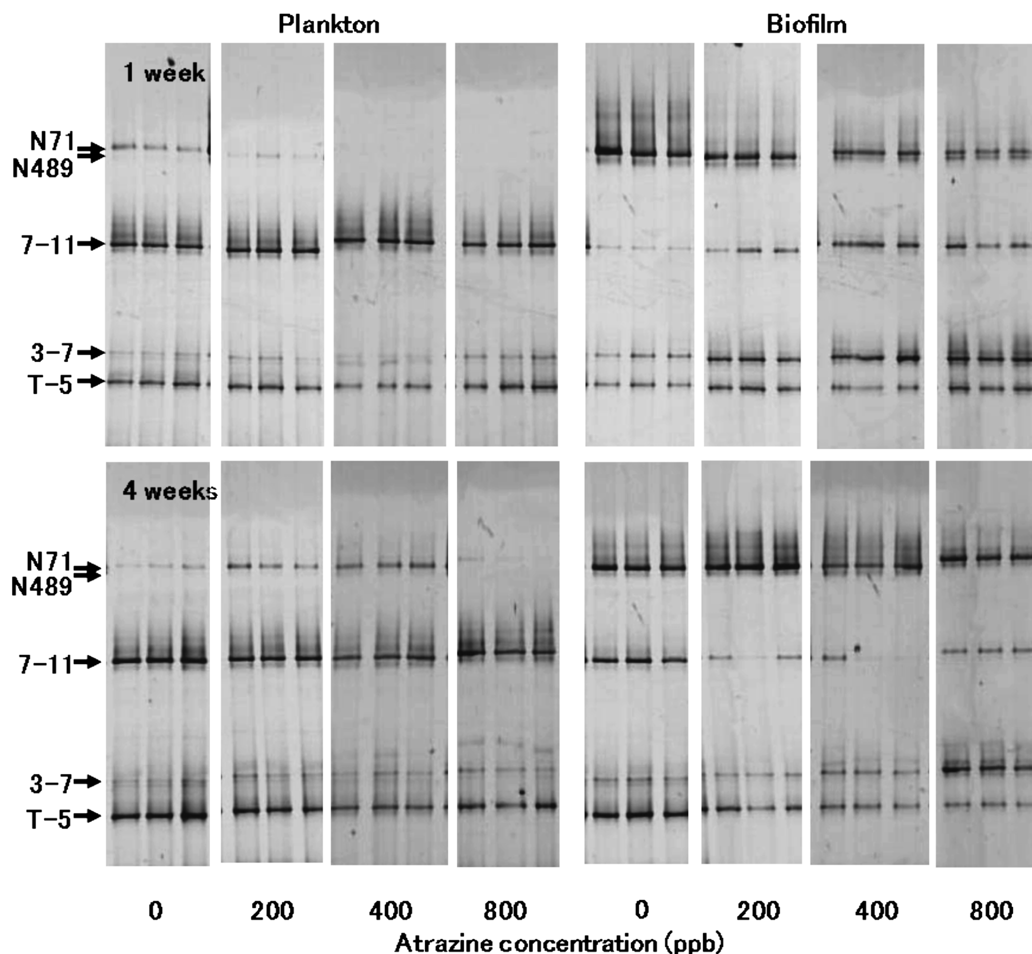

Fig. 2. Effect of atrazine on DGGE profiles of 16S rRNA genes of plankton and biofilm in the formation of model biofilm. The model biofilm was constructed using two diatom strains, *Achnanthes minutissima* N71 and *Nitzschia palea* N489, and three bacterial strains, *Pedobacter* sp. 7–11, *Stenotrophomonas* sp. 3–7 and *Aquaspirillum* sp. T-5. Profiles of triplicate cultures are shown.

These results indicate that atrazine directly inhibited diatom growth initially, leading to the reduction of photosynthetic diatom products that may act as a nutrient for bacteria, and then bacterial growth was indirectly inhibited, resulting in the retarded development of biofilm. Indirect effects of diuron on the bacterial population and activity were also reported in a biofilm constructed on a glass substrate using natural river water.[22]

The amount of chlorophyll *a* in the biofilm increased exponentially as with the diatom number (Fig. 1), the increase rates at 0, 200, 400, 800 ppb were calculated from the data during 0–1, 0–2, 0–3 and 0–4 weeks, respectively, and the EC_{50} value was determined to be 210 ppb (Fig. 3).

In spite of the exponential growth of bacteria in the biofilm, the esterase activity increased linearly for 4 weeks, except for 400 ppb at 4 weeks (Fig. 1). The increase rates of esterase activity in the biofilm were calculated based on the data during 4 weeks of incubation, except that the data during 3 weeks were used for 400 ppb. Considering the low relative increase rate (10%) at 400 ppb (Fig. 3), it was expected that esterase activity would be completely inhibited at less than 800 ppb. Thus, the EC_{50} value was calculated to be 215 ppb based on the data at 200 and 400 ppb. It was found in the previous study that bacteria contributed to almost all esterase activity in the model biofilm[18]; therefore, the reason for the discrepancy between the bacterial number and esterase activity is unclear.

Microbial community structure in the biofilm showed that all of the bands corresponding to the inoculated microbial strains were detected throughout the incubation period but the intensities of the bands were changed (Fig. 2). At 1 week, the relative intensity of bands 7–11 and 3–7 increased with higher concentrations of atrazine, while that of T-5 was not affected by atrazine. At 4 weeks, the relative intensity of band 3–7 increased with a higher concentration of atrazine, while those of 7–11 and T–5 decreased by the addition of atrazine. In diatom strains, the intensity of band N71 was stronger than N489, and the relative intensity of band N71 decreased with a higher concentration of atrazine, while that of N489 was not affected by atrazine during the incubation period. These results suggested that atrazine affected the microbial community structure in the model biofilm, but it was difficult to evaluate the effects quantitatively.

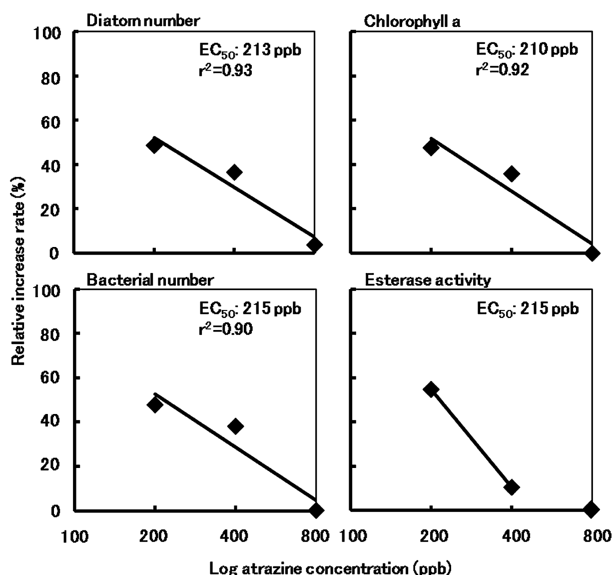

Fig. 3. Relative increase rate-log atrazine concentration plots of biological parameters in the formation of model biofilm. EC_{50} value and correlation coefficient of the regression line are shown in each plot. Increase rates were calculated from data in which the linearity of the increase was observed.

Changes in the biological parameters during the incubation period of biofilm formation in the control in this study were comparable with those in a previous study [18] and the variations in triplicate experiments were small (Fig. 1), indicating that the model biofilm could be constructed with good reproducibility and used to assess pesticide effects on river biofilms. High correlation coefficients (r^2=0.90–0.93) between the relative increase rate and log atrazine concentration were observed in all parameters except that it could not be calculated for esterase activity due to the small number of plots (Fig. 3). In addition to the good correlation, similar EC_{50} values (210–215 ppb) were tentatively obtained for all of the biological parameters except for the microbial community structure; therefore, the EC_{50} values of biological parameters seem to be a suitable index as an endpoint. Additional experiments with a wider range of concentration are necessary to obtain more reliable results and other endpoints such as the no observed effect concentration (NOEC).

Guasch *et al.*[13–16] examined the toxicity of atrazine on the photosynthetic activity of river biofilm which formed on a glass slide set in European rivers. The EC_{50} values obtained in these studies ranged with almost even distribution from 41 to 716 ppb (n=67) depending on the river, season, stage of biofilm development, and light intensity during exposure. Concerning the toxicity of atrazine on a diatom strain, it has been reported that the EC_{50} values for the growth rates of *Asterionella formosa*,[23] *Navicula accomoda*,[23] *Nitszchia* sp.[23] and *Chaetoceros* sp.[24] were >2160, 164, 412 and 43 ppb, respectively. In our preliminary experiment, the EC_{50} values for *A. minutissima* N71 and *N. palea* N489 were 255 and 207 ppb, respectively, as described above; therefore, the EC_{50} values obtained by the model biofilm (210–215 ppb) were the same as those for natural river biofilms and the number of diatom strains.

For the ecological risk assessment of pesticides on river biofilms, it is necessary to consider the variability of natural river biofilms because several factors, such as the microbial composition and developmental stage of natural river biofilms and their different sensitivity to pesticides, and environmental conditions of river water, are expected to affect toxicity. In the above studies of Guasch *et al.*,[13–16] the largest difference in the EC_{50} values of atrazine for natural river biofilms was about 20 times. Based on the data, the safety factor for ecological risk assessment might be tentatively set at 20 considering the variability among natural river biofilms, but more data from other pesticides and river biofilms are necessary to obtain a more reasonable value. The model biofilm seems to be applicable to use for analyzing environmental factors causing such variability observed in the data from natural river biofilms because the experimental conditions could be intentionally controlled. In addition, the selection of different microorganisms to develop a model biofilm could provide information on a range of sensitivities of river biofilms to pesticides.

It was demonstrated that this model biofilm can detect indirect effects, which cannot be detected by toxicological tests with individual microorganisms. This model biofilm is superior to biofilms that have been previously constructed using natural river water for the evaluation of pesticide effects, because good reproducibility of microbial composition and parameters can be obtained in this model biofilm under controlled conditions. Although this model biofilm is not the same as natural river biofilm, it showed potential to assess pesticide effects on natural river biofilms. Further studies with other pesticides and experimental conditions are necessary to make this model biofilm a more reliable test system for ecological risk assessment of pesticides on river biofilms.

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