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Research Article
Microbial Degradation of Fenitrothion in Kurose River Water, Hiroshima Prefecture, Japan

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Abstract

Background and Objectives: Identification of microbial community composition is very important for understanding the degradation kinetics of pollutants in water. This study was conducted to isolate and to identify the microbial communities of Kurose River water and to investigated the biodegradation kinetics of fenitrothion by identified microbial strains. **Materials and Methods:** Microbial community composition was investigated using differential display-denaturing gradient gel electrophoresis (DGGE). Identification of microbial isolates was carried out using 16S rRNA gene sequence analysis. Biodegradation of fenitrothion was carried out using High-performance liquid chromatography and gas chromatography-mass spectrometry analysis. **Results:** The results showed that the degradation of fenitrothion in Kurose River water occurred because of various bacterial isolates. Moreover, the degradation rate of fenitrothion in Kurose River water at different sampling sites depended on the microbial content and the chemical composition of the water. The bacteria isolated from the Kurose River identified as *Pseudomonas putida* and *Flavobacterium* sp. The degradation rate of fenitrothion by the isolated bacteria was lower in river water than in artificial growth media. The identified degradation products of fenitrothion were amino-fenitrothion and 3-methyl-4-nitrophenol. **Conclusion:** The microbial community and chemical composition of the Kurose River water significantly affect the fate and biodegradation kinetics of fenitrothion. Molecular identifications of microbial community structures of aquatic systems are very useful for interpretation of the fate and degradation kinetics of fenitrothion.

Key words: Gradient gel, biodegradation, fenitrothion, bacteria, identification

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The extensive and widespread use of pesticides for control of agricultural pests and public health protection has resulted in severe environmental pollution and adverse effects to non-target organisms[1]. Therefore, it is necessary to conduct intensive investigation on the fate of these pesticides after application and their impact on the environment and public health. Organophosphorus compounds comprise some of the most used insecticides and accounting for about 50% of those used globally[2]. Some organophosphorus pesticides (OPs) are highly toxic and their wide use has caused serious concerns over food safety and environmental pollution[3].

In Japan, fenitrothion is one of the most widely used organophosphorus insecticides for various purposes, mainly for control of rice stem bores in the rice crop, the main crop grown in Japan. As a result of its intensive use, fenitrothion is a common contaminant in river water and has been detected in river water throughout Japan[4-8]. Studies have shown that fenitrothion is a highly toxic pesticide to fish and to cause behavioral changes in some species like guppies, *Poecilla reticulate*[9].

In addition, fenitrothion, after application and access to water, is affected by many degradation processes that occur in the aquatic environment, such as photolysis, hydrolysis and bioegradation[10,11]. Breakdown of fenitrothion can lead to the generation of degradation products that are even more toxic[10,11], such as fenitrooxon and 3-methyl-4-nitrophenol[11-12]. Consequently, due to undesirable effects on the aquatic environment and in human health of this pesticide and its byproducts[10,11,13-15], it became necessary to understand the degradability and biodegradation of fenitrothion and its byproducts in water.

Several aspects are involved in the fate and behavior of OPs in the environment; namely, effluent irrigation[16], photodecomposition mechanisms[17], volatilization[18] and biodegradation[16,19]. Because of their toxicity, organophosphates biodegradation is being investigated worldwide[20]. In Japan, the biodegradation of organophosphorus insecticides in water has been reported by Kaonga *et al.*[21]. This, besides the degradability of pesticides by microorganisms in water, is considered to be the largest factor in measuring the purification ability of environmental water[22].

Identification of microbial community compositions is very important to understanding the degradation kinetics of pollutants in water. Moreover, it is important to evaluate changes in the microbial community structures of aquatic systems because microbial communities are the foundation of biogeochemical cycles[23,24]. Complex microbial communities exhibit large diversity, hampering differentiation by DNA fingerprinting. Herein, differential display-denaturing gradient gel electrophoresis (DGGE) is proposed. Currently, DGGE is probably the most commonly used method for typing and comparing microbial communities. This method is capable of separating DNA of the same chain length based on differences in sequences[25]. Accordingly, it is possible to determine the profile and pattern of a microbial community by analyzing PCR amplified fragments of 16S rDNA by DGGE and comparing the band patterns, which reflect the microbial community structure[24].

This study was conducted to investigate the biodegradation of fenitrothion in Kurose River water at different sampling sites, to isolate and identify bacterial isolates involved in the degradation of fenitrothion in Kurose River water, to compare the degradation of fenitrothion by the identified microbial isolates in different growth media and river water and to identify the biodegradation mechanism and main biodegradation products of fenitrothion in river water.

MATERIALS AND METHODS

Sampling sites: The Kurose River is located in Hiroshima Prefecture and extends for approximately 43 km. This river runs through urban and agricultural areas of the Kamo plateau, including the cities of Higashi-Hiroshima and Kure and then flows into the SetoInland Sea[26]. Water samples were collected from 3 sites in the Kurose River, Namitakiji, Izumi and Hinotsume (Fig. 1). Namitakiji (St1) is located in the uppermost part of the Kurose River, Izumi (St2) is located in the downtown area of Higashi-Hiroshima and Hinotsume (St3) is located in the agricultural area of Higashi-Hiroshima. Surface water was collected using a bucket made of polyethylene and placed in a brown glass container that had been sterilized with dry heat for 3 h at 150°C, then immediately returned to the laboratory and stored in a refrigerator for testing.

Reagents: The growth media used in this study (standard agar medium, mineral medium and C-medium) is described in Table 1. Sterile saline solution was obtained from Katayama Chemical Industries Co., Ltd. Dichloromethane, acetone, sodium sulfate anhydrous and sodium chloride were obtained from Wako Pure Chemical Industries, Ltd. Standards of fenitrothion and its byproducts (3-methyl-4-nitrophenol and 3-methyl-4-nitroanisole) were obtained from Kanto Chemical Co., Inc.

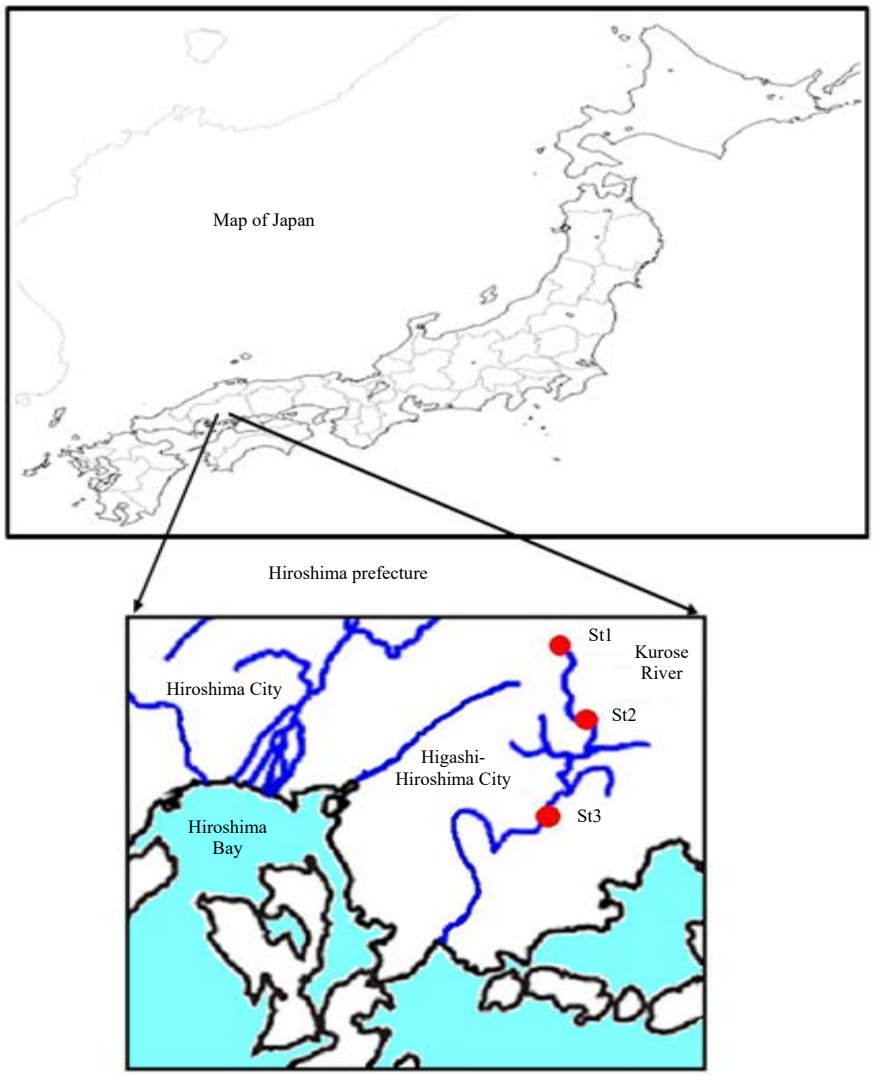

Fig. 1: Location of sampling sites in the Kurose River

Water quality analysis: At the sampling sites, conductivity and pH were measured using a D-24 pH/conductivity meter (Horiba, Ltd., Japan), while water and air temperature were measured with a mercury thermometer and the dissolved oxygen (DO) and biochemical oxygen demand (BOD) were measured as described by the American Public Health Association[27]. Anions (Cl^-, NO_2^-, NO_3^- and SO_4^{2-}) and cations (Na^+, K^+, NH_4^+, Ca^{2+} and Mg^{2+}) were measured by DX 500 ion chromatography (Dionex Co., USA) using an IonPac AS11 column for anions and an IonPac CS12A column for cations. Dissolved organic carbon (DOC) was measured using a TOC-5000 A (Shimadzu Co., Japan).

Primary test for fenitrothion biodegradation in river water: The collected river water was added to the triangular flask and spiked with fenitrothion to give a final concentration of 5 mg L^{-1}, after which the solution was kept in an incubator (Biotron LH-24, Nippon Medical and Chemical Instruments Co., Ltd., Japan) at 21±1°C. All glassware used for this experiment was sterilized for 3 h at 150°C before use. River water that had been sterilized by autoclaving at 121°C for 20 min was used as a blank[28]. Water (8 mL) was collected at different intervals to monitor the degradation of fenitrothion with time followed by high performance liquid chromatography (HPLC) analysis according to method described by Kiso *et al.*[29]. Water was filtered before HPLC analysis using a syringe filter with a pore size of 0.45 μm. To calculate the recovery, water spiked with fenitrothion at 5 μg L^{-1} and was analyzed in the same manner as the normal sample and the mean recovery percentage was 90.65±1.2%.

Table 1: Composition of used media in this study

Standard agar medium	Weight	Company name
Yeast extract	2.5 g	Eiken Chemical Co., Ltd., Japan
Tryptone	5.0 g	Eiken Chemical Co., Ltd., Japan
Glucose	1.0 g	Eiken Chemical Co., Ltd., Japan
Agar	15.0 g	Eiken Chemical Co., Ltd., Japan
DW	1000 mL	
pH	7.1±0.2	
M-medium (mineral medium)		
K_2HPO_4	0.5 g	Sigma Chemical Co., Ltd., USA
$NaNO_3$	0.5 g	Eiken Chemical Co., Ltd., Japan
$MgSO_4·7H_2O$	0.2 g	Eiken Chemical Co., Ltd., Japan
$FeSO_4·7H_2O$	1 mg	Eiken Chemical Co., Ltd., Japan
DW	1000 mL	
pH	7.0	
C-medium (M-medium+glucose)		
K_2HPO_4	0.5 g	Sigma Chemical Co., Ltd., USA
$NaNO_3$	0.5 g	Eiken Chemical Co., Ltd., Japan
$MgSO_4·7H_2O$	0.2 g	Eiken Chemical Co., Ltd., Japan
$FeSO_4·7H_2O$	1 mg	Eiken Chemical Co., Ltd., Japan
D-glucose	0.1 g	Eiken Chemical Co., Ltd., Japan
DW	1000 mL	
pH	7.0	
Solid media to the liquid medium 1 L, it was added as follows:		
Agar	1.5 g	Wako Pure Chemical Co., Ltd., Japan

Isolation of bacteria from different sites in Kurose River: The inorganic salt medium described in Table 1 was sterilized in an autoclave at 121°C for 20 min, after which fenitrothion was added to the sterilized inorganic salt medium at a final concentration of 5 mg L^{-1} and allowed to cool to 40-50°C. Next, 15 mL of the medium spiked with fenitrothion was poured in each Petri dish. After the agar solidified, 1 mL aliquots of water samples from different sites were plated and samples were cultured for 7 days at 21±1°C in an incubator. After culturing, the different bacterial isolates on the medium were streaked for isolation. The purified bacterial isolates were subsequently named and tested for the ability to biodegrade fenitrothion.

Culturing of isolated bacteria on artificial growth medium with and without fenitrothion: For culturing of isolated bacteria on artificial growth medium without fenitrothion, 10 mL of inorganic salt liquid medium supplemented with glucose as an energy source (C-medium) were added to a test tube, then inoculated with the isolated bacterial strains and shaken at 80 rpm under 21±1°C for pre-culture. After 3 days of incubation, 5 mL of the culture solution was centrifuged (9000 rpm, 10 min), after which the bacteria were collected. After suspending the bacterial cell pellet in sterile saline and washing it twice, the cells were again centrifuged. Next, the pellet was again suspended in sterile saline, after which the turbidity (OD 610 nm) was adjusted to 0.2 to create a bacterial suspension. For culturing of isolated bacteria on artificial growth medium spiked with fenitrothion, the same procedure was carried out as mentioned above in the presence of fenitrothion.

Total number of viable bacteria in Kurose River water at different sites: The viable bacterial count was determined by the conventional agar plate method as described by Yoshikura *et al.*[30]. The number of bacteria at different Kurose River water sites was determined by culturing microorganisms that might be available in river water on standard agar medium for 24±2 h at 35-37°C. Specifically, 1 mL aliquots of water samples were diluted 10-fold with ultrapure water that had been sterilized by autoclaving at 121°C for 20 min in a test tube and shaken well. Next, 1 mL aliquots of the serially diluted samples were transferred onto Petri dishes (90×15 mm, IWAKI) containing 15 mL of sterilized standard agar that had not yet solidified. The Petri dishes were then gently rotated to the left and right to mix the sample with the medium. After solidification of the agar, Petri dishes were incubated (Clean Hot Air Circulating Dryer CHD-200) upside down for 24±2 h at 37°C, after which the number of colonies in each Petri dish was counted and the average number/1 mL of each sample was determined.

Microbial community compositions in Kurose River water at different sites: To investigate the microbial community composition in Kurose River water from different sampling sites, DNA of isolated bacteria was extracted and amplified by PCR using 16S rRNA gene sequence analysis. The DNA fragments of bacteria as PCR products were then separated by DGGE as previously described[25]. A seawater lane was used for comparison. It should be noted that no markers were used because we only wanted to show the microbial community composition of different sites. The DGGE analysis was conducted by Takara Bio Co. Dragon Genomics Center (Mie, Japan).

Identification of fenitrothion biodegradation products using GC-MS: Biodegradation of fenitrothion by isolated bacteria was conducted in river water collected at Izumi (St2) in the Kurose River and in different artificial growth media (C and M media). Fenitrothion at a concentration of 5 mg L^{-1} was incubated with different isolated bacteria either in the river or artificial media at 21±1°C in an incubator. The isolate-free control treatment underwent minimal degradation of fenitrothion (<7%). Organophosphorus pesticides hydrolysis occurs at pH 11 or more[31]; therefore, we maintained the pH at 6.5-7.4 during the culture period so that chemical hydrolysis of fenitrothion was not expected.

Extraction of fenitrothion and its possible degradation products from water or growth media samples after biodegradation was conducted as follows. About 1 N-HCl was added to the recovered medium (10 mL) until the pH was adjusted to 3. Next, sodium chloride for pesticide residue analysis (0.5 g) was added and the solution was transferred to a 100 mL separatory funnel. Dichloromethane (5 mL) was then added and the separatory funnel was shaken for 10 min, after which the dichloromethane layer was collected. The extraction operation was then repeated 3 times and the resulting dichloromethane layer was combined, after which the dichloromethane layer was dehydrated with anhydrous sodium sulfate. Finally, samples were gently dried under a nitrogen stream and phenanthrene-d10 dissolved in acetone was added as an internal standard.

Samples were analyzed using a Hewlett-Packard HP 6890 series gas chromatography equipped with an HP 6890 mass selective detector and a refused silica capillary HP-5MS column (30 m length×0.25 mm i.d., film thickness 0.25 μm). The oven temperature was programmed to hold for 2 min at 60°C, then increase to 280°C at 8°C min^{-1}, where it was held for 8 min. The injector and transfer line temperatures were 250 and 280°C, respectively. The carrier gas (helium) was applied at a constant flow rate of 1 mL min^{-1}. The standard and extracted solutions were injected in splitless mode according to the method described by Durand *et al.*[32] with some modifications. For quality control of the analytical method, the detection limits of fenitrothion and 3-methyl-4-nitrophenol were 1.4 and 5.1 μg L^{-1}, while the quantification limits were 4.6 and 17 μg L^{-1}, respectively. Also, the mean recovery of fenitrothion and 3-methyl-4-nitrophenol in media were 89.0±4.2 and 74.2±3.8%, respectively, while for river water they were 70.8±3.2 and 83.4±3.8%.

Identification of isolated bacteria: The bacterial strains isolated and purified from different Kurose River water sites were identified using the 16S rRNA base sequence analysis by Takara Bio Co. Dragon Genomics Center (Yokkaichi, Mie Prefecture). The strain as a template was subjected to PCR to amplify a portion of the highly variable region of 16S rRNA. The PCR product was processed and the nucleotide sequence was read using a DNA analyzer. The sequence data of 500 bp were surveyed by BLAST searches of the GenBank non-redundant database for identification of the isolated strains.

Statistical analysis: For analysis of variance (ANOVA) of obtained data, XLSTAT PRO statistical analysis software (Addinsoft) was used. Fisher's least significant difference (LSD) test was used to separate the mean of each treatment. All analysis were performed at a significance value of $p \leq 0.05$.

RESULTS

Primary biodegradability of fenitrothion in Kurose River water: The results of the biodegradation test of fenitrothion in Kurose River water are shown in Fig. 2a. The results also showed that there was low degradation of fenitrothion in water collected from the Namitakiji site located in the portion of the Kurose River furthest upstream. However, the degradation of fenitrothion in water collected from the Izumi and Hinotsume sites located in the middle reaches was 96 and 43% after 28 days, respectively. A higher bacterial abundance was also found in water collected from the Izumi site followed by the Hinotsume and Namitakiji sites. However, the measured anions were higher in water collected from the Hinotsume site followed by the Izumi and Namitakiji sites. As shown in Fig. 2b, the average number of bacteria in water

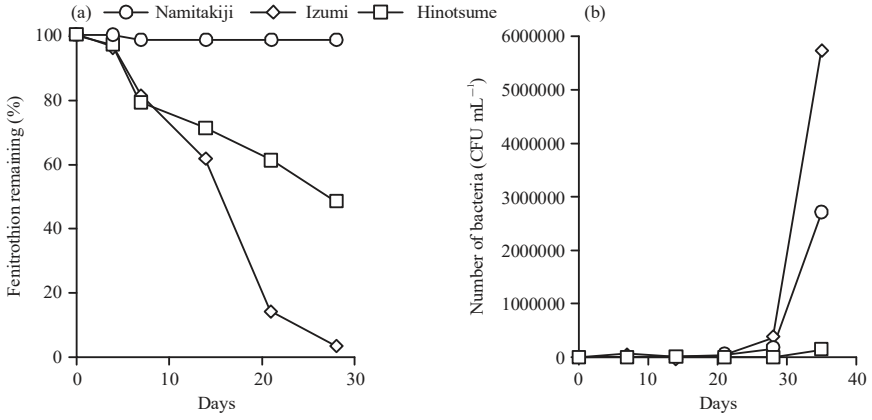

Fig. 2(a-b): (a) Biodegradation of fenitrothion and (b) Changes in bacterial counts in water collected from different sites in the Kurose River

Table 2: Water quality of the Kurose River

Sampling sites	Water temp. (°C)	pH	BOD (mg L^{-1})	DO (mg O_2 L^{-1})	DOC mg C L^{-1}	Cl^- (μM)	NO_2^- (μM)	NO_3^- (μM)	SO_4^{2-} (μM)	Number of bacteria CFU mL^{-1})
Namitakiji	4.0±0.1[a]	6.86±0.3[a]	2.1±0.1[a]	12.2±0.7[a]	1.4±0.1[a]	159.0±1.2[a]	ND	4±0.2[a]	34±1.1[a]	7±0.2[a]
Izumi	6.0±0.2[b]	7.36±0.5[b]	5.4±0.1[b]	11.6±0.6[b]	2.9±0.1[b]	809.0±2.1[b]	3±0.1[a]	128±1.2[b]	141±2.1[b]	1.5×10^4±9.2[b]
Hinotsume	5.5±0.1[b]	7.68±0.3[b]	6.0±0.2[b]	11.3±0.4[b]	2.8±0.1[b]	16145.0±5.2[c]	18±0.2[b]	133±1.5[b]	613±5.2[c]	3.1×10^2±7.4[c]

BOD: Biochemical oxygen demand, DO: Dissolved oxygen, DOC: Dissolved organic carbon, ND: Not detected, statistical comparisons were made among treatments within a single column, different letters represent significant differences using Fisher's LSD test at $p \leq 0.05$, each mean value came from three replicates

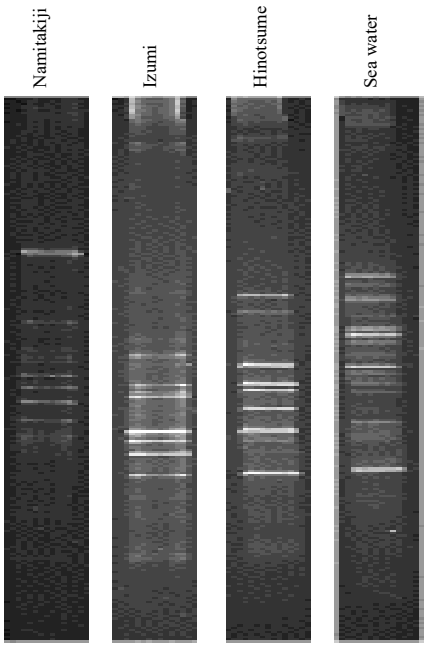

Fig. 3: DGGE profile pattern of microbial flora in water from different sampling sites of the Kurose River
Seawater lane was used for comparison

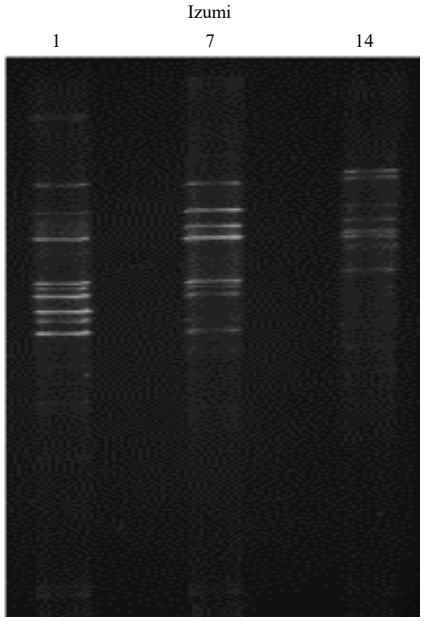

Fig. 4: Changes in DGGE pattern of microbial flora with time at the Izumi site

collected from the Namitakiji site was 7 CFU mL^{-1}, while it was 310 CFU mL^{-1} in water collected from the Hinotsume site. However, the number of bacteria in water collected from the Izumi bridge was 15,000 CFU mL^{-1}.

Isolation of bacteria in Kurose River water in the presence of fenitrothion: No bacterial colonies formed in the sterilized medium containing fenitrothion, but colonies formed in the medium containing water collected from the Izumi and Hinotsume stations and spiked with fenitrothion. Seven strains were isolated from this spiked medium and subjected to a preliminary decomposition test. Based on this screening biodegradation test, we selected 3 strains with a high decomposition rate of fenitrothion for further degradation experiments. Two of these 3 strains were isolated from the Izumi site, while 1 was from the Hinotsume site. The bacteria isolated from the Izumi site were denoted Izumi-1 and Izumi-2, while that from the Hinotsume site was denoted Hinotsume-1.

Water quality analysis: Analytical results of water quality in the Kurose River water collected from different sites are shown in Table 2. The results showed that the DOC, which is important for the growth of microorganisms, was higher in Izumi than at other sites. Moreover, the BOD values were found to be higher in Izumi and Hinotsume compared to the Namitakiji site.

The microbial flora composition after the addition of fenitrothion in river water collected from different sampling sites was examined by DGGE (Fig. 3). The results showed that the total number of bands and band distribution of the microbial community differed among sampling sites, implying that the microbial community composition at each site differed. In addition, the bands corresponding to the microbial flora in water collected from the Izumi site changed with time (from 0-7 to 14 days), indicating that the bacterial population changed with time (Fig. 4).

Decomposition of fenitrothion by isolated bacteria in growth media and river water: The results showed that the isolate-free control treatment underwent minimal

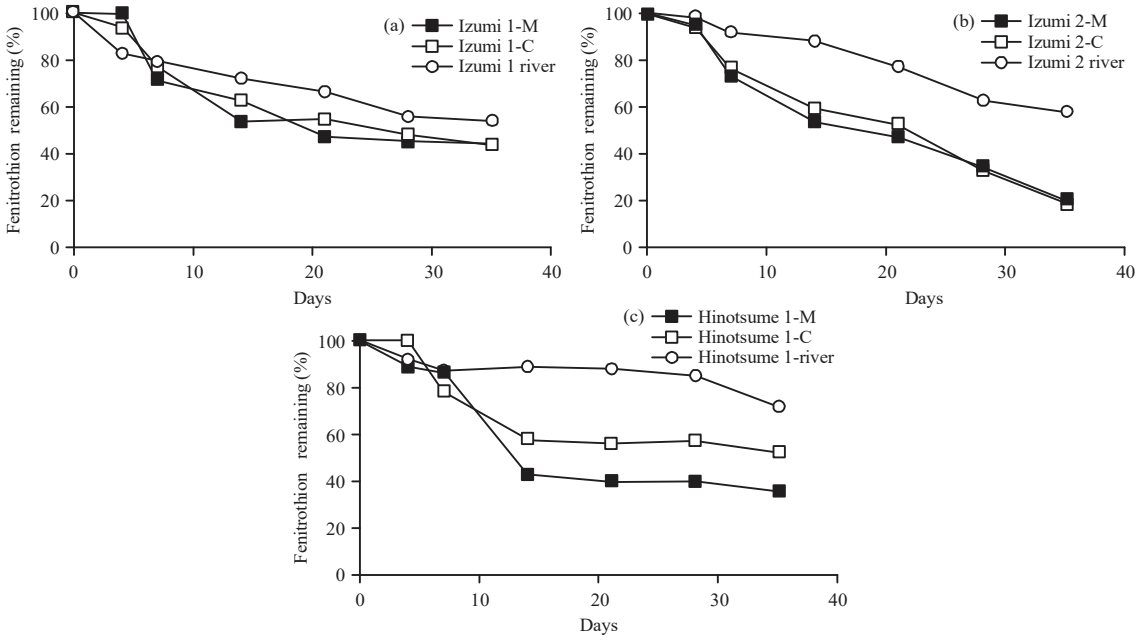

Fig. 5(a-c): Degradation of fenitrothion in different growth media and in river water using (a) Izumi-1, (b) Izumi-2 and (c) Hinotsume isolates

degradation of fenitrothion (<7%). Organophosphorus pesticides hydrolysis occurs at pH 11 or more; therefore, the pH was maintained at 6.5-7.4 during the culture period so that chemical hydrolysis of fenitrothion was not expected. The degradation of fenitrothion in the medium by bacteria Izumi-1 (Fig. 5a), Izumi-2 (Fig. 5b) and Hinotsume (Fig. 5c) isolated from different sampling areas is shown in Fig. 5. The results showed that degradation of fenitrothion by Izumi-1 and Izumi-2 isolates did not differ between M and C growth media (Fig. 5a and b). However, Hinotsume-1 degraded fenitrothion more rapidly in M-medium than C-medium (Fig. 5c). Moreover, the degradation of fenitrothion by Izumi-2 isolate was higher than that by Izumi-1 and Hinotsume-1 isolates in both growth media (M and C media). Under all tested bacterial isolates, the degradation of fenitrothion in the first ten days was very fast, then gradually became slow.

The degradation of fenitrothion in real river water collected from the Izumi site by isolated bacteria was evaluated. In the bacteria-free control treatment, almost no decrease in fenitrothion was observed (<5%). During the culture period, the pH of the river water ranged from 7-7.2, suggesting that chemical hydrolysis did not occur. As shown in Fig. 5, all 3 strains degraded fenitrothion in river water. Moreover, the highest degradation rate of fenitrothion in river water was by Izumi-1, followed by Izumi-2 and then Hinotsume-1. After 35 days, the Izumi-1 and Izumi-2 isolates decomposed 46 and 42% of the fenitrothion, respectively. However, a mixture of the Izumi-1 and Izumi-2 isolates in equal amounts (hereafter, Izumi isolate) resulted in about 52% decomposition after 35 days.

Identification of fenitrothion degradation products in growth media and river water: Identification of fenitrothion biodegradation products was conducted by comparing the mass spectrum of standardly available metabolites (Fig. 6, 7) of fenitrothion that are known as its degradation products and those of samples using GC-MS analysis (Fig. 6, 7). The results showed that after 14 days of fenitrothion incubation with Izumi-1 isolate in M-medium, a peak with characteristic ions (m/z 247, 138 and 122) corresponding to amino fenitrothion (Fig. 7) was observed, indicating that this compound was one of the degradation products of fenitrothion. However, when fenitrothion was incubated with Izumi-1 isolate on C-medium (Izumi 1-C), this peak was not seen. Furthermore, after 21 days of fenitrothion incubation with Izumi-1 in both types of growth media (M and C), a peak with characteristic ions (m/z 136, 77 and 153) corresponding to 3-methyl-4-nitrophenol (Fig. 7) was observed. Moreover, during degradation of fenitrothion by Izumi 1-C isolate, 3-methyl-4-nitrophenol formation increased over time (Fig. 8), but this product was not observed in response to fenitrothion degradation or the formation rate was very low or almost stable with the time when we use Izumi-1 isolate on M-medium (Izumi 1-M) (Fig. 8b). The results

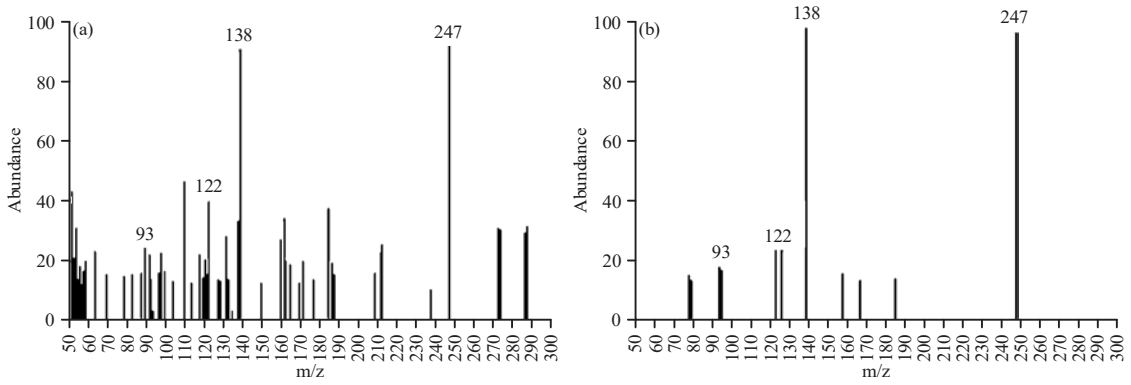

Fig. 6(a-b): Mass spectrum of amino fenitrothion as degradation product by (a) Izumi-1 isolate and (b) As standard

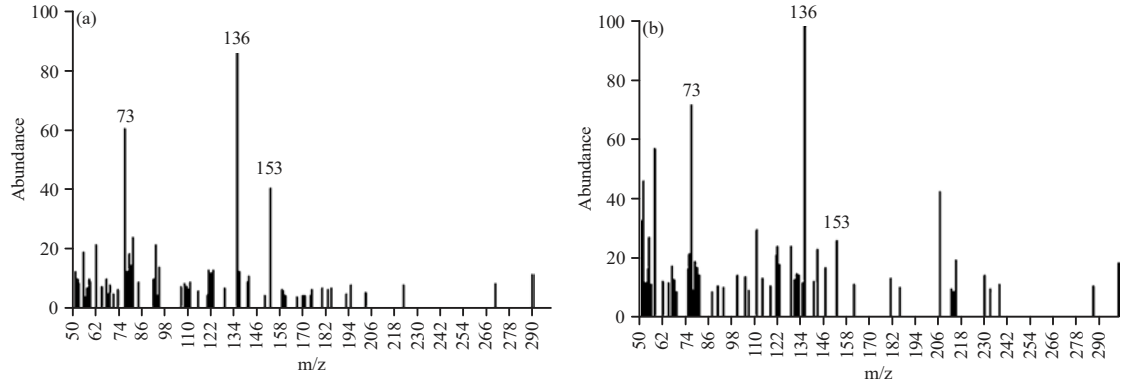

Fig. 7(a-b): Mass spectrum of 3-methyl-4-nitrophenol as degradation product by (a) Bacterial isolates and (b) As standard

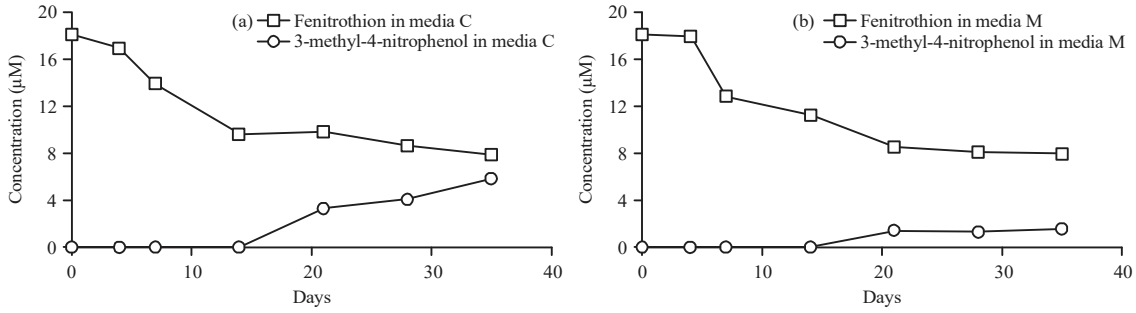

Fig. 8(a-b): Concentration of fenitrothion and 3-methyl-4-nitrophenol during degradation by (a) Izumi-1 in C-medium and (b) M-medium

also showed that there was a lag between the detection of 3-methyl-4-nitrophenol from the beginning of the degradation of fenitrothion. Therefore, a separate experiment to check the degradation of the 3-methyl-4-nitrophenol itself was conducted under the same conditions as for fenitrothion degradation. After 35 days, approximately 28% of this degradation product was decomposed by Izumi 1-M, while 25% was degraded by Izumi 1-C (Fig. 9). The degradation of fenitrothion by the Izumi-2 isolate generated a peak with characteristic ions (m/z 136, 77 and 153) that were identified as 3-methyl-4-nitrophenol. Moreover, this degradation product (3-methyl-4-nitrophenol) was detected from day 14 of incubation of fenitrothion with Izumi-2 isolate in the M-medium (Fig. 10a) and C-medium (Fig. 10b). Over time, the concentration of 3-methyl-4-nitrophenol increased, while that of fenitrothion decreased (Fig. 10a and b). However,

3-methyl-4-nitrophenol was considered to have accumulated without being able to further decompose, while the presence of other degradation products could not be confirmed. During the degradation of fenitrothion by Hinotsume-1, no decomposition products were detected. Additional examinations were conducted, but the results were similar.

During the degradation of fenitrothion by Izumi-1 in river water, 3-methyl-4-nitrophenol was not detected, but a peak identified as amino fenitrothion was found from day 14. However, during the degradation of fenitrothion by Izumi-2 isolate in river water, a peak identified as 3-methyl-4-nitrophenol was generated. During the degradation of fenitrothion in river water by the Izumi isolate (a mixture of Izumi-1 and Izumi-2), a peak identified as aminofenitrothion was detected from day 21 of incubation. During degradation of fenitrothion in river water using Hinotsume-1, no degradation products were identified even though this isolate showed a decomposition percentage of about 29% after 35 days.

Identification of isolated bacteria: The identification of bacteria isolated from different sampling sites in the Kurose River was conducted using their 16S rRNA nucleotide sequences and the results are summarized in Table 3. The bacterial strains (Izumi-1 and Hinotsume-1) isolated from water collected from the Izumi and Hinotsume sites were identified as *Flavobacterium* sp. However, comparison of the homology of the 16S rRNA base sequence indicated that the 2 identified strains (Hinotsume-1 and Izumi-1) were different from the congeners and the colonies formed were different. Izumi-2 was identified as *Pseudomonas putida*.

DISCUSSION

In this study, the results showed that there were differences in the degradability of fenitrothion between the 3 sampling sites that may have reflected differences in microflora composition in the water at different sampling sites (abundance and diversity). This finding is in agreement with those of Nishihara *et al.*[33], who reported that the structure of a bacterial community in an aquatic environment is dependent on both the physical and chemical conditions of their habitats. Moreover, the water quality data revealed higher DOC concentrations (which are an important source of carbon for microbial growth) at the Izumi and Hinotsume sites than the Namitakiji site, which reflects the faster biodegradation of fenitrothion in these 2 sites. Furthermore, the BOD values in Izumi and Hinotsume water were higher than in water from Namitakiji, which is considered a good indicator of a high level of microorganisms and reflects the faster degradation of fenitrothion in water from these 2 sites compared to the Namitakiji site[34]. The results also showed that the microbial community in Izumi site changed with the time and this agree with findings of Massana *et al.*[35], who demonstrated that bacterial communities change dramatically during long-term (8-10 days) incubations. However, the changes in community composition with time may result from the modification of substrate quality and quantity over time as reported by Gattuso *et al.*[36].

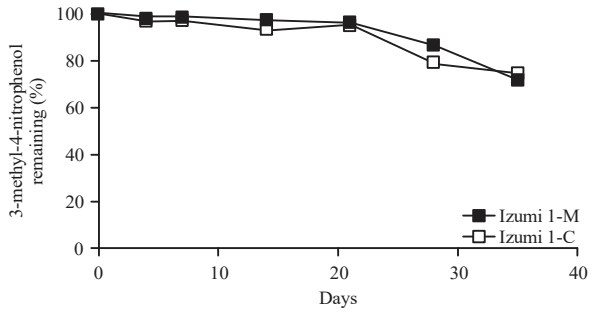

Fig. 9: Decomposition of 3-methyl-4-nitrophenol by Izumi-1 in different growth media

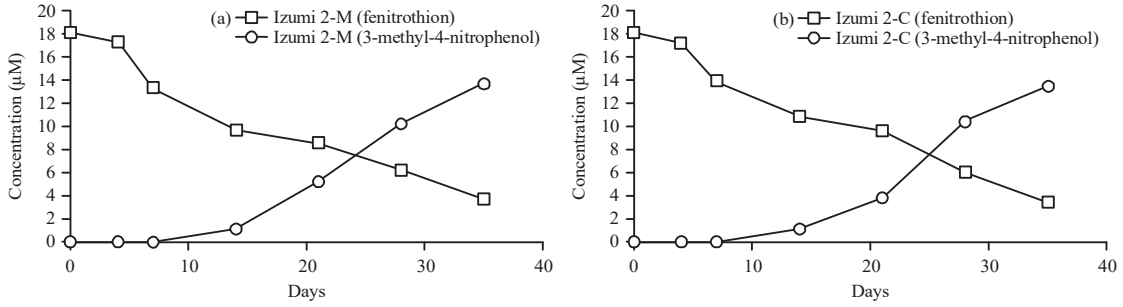

Fig. 10(a-b): Concentration of fenitrothion and 3-methyl-4-nitrophenol during the degradation of fenitrothion by (a) Izumi-2 in M and (b) C growth media

Table 3: Nucleotide sequence of 16S rRNA of different isolated bacterial strains

Bacterial isolates	Nucleotide sequence
Izumi-1	CGAGGGGTAGGGNTCTTCGGNNNCTGAGACCGGCGCACGGGTGCGTAACG
	CGTATGCAATCTGCCTTTCACAGAGGGATAGCCCAGAGAAATTTGGATTA
	ATACCTCATAGCATTGGAGAAGGGCATCCTTTTCCAATTAAAGTCACAAC
	GGTGAAAGATGAGCATGCGTCCCATTAGCTAGTTGGTAAGGTAACGGCTT
	ACCAAGGCGACGATGGGTAGGGGTCCTGAGAGGGAGATCCCCCACACTGG
	TACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTG
Izumi-2	AGCTTGCTCTCTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCT
	GCCTGGTAGTGGGGGACAACGTCTCGAAAGGGACGCTAATACCGCATACG
	TCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGC
	CTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGAT
	CCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGT
	CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAA
Hinotsume-1	ACTTNGGTGCTAGAGACCGGCGCACGGGTGCGTAACGCGTATGCAATCTG
	CCTTTCACAGAGGGATAGCCCANAGAAATTTGGATTAATACCTCATAGTA
	TTATGTTTCGGCATCGAGATATAATTAAAGTCACAACGGTGAAAGATGAG
	CATGCGTCCCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCTACGA
	TGGGTAGGGGTCCTGAGAGGGAGATCCCCCACACTGGTACTGAGACACGG
	ACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGGCGCA

In this study, three bacterial strains involved in the decomposition of fenitrothion and isolated from Kurose River water were Izumi-1 and Izumi-2 strains which identified as *Flavobacterium* sp. and *Pseudomonas putida*, respectively, while Hinotsume-1 strain identified was as *Flavobacterium* sp. The isolated and identified bacteria in Kurose River water were in agreement with the findings of Hashizume *et al.*[28], who reported that *Flavobacterium* sp. and *Pseudomonas* sp. are widespread in the environment and involved in the decomposition of environmental pollutants such as fenitrothion.

Evaluation of fenitrothion degradation in river water and growth media by the isolated bacterial strains revealed that it was about 10-40% of fenitrothion degradation lower in river water than in the growth media. This difference may have been because the chemical composition of media used to cultivate the microorganisms differed from that of the river water, indicating that decomposition of fenitrothion in the medium does not necessarily occur as in the river water. The results also showed that the degradation of fenitrothion in C-medium was also lower than that in M-medium, which may have been because fenitrothion was the only energy source in the inorganic salt medium (M-medium), while the C-medium contained fenitrothion and glucose as energy sources. As a result, bacterial isolates in the C-medium first used glucose as an energy source, after which they used fenitrothion, while in M-medium they used fenitrothion directly as an energy source from the onset of the experiment, which led to faster degradation of fenitrothion by bacterial isolates.

Regarding the degradation products of fenitrothion, the results confirmed that 3-methyl-4-nitrophenol and aminofenitrothion are known as the major biodegradation products of fenitrothion in growth media by Izumi-1 strain (*Flavobacterium* sp). These results are in agreement with Hashizume *et al.*[28] who reported that the products of fenitrothion degradation by *Flavobacterium* sp. were 3-methyl-4-nitrophenol and aminofenitrothion. On the other hand, the degradation of fenitrothion by Izumi-2 strain (*P. putida*) produced 3-methyl-4-nitrophenol only as the main product. However, Hinotsume-1 (*Flavobacterium* sp.) had an unknown mechanism of fenitrothion decomposition. These results agree with those of Maya *et al.*[37] and Li *et al.*[38], who reported that most bacterial strains that can degrade OPs belong to the genera *Pseudomonas*, *Bacillus*, *Serratia* and *Agrobacterium*. Moreover, *Pseudomonas* sp. has been reported to be involved in the degradation of environmental pollutants and some species of *Pseudomonas* have been confirmed to decompose fenitrothion[28]. On the other hand, the results of this study showed that the main biodegradation product of fenitrothion in river water was 3-methyl-4-nitrophenol. These findings are in accordance with those of another study that found -methyl-4-nitrophenol was one of the main fenitrothion degradation products in river water[6].

Therefore, the metabolic pathway of fenitrothion based on the obtained degradation products, as illustrated in Fig. 11. Firstly, fenitrothion degrade to produce aminofenitrothion and 3-methyl-4-nitrophenol and then 2 compounds may undergo for further degradation processes and finally

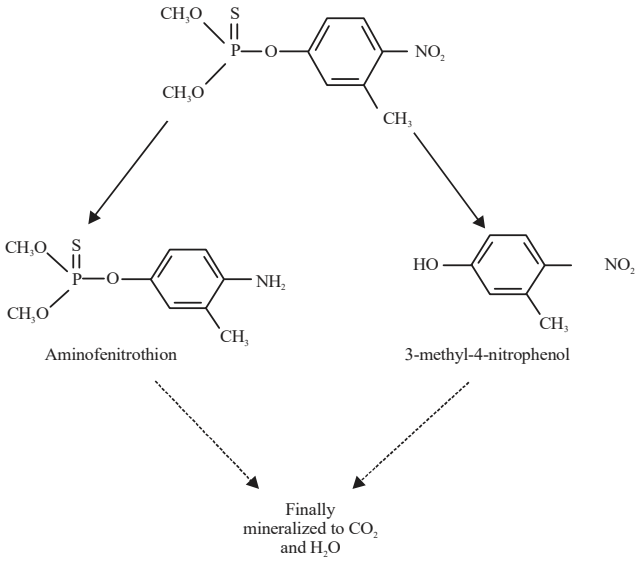

Fig. 11: Biodegradation pathway of fenitrothion

mineralized to carbon dioxide and water (Fig. 11). A possible mechanism for the formation of 3-methyl-4-nitrophenol byproduct may be through the hydrolase (OPH) enzyme, which hydrolyzes the P-O and P-S bonds of fenitrothion, resulting in the formation of 3-methyl-4-nitrophenol[39-41]. However, aminofenitrothion was formed by reduction of the nitro group in fenitrothion to give an amino group and considered a degradation product by *Acinetobacter* sp., *Pseudomonas* sp., *Alcaligenes* sp. and *Flavobacterium* sp. isolated from the Tenpaku River, Japan[28]. The presence of 3-methyl-4-nitrophenol as degradation products in river water may pose a risk to the aquatic organisms since 3-methyl-4-nitrophenol is more toxic to aquatic organisms than its parent compound (fenitrothion)[42] and its mutagenic formation potential is higher than that of fenitrothion[43]. Moreover, 3-methyl-4-nitrophenol have been reported to be potential endocrine disrupting substances[11,13,14,44].

CONCLUSION

Biodegradation of fenitrothion in Kurose River water was confirmed and the degradation rate at different sampling sites was found to depend on the microbial content and chemical composition of water. The bacteria identified from Kurose River water were *Pseudomonas putida* and *Flavobacterium* sp. The degradation rate of fenitrothion by the isolated bacteria was lower in river water than in the artificial growth media. The main degradation products of fenitrothion were amino-fenitrothion and 3-methyl-4-nitrophenol. Microbial community compositions and chemical characters of water considered very important factors to identify biodegradation kinetics and fate of pesticides in natural water.

SIGNIFICANCE STATEMENT

Earlier studies investigate biodegradation of fenitrothion or the microbial communities in natural water, however, this study investigated both biodegradation and microbial compositions. This study reflects the important role of that microbial community compositions and chemical characters of water on the fate and biodegradation kinetics of pesticides in natural water. This study open the way for further scientific investigation for isolation of biodegradation responsible genes and using for acerbation of fenitrothion biodegradation rate through gene cloning.

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