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Multiple Detection of Occurrence of Bacterial Pathogens in Two Rivers in the Kinki District of Japan with a DNA Microarray

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Abstract

Comprehensive understanding of the occurrence of pathogens in the aquatic environment is important for assessing possible biological health risks associated with water usage. The occurrence of bacterial pathogens in the Yodo and Kita Rivers, Kinki district, Japan, was investigated by using a DNA microarray targeting 1012 species/groups of bacterial pathogens infectious to human, animals, plants, fish, and shellfish. Eighty-seven pathogens were detected in 24 river water samples collected from two rivers, with more than half present in both rivers. The pathogen profile in the river waters varied primarily seasonally. Effluent from wastewater treatment plants, a well-known possible pathogen source, did not significantly affect the occurrence of bacterial pathogens in the monitored basins. Moreover, some of the detected pathogens, particularly non-fecal ones, did not positively correlate with the total coliform count, a conventional hygienic indicator. Therefore, the conventional hygienic indicator for fecal contamination is inadequate for comprehensive determination of the health risks associated with contamination of river water by bacterial pathogens.

Key words: bacterial pathogen; DNA microarray; hygienic indicator; river water; total coliform count

INTRODUCTION

Surface freshwater is a primary source of drinking water for the majority of the world's human population. In FY2001, of the approximately 17 billion m^3 of drinking water consumed in Japan, 72.3% originated from surface water sources such as reservoirs, rivers, and lakes (http://www.jwwa.or.jp/english/water_en/frame-e02.html). The appropriate assessment and management of surface water quality are of great importance to protect against potential health risks associated with unintentional ingestion of microbiologically contaminated surface water. Nevertheless, during the last decade, 21 health hazard cases in Japan were caused by pathogenic microorganisms, including *Escherichia coli, Campylobacter jejuni, Shigella sonne, Plesiomonas shigelloides, Yersinia enterocolitica, Leptospira* spp., *Clostridium botulinum*, norovirus, and rotavirus, in drinking water supplied from public or private water supply systems[1]. Plant and fish and shellfish pathogens have also caused serious damage to agriculture and fisheries and have disrupted natural ecosystems. To reduce the incidence of disease and problems caused by pathogenic microorganisms, more adequate prediction of pathogenic contamination of water sources is required, in addition to improvement of water resource management and disinfection systems.

To date, several systematic studies have

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been undertaken to determine the occurrence of bacterial pathogens in environmental waters[2-6]. These investigations targeted well-known pathogens such as *E. coli, Bacteroides, Campylobacter*, and *Salmonella*. Nevertheless, emerging and reemerging infectious diseases have been increasing yearly worldwide, and in Japan the causative microorganisms of diseases have included pathogens not targeted by previous studies[1]. It is obvious that the comprehensive detection and monitoring of multiple pathogens, including those not targeted in previous surveys, is very important for assessing the possible health risks associated with waterborne pathogens; i.e., it is urgent that the kinds and numbers of pathogens present in surface waters used as water sources be fully elucidated.

Microarray analysis has recently emerged as a promising tool that allows the simultaneous and specific detection of tens of thousands of genes on small glass slides. By application of the microarray technique, the pathogens present in the aquatic environment can be efficiently monitored and characterized, providing the informative data necessary for establishing effective strategies for bacterial pathogen control. This technique should allow those pathogens that need special attention, although ignored by current water safety assessment methods, to be identified. Moreover, the correlation of these newly identified important pathogens with one or more, possibly novel, hygienic indicators, would contribute much to advance the management of the aquatic environment. However, no DNA microarray analysis data on the comprehensive monitoring of a very wide range of pathogens have been published, and no report has completely characterized the pathogen risk in an aquatic environment such as a river basin. Several types of microarrays for the detection of pathogens have been designed[7-11]. However, the target organisms in these studies were limited to specific well-known pathogen groups (fewer than 100 different pathogens); the simultaneous analysis of hundreds or thousands of pathogens in the aquatic environment by the microarray technique has not been reported.

In this study, we performed a DNA microarray analysis targeting 1012 species/groups of bacterial pathogens infectious to humans, animals, plants, fish, and shellfish to comprehensively understand the occurrence and behavior of multiple bacterial pathogens in surface waters of two rivers in the Kinki district of Japan as a case study. We then assessed whether pathogens detected in multiple samples correlated with total coliform test results, a hygienic water quality indicator.

MATERIALS AND METHODS

River water samples A total of 24 subsurface water samples (30- to 50-cm depth) were collected from four stations on the Yodo River (Y1 to Y4, upstream to downstream) and two stations on the Kita River (K1 and K2, upstream and downstream, respectively) in the Kinki district of Japan (Fig. 1) in October 2005, August 2006, and January and May 2007. The Yodo River is the largest watershed in the Kinki district of Japan, with a catchment area of 8240 km^2. It is the main drinking water source of the more than 14 million residents of Osaka City and its 24 circumjacent cities. The Kita River is among the Japanese rivers with the best water quality, based on the biochemical oxygen demand (BOD) level. The river flows from northwestern Shiga Prefecture through southwestern Fukui Prefecture, and its catchment area is 842 km^2. The collected river water samples were transported on ice to the laboratory and subjected to water quality analysis on the same day and DNA extraction within 12 h of collection.

DNA Microarray We purchased a microarray for detecting bacterial pathogens from AMR Inc. (Gifu, Japan). Oligonucleotide probes for the 16S rRNA genes of 1012 bacterial pathogens infectious to humans, animals, plants, fish, and shellfish are mounted on this microarray. The target pathogens include all biosafety level (BSL) 2 and 3 pathogens in the classification of Japanese Society for Bacteriology[12] and other opportunistic pathogens. This microarray was developed by Prof. Takayuki Ezaki and his coworkers at Gifu University, Japan, for the comprehensive, accurate, and rapid testing of the causative pathogens of infectious diseases, including emerging/reemerging diseases,

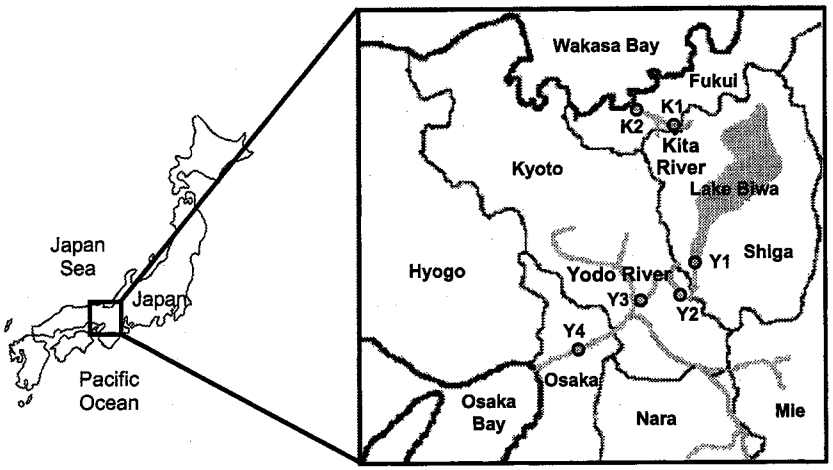

Fig. 1 Locations of sampling stations.

which are increasing in number and becoming more globalized. Applicability of the microarray to environmental samples had been assured by the developers.

Microarray analysis DNA was extracted from river water samples as described elsewhere[13]. The conserved region (ca. 510 bp) of eubacterial 16S rDNA was PCR amplified using the 8UA (5'-AGA GTT TGA TCM TGG CTC AG-3') and 519B (5'-GTA TTA CCG CGG CKG CTG-3') primer set with a Mastercycler Standard (Eppendorf, Tokyo, Japan). The 5'-end of the reverse primer was labeled with Cy3 dye to fluorescently label the PCR products. Amplified products were purified by ethanol precipitation.

Microarray hybridization was performed in accordance with the manufacturer's instructions. Cy3-labeled target DNA (35 μg) was dissolved in a 50 μl hybridization buffer (5× SSC, 0.5% sodium dodecyl sulfate), denatured at 90 ℃ for 1 min, cooled to 55 ℃, and hybridized with the prehybridized array in a hybridization chamber (DNA Chip Research Inc., Kanagawa, Japan) at 55 ℃ for 16 h. Following hybridization, the microarray slides were scanned with an arrayWoRx (GE Healthcare UK Ltd., Buckinghamshire, England). Scanned images were then processed with Array Vision ver. 8.0 (GE Healthcare UK Ltd.). After subtraction of the background intensity, the signal intensities of the spots were normalized in relation to the intensity of the positive Cy3 spots. Test spots whose relative signal intensity (RSI) exceeded 0.25 were considered positive and used for further analysis.

Water quality measurements Water temperature, electrical conductivity, pH, and dissolved oxygen (DO) were recorded at the sampling site. Concentrations of dissolved organic carbon (DOC), total nitrogen (T-N), heterotrophic bacteria, eubacterial 16S rDNA, and total coliforms were analyzed in the laboratory. DOC was analyzed with a total organic carbon analyzer (TOC-5000A; Shimadzu, Kyoto, Japan). Concentrations of T-N were measured by the standard method[14]. Heterotrophic bacteria were determined by plating using a 1/10 diluted CGY medium[15]. The eubacterial 16S rDNA number was quantified from a DNA template prepared as described above by most-probable-number (MPN)-PCR[16] using the EUBf933 and EUBr1387 primer set[17]. Total coliforms were quantified by the MPN method using a slightly modified standard coliform medium (lactose 5 g/*l*, bonito extract 3 g/*l*, peptone 10 g/*l*, pH 7.0).

Statistical analysis Correlation analysis between the RSI of each pathogen and the total coliform count relative to the total number of heterotrophic bacteria was performed with Microsoft Excel 2002 (Microsoft Corporation, Redmond, WA, USA). Principal component analysis (PCA) against the occurrence pattern (presence/absence) of the pathogens in the river water samples was carried out with SPSS 15.0 for Windows

(SPSS Inc., Chicago, IL, USA).

RESULTS

Physiological and biological water quality parameters Physiological and biological water quality parameters in the river water samples are listed in Table 1. Water temperature varied according to the season. Although electrical conductivity was low in almost all samples, it was exceptionally high at station K2 in spring, summer, and autumn, indicating that a brackish water environment had developed as a result of the backflow of marine water. Concentrations of DOC and T-N tended to increase between Y2 and Y3 in the Yodo River, possibly related to effluents from wastewater treatment plants (WWTPs) along the river. Heterotrophic bacteria occurred in river water samples in quantities on the order of 10^3 to 10^5 CFU/m*l*. Total coliform counts in spring, summer, and winter samples varied from $<3.6 \times 10^1$ to 9.3×10^3 MPN/100 m*l*. Spring and summer samples tended to have higher total coliform counts than winter samples.

Pathogen profile in river water samples A total of 87 bacterial pathogen species/groups, including 21 BSL2 pathogens, were detected by the DNA microarray analysis of 24 river water samples (Table 2). Forty-nine of the 87 pathogens (listed in Table 3) were present in two or more samples, and 45 of these pathogens were found in both rivers. Furthermore, 27 of the 49 pathogens were present in two distinct seasons, whereas the other 22 were detected in only one sampling period. The distribution of the other 38 pathogens, which each occurred in only one sample, was as follows: 3 at Y2, 6 each at Y1, Y3, and Y4, and 17 at K2. Although most of the detected pathogens were human or animal pathogens, two fish/shellfish-infectious pathogens, *Pseudoalteromonas atlantica*[18] and *Vibrio cholerae/mimicus*[19], and two plant

Table 1 Physicochemical and biological water quality parameters of the river water samples in this study[a]

Sampling date	Sampling station	Temp (℃)	pH	DO (mg/*l*)	Conductivity (mS/cm)	DOC (mg/*l*)	T-N (mg/*l*)	Heterotrophic bacteria (CFU/m*l*)	16S rDNA (MPN-copies/m*l*)	Total coliforms (MPN/100 m*l*)
October 2005	Y1	21.2	8.1	7.9	0.1	1.5	1.1	2.7×10^4	2.4×10^5	na[a]
	Y2	21.5	7.2	6.8	0.1	1.1	1.4	7.1×10^3	1.5×10^5	na
	Y3	22.0	7.3	7.4	0.2	1.9	2.0	1.3×10^4	2.1×10^5	na
	Y4	21.8	6.6	6.4	0.2	2.0	1.6	3.3×10^4	2.4×10^4	na
	K1	13.4	6.7	9.5	0.1	0.36	2.4	5.7×10^3	2.3×10^3	na
	K2	11.8	6.5	6.5	13.9	1.8	2.6	1.9×10^5	7.0×10^3	na
August 2006	Y1	30.0	9.0	6.9	1.4	na	na	1.1×10^4	2.3×10^4	4.3×10^2
	Y2	28.6	7.3	4.8	0.1	na	na	1.4×10^4	2.3×10^4	9.0×10^1
	Y3	29.8	7.5	6.1	0.2	na	na	4.0×10^4	9.3×10^4	9.3×10^2
	Y4	30.2	8.5	6.3	0.1	na	na	4.0×10^4	9.3×10^4	9.3×10^2
	K1	21.6	8.2	9.3	<0.1	na	na	6.3×10^3	2.4×10^4	1.4×10^2
	K2	30.0	7.8	3.3	50.1	na	na	1.1×10^5	1.5×10^4	4.3×10^3
January 2007	Y1	7.2	7.5	7.8	0.1	6.3	0.87	4.6×10^4	9.3×10^3	3.6×10^1
	Y2	6.5	7.6	8.1	0.1	5.5	0.80	7.8×10^3	4.3×10^3	$<3.6 \times 10^1$
	Y3	13.1	7.1	5.9	0.3	11.3	4.6	1.7×10^4	2.4×10^4	7.4×10^2
	Y4	8.4	7.5	4.2	0.1	6.1	1.7	4.4×10^4	9.3×10^3	2.1×10^2
	K1	6.6	7.2	6.3	<0.1	2.8	0.67	4.1×10^3	1.5×10^3	3.6×10^1
	K2	7.1	6.9	6.8	0.1	4.8	0.50	4.4×10^4	2.1×10^3	2.4×10^3
May 2007	Y1	19.8	7.7	9.1	0.1	2.4	0.34	4.6×10^4	2.4×10^6	2.3×10^2
	Y2	19.4	7.4	7.6	0.1	2.7	0.69	7.9×10^3	2.4×10^4	2.9×10^2
	Y3	21.0	7.1	7.0	0.2	8.2	1.9	4.7×10^3	9.3×10^6	1.5×10^3
	Y4	20.5	7.4	7.9	0.1	4.5	1.5	1.8×10^4	9.3×10^4	2.1×10^2
	K1	12.8	7.2	8.8	0.1	8.2	0.88	4.5×10^3	2.4×10^4	na
	K2	16.8	6.6	6.2	7.0	3.3	0.90	5.7×10^4	9.3×10^4	9.3×10^3

[a] na, not analyzed.

Table 2 Relative signal intensity of positive probes in 24 river water samples[a]

Pathogen species/group	October, 2005						August, 2006						January, 2007						May, 2007					
	Y1	Y2	Y3	Y4	K1	K2	Y1	Y2	Y3	Y4	K1	K2	Y1	Y2	Y3	Y4	K1	K2	Y1	Y2	Y3	Y4	K1	K2
Acetivibrio cellulosolvens	0.62	0.25	0.51	0.30		0.60					0.31	1.42												
Acinetobacter aceti							0.30																	
Acinetobacter anitratum						0.56																		
Acinetobacter baumannii						0.56																		
Acinetobacter haemolyticus						0.54																		
Acinetobacter johnsonii						0.61																		
Acinetobacter junii						0.57																		
Acinetobacter lwoffii						0.41																		
Acinetobacter radioresistens						0.25																		
Actinobacillus muris													0.61	0.43	0.64	0.62	0.87	0.65	0.57	0.56	0.63	0.81	0.57	
Actinobacillus pleuropneumoniae													1.06	0.76	0.95	0.82	0.82	0.79	1.05	0.58	0.65	0.93	0.63	0.57
Actinomadura spp.	0.71					0.57																		0.83
Aegyptianella pullorum	0.29					0.49																		
Agrobacterium tumefaciens group						0.43																		
Anaplasma marginale/centrale	0.35					0.29																		
Anaplasma phagocytophila	0.30					0.37																		
Arcobacter genus	0.68	0.26	0.60	0.61		0.59																		
Arcobacter sp.	0.28																							
Bacteroides distasonis																						0.47	0.30	0.62
Bacteroides fragilis																								0.97
Bacteroides urealyticus	0.66	0.54	0.58	0.69		0.64					0.54													
Balneatrix alpica													0.57	0.49	0.90	0.62	0.79	0.63	0.54	0.55	0.64	0.58	0.59	0.97
Bordetella avium group	0.26																							
Borrelia burgdorferi/valaisiana									0.44															
Brevundimonas diminuta	0.75	0.37	0.63	0.56		0.66					0.72													
Brevundimonas group	0.68	0.43	0.58	0.62		0.63					0.49													
Campylobacter concisus	0.71	0.52	0.63	0.30		0.54																		
Campylobacter fetus group	0.33		0.26			0.34																		
Campylobacter jejuni group	0.69		0.61	0.27		0.58																		
Campylobacter rectus									0.57			0.50												
Campylobacter sputorum	0.59					0.40																		
Centipeda periodontii																					0.39			
Chromobacterium violaceum									0.42															
Chryseobacterium meningosepticum group (1)[b]	0.59	0.33	0.52	0.30		0.56					0.29													
Chryseobacterium meningosepticum group (2)								0.27	0.95															
Chryseobacterium proteolyticum						0.36																		
Chryseobacterium scophthalmum						0.30																		
Clavibacter michiganensis							1.19	1.19	0.91	1.42		1.35												
Corynebacterium mycetoides						0.31																		
Eggerthella lenta group										0.33														
Eperythrozoon spp.							1.15	1.13	1.30	0.61		1.04												
Erysipelothrix spp.						0.31																		
Erysipelothrix rhusiopathiae	1.29	0.38	0.63	0.81		0.67					0.65													

Pathogen species/group	October, 2005						August, 2006						January, 2007						May, 2007					
	Y1	Y2	Y3	Y4	K1	K2	Y1	Y2	Y3	Y4	K1	K2	Y1	Y2	Y3	Y4	K1	K2	Y1	Y2	Y3	Y4	K1	K2
Erysipelothrix tonsillarum	0.65	0.29	0.37	0.66		0.57					0.56													
Eubacterium combesii									0.37															
Ewingella americana							1.17	1.17	1.03	1.33		1.37												
Haemophilus haemolyticus												0.33												
Haemophilus influenzae (1)													0.87	0.82	0.65	0.63	0.73	0.73	0.62	0.64	0.66	0.58	1.04	0.81
H. influenzae (2)													1.06	0.56	0.86	0.82	0.74	0.79	1.04	0.55	1.05	0.97	0.64	0.76
Haemophilus parasuis (1)													0.89	0.76	0.90	0.56	0.59	0.65	0.98	0.61	1.05	0.61	1.01	0.94
H. parasuis (2)													0.63	0.68	0.62	0.81	0.78	0.78	0.57	0.58	0.63	0.96	0.61	0.58
Hafnia alvei							1.22		0.38	0.38		0.89												
Klebsiella oxytoca group							0.38		0.26			0.23												
Kluyvera ascorbata	0.68	0.50	0.56	0.51		0.59					0.32													
Kluyvera cryocrescens	0.62		0.32	0.28		0.57					0.28													
Lactobacillus spp.	0.30		0.26																					
Legionella brunensis										0.27														
Legionella spiritensis										0.31														
Leptospira noguchii							0.46	1.22	1.46	1.24		1.36												
Leptospira parva							0.28		0.44	0.29														
Leptospira santarosai									0.49			0.32												
Mannheimia granulomatis													0.97	0.81	0.89	0.53	0.46	0.69	0.57	0.57	0.63	0.57	0.60	0.55
Mannheimia haemolytica													1.06	0.89	0.94	0.85	0.77	0.79	0.63	1.02	1.07	0.61	1.07	0.77
Marinospirillum megaterium													1.03	0.74	0.94	0.77	0.63	0.60	0.60	1.01	1.01	0.80	1.04	0.93
Moraxella caviae													1.06	0.58	0.64	0.94	0.86	0.62	0.65	1.05	1.07	0.90	1.06	0.94
Moraxella lacunata group													1.03	0.89	0.51	0.50	0.41	0.34	1.04	1.02	1.05	0.89	1.06	0.94
Mycobacterium mucogenicum													0.27		0.34					0.33		0.80	0.35	0.69
Mycobacterium nonchromogenicum									0.42															
Mycoplasma kahnei						0.34																		
Olsenella uli	1.11		0.31			0.48																		
Pasteurella bettyae													1.06	0.58	0.97	0.74	0.54	0.71	0.85	1.01	1.05	1.02	1.06	0.85
Pasteurella caballi														0.99	0.85	0.57	0.44	0.65	0.82	0.94	0.91	0.59	1.00	0.85
Pasteurella pneumotropica (1)													1.06	0.92	0.96	0.80	0.78	0.83	1.04	1.02	1.05	0.96	1.06	0.84
Pasteurella pneumotropica (2)													1.03	1.05	0.47	0.58	0.80	0.79	0.99	0.65	1.06	0.64	1.02	0.89
Peptoniphilus asaccharolyticus										0.30														
Porphyromonas cangingivalis								0.81																
Proteus vulgaris							0.26																	
Pseudoalteromonas atlantica group								0.31																
Rhodococcus equi								0.40																
Selenomonas ruminantium												1.37												
Slackia heliotrinreducens	0.39																							
Sphingobacterium multivorum									0.29															
Sphingobacterium thalpophilum				0.26																				
Sphingomonas paucimobilis	0.28																							
Staphylococcus capitis/caprae										0.27														
Treponema denticola							0.35	0.51	1.33	0.67		1.14												
Vibrio cholerae/mimicus																								0.46

[a]Blank entries indicate a negative result (relative signal intensity < 0.25).
[b]Numbers in parentheses following the species name indicate that probes were designed based on different sequences of the same species.

pathogens, *Agrobacterium tumefaciens*[20] and *Clavibacter michiganensis*[21], were also detected, each in only one of the 4 sampling periods.

The number of pathogen species/groups found varied among samples (Fig. 2). Similar numbers of pathogens (16 to 20 species) were detected at all six sampling stations in spring and winter. Even the pathogen profile was almost identical in the 12 samples collected in these seasons; the following species were present in all 12 samples: *Actinobacillus pleuropneumoniae*, *Balneatrix alpica*, *Haemophilus influenzae* and *H. parasuis*, *Mannheimia granulomatis* and *M. haemolytica*, *Marinospirillum megaterium*, and *Moraxella caviae* and *M. lacunata*. Summer samples contained the lowest numbers of bacterial pathogen species (9 to 16 species) among the four seasons. In autumn, 10, 15, and 13 pathogen species occurred at stations Y2, Y3, and Y4, respectively. In contrast, in the same season, 24 and 32 different pathogens occurred at stations Y1 and K2, respectively. Exceptionally, all 1012 bacterial pathogens

Table 3 Biosafety level of pathogens detected in two or more samples[a]

Pathogen species/group	BSL[b]	Pathogen species/group	BSL	Pathogen species/group	BSL
Acetivibrio cellulosolvens	–	*Campylobacter sputorum*	1	***Leptospira noguchii***	1
Actinobacillus muris	–	*Chryseobacterium meningosepticum* group (1)[c]	2	***Leptospira parva***	–
Actinobacillus pleuropneumoniae	1	***C. meningosepticum* group (2)**	2	***Leptospira santarosai***	1
Actinomadura spp.	2	***Clavibacter michiganensis***	–	***Mannheimia granulomatis***	1
Aegyptianella pullorum	1	***Eperythrozoon* spp.**	1	***Mannheimia haemolytica***	1
Anaplasma marginale/centrale	1–2	*Erysipelothrix rhusiopathiae*	2	***Marinospirillum megaterium***	–
Anaplasma phagocytophila	2	*Erysipelothrix tonsillarum*	–	***Moraxella caviae***	–
Arcobacter genus	1	***Ewingella americana***	1	***Moraxella lacunata* group**	1
Bacteroides distasonis	1	***Haemophilus influenzae* (1)**	2	***Mycobacterium mucogenicum***	2
Bacteroides urealyticus	1	***H. influenzae* (2)**	2	*Olsenella uli*	1
Balneatrix alpica	1	***Haemophilus parasuis* (1)**	2	***Pasteurella bettyae***	1
Brevundimonas diminuta	1	***H. parasuis* (2)**	2	***Pasteurella caballi***	–
Brevundimonas group	1	***Hafnia alvei***	1	***Pasteurella pneumotropica* (1)**	2
Campylobacter concisus	1	***Klebsiella oxytoca* group**	2	***P. pneumotropica* (2)**	2
Campylobacter fetus group	2	*Kluyvera ascorbata*	1	***Treponema denticola***	1
Campylobacter jejuni group	1–2	*Kluyvera cryocrescens*	1		
Campylobacter rectus	1	*Lactobacillus* spp.	1		

[a] Pathogen species/groups shown in boldface were assessed for their correlation with total coliforms.
[b] Biosafety level (BSL) according to the Japanese Society for Bacteriology[12].
[c] Numbers in parentheses following the species name indicate that probes were designed based on different sequences of the same species.

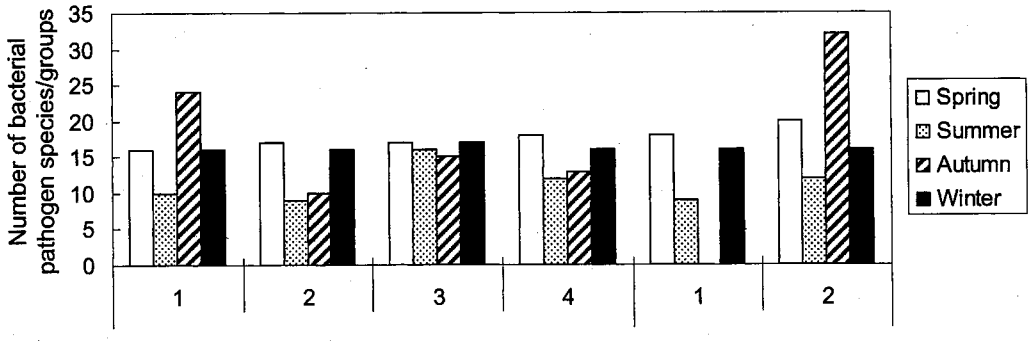

Fig. 2 Spatial and temporal variations in the number of pathogenic bacterial species in the Yodo and Kita rivers. Probes with relative intensity > 0.25 in the microarray analysis were judged as positive.

targeted were below the detection limit in autumn at station K1.

In the Yodo River, 16, 5, 9, and 15 species/groups were detected at all four stations in spring, summer, autumn, and winter, respectively (Table 2; Fig. 2). The number of pathogen species increased from Y2 to Y3 and slightly decreased from Y3 to Y4 in summer and autumn. In summer, 7 and 5 species/groups that were never detected at the upstream stations Y1 and Y2 were found at Y3 and Y4, respectively. In the Kita River, the pathogens detected at stations K1 and K2 were completely different between stations in summer and autumn although almost identical in spring and winter, as mentioned above.

In the PCA against the occurrence pattern of bacterial pathogens in the river water samples, excluding the autumn sample from K1, in which no pathogen was detected, 73.0% of the total variation was explained by the first (PC1) and second (PC2) principal components. Scatter plot based on PC1 and PC2 revealed that the 23 analyzed samples fell into three distinct groups A, B, and C, depending basically on the season of sample collection (Fig. 3); group A consisted of all of the spring and winter samples, group B consisted of the autumn samples from all stations on the Yodo River and station K2 on the Kita River and a summer sample from K1, and group C consisted of the summer samples from all stations on the Yodo River and from K2 on the Kita River.

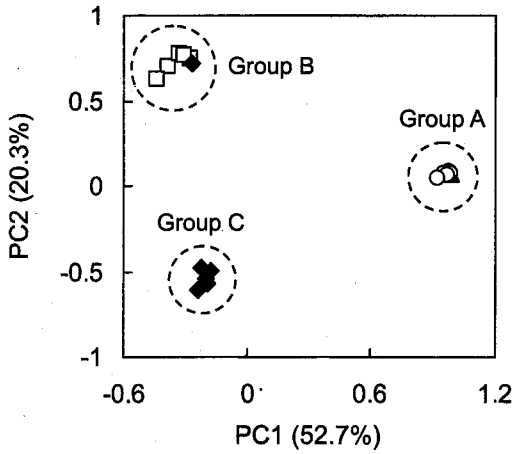

Fig. 3 Ordination produced from a principal component analysis based on pathogen profiles of river water samples collected from the Yodo and Kita rivers in spring (open circles), summer (closed diamonds), autumn (open squares), and winter (closed triangles).

Correlation between pathogens detected in microarray analysis and total coliforms The correlation between the RSIs of the 30 pathogens detected in two or more samples in spring, summer, or winter (shown in boldface in Table 3) and the relative total coliform count was assessed. The RSI of 19 species increased with the relative total coliform count (e.g., Fig. 4 A–C). In contrast, the remaining 11 pathogenic bacteria did not show a positive correlation with the relative total coliform count (e.g., Fig. 4 D–F). The latter group included 4 fecal bacteria (*Campylobacter rectus, Leptospira noguchii, Leptospira parva, Leptospira santarosai*) and 7 non-fecal bacteria (*Actinobacillus pleuropneumoniae, Eperythrozoon* spp., *Haemophilus influenzae, Haemophilus parasuis, Klebsiella oxytoca* group, *Mannheimia granulomatis*, and *Pasteurella pneumotropica* [2 targets]).

DISCUSSION

We reported the simultaneous detection of multiple pathogens in surface waters from the Yodo and Kita Rivers in the Kinki district of Japan. Of the two monitored rivers, the Yodo River is a relatively polluted urban river, whereas the Kita River is a clean, rural river. The geographical features of the two river basins suggest that neither has any potential fecal sources other than WWTPs. No unusual abundance of fecal indicator bacteria in the WWTP effluents or in the surface waters of either river has been reported in recent years. Therefore, the health risk associated with waterborne pathogens does not appear to be easily predictable by use of the conventional fecal indicators in these basins. Nevertheless, we found a total of 87 pathogen species/groups in our survey. In addition, more than half were present in both rivers, and one-third occurred in two seasons. These results suggest that specific groups of bacterial pathogens may be commonly present in surface waters in our monitoring region. Furthermore, the detection of fish/shellfish

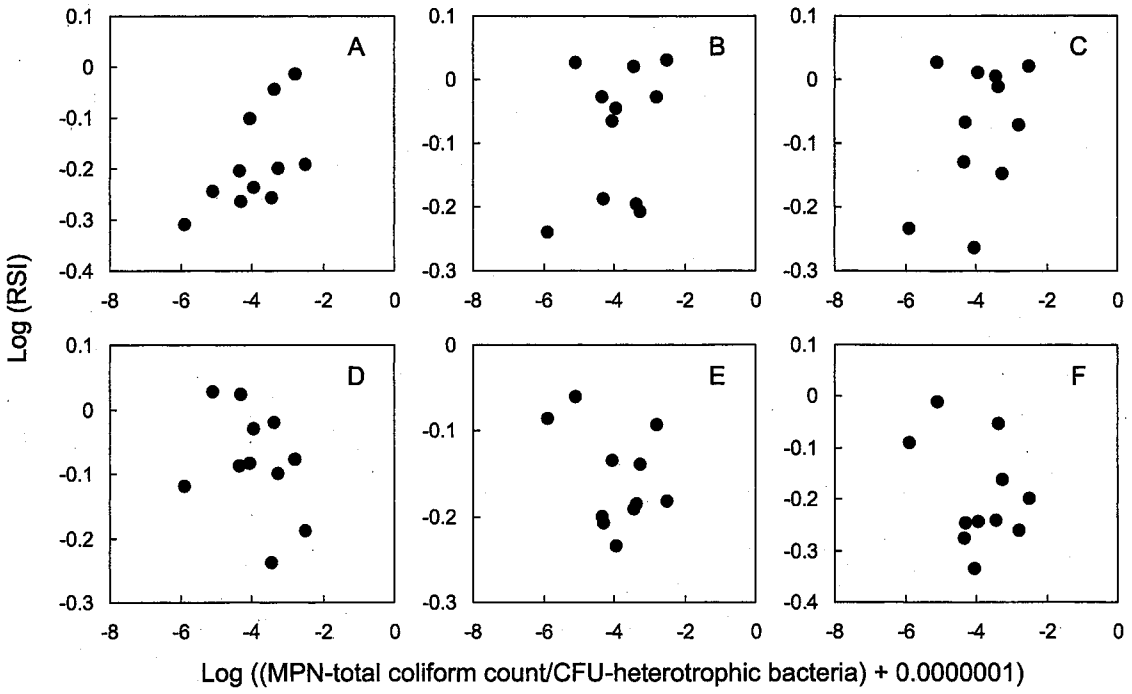

Fig. 4 Examples of correlations between the coliform count and the relative signal intensity of pathogen probe detected. The total coliform count relative to the total number of heterotrophic bacteria was used. A, *Balneatrix alpica*; B, *Moraxella caviae*; C, *Pasteurella bettyae*; D, *Actinobacillus pleuropneumoniae*; E, *Haemophilus influenzae* (1); F, *Mannheimia granulomatis*. A, B, and C are examples of a positive correlation, and D, E, and F are examples without any positive correlation.

and plant pathogens, in addition to human and animal pathogens, indicates that the surface waters may pose health hazards to various organisms, with consequent economic and ecological damage.

The pathogen profile in the surface waters of the monitored rivers varied primarily according to the season. Seasonal variables are well known to be one of the most important factors determining bacterial survival in the natural environment. Many previous studies have reported that the composition of the bacterial community in river environments is season dependent[22,23]. Thus, the pathogen profile in the monitored rivers can be expected to change in accordance with the natural, season-dependent appearance/disappearance of microorganisms. It has been also reported that the incidence of some kinds of pathogens in river environments is influenced by seasonal variables, particularly water temperature[24-27].

Bacterial pathogens enter surface waters from both point and non-point sources, including in raw sewage, effluent from WWTPs, and run-off from agriculture and livestock farming[4,28-30]. Among these potential sources, effluent from WWTPs is recognized as an important pathogen source that can alter the pathogen profile along a river's course. In our monitoring area, several WWTPs are located between stations Y2 and Y4 on the Yodo River. The number of pathogen species/groups became marginally elevated between Y2 and Y3 in summer and autumn, and several pathogens that were absent at the upstream stations emerged at Y3 and Y4 in summer. From these results, we inferred that effluent from WWTPs slightly impacted the pathogen profile of the Yodo River in summer and autumn. However, no noticeable impact was observed in spring or winter, suggesting that the influence of WWTPs on the pathogen profile in the Yodo River is marginal, despite the input of a large amount of WWTP effluent by repeated use of river water (by the time it reaches the river's mouth the downstream, the water has been used five times)[31], compared with the predominant impact by seasonal factors. In

the Yodo River basin, coverage of the sewer system was nearly 90% in FY2004. In addition, WWTPs within the basin are equipped with satisfactory disinfection units. These facts suggest that WWTPs are not a significant pathogen source in the basin because the public sewer system has been sufficiently improved, even though the Yodo River basin is relatively polluted according to the BOD level.

The bacterial species diversity in river water increases at the mouth, where a brackish water environment results from the mixing of river water and seawater[32,33]. Thus, we speculated that the pathogen diversity would also be higher at the mouth of a river than further upstream where no mixing occurred. As expected, our results showed that the pathogen species richness in the Kita River was greater at K2, near the mouth of the river (nearly 500 m upstream from the mouth), than at K1. The occurrence of the highest number of distinct pathogens at K2 presumably also resulted from the unusual condition of a brackish water environment, namely, characteristics intermediate between those of freshwater and seawater. Therefore, waters at the river mouth, under brackish water conditions, are likely to serve as a reservoir of diverse bacterial pathogens even in the case of a less-polluted river like the Kita River.

Recent studies have shown that conventional hygienic water quality indicators are not well correlated with feces-related bacterial pathogens such as the *Bacteroides-Prevotella* group[34], *Campylobacter* spp.[3], *Salmonella* spp.[4], and *Yersinia* spp.[35] or eukaryotic pathogens such as *Cryptosporidium* spp.[3,4,35]. In this study, we also observed that 11 of the 30 pathogen species/groups assessed did not show a significant positive correlation with total coliforms. These species/groups included not only opportunistic but also BSL2 pathogens (*Haemophilus influenzae, Haemophilus parasuis, Klebsiella oxytoca,* and *Pasteurella pneumotropica*), and more than half were non-fecal. To our knowledge, this is the first report suggesting the possibility that multiple pathogens, including both fecal and non-fecal ones, show a low correlation with the conventional hygienic indicator. The lack of a positive correlation between these pathogens and the conventional fecal indicator may reflect a dissimilarity in the environmental behavior between some bacterial pathogens and the indicator bacteria[3,4]. Moreover, with respect to non-fecal pathogens, the source and route of their discharge into surface waters differ from those of the fecal indicator bacteria, which may be another important reason for the low correlation. Because species of *Leptospira,* which have in fact caused a human health hazard via the drinking water supply in Japan[1], were among the pathogens that did not correlate with total coliforms, it is clear that the conventional hygienic indicators cannot necessarily predict the occurrence of significant bacterial pathogens. Therefore, systematization of a new set of indicators that can comprehensively predict the occurrence of various pathogens is strongly required for assessment of the health risks associated with waterborne pathogens. Among the pathogens that did not positively correlate with the total coliforms in this study, high-risk (BSL2) and non-fecal pathogens may be candidates for new indicators. From the viewpoint of preventing damage to agriculture and fisheries as well as ecological damage, pathogens infectious to fish/shellfish and plants should also be considered as candidate indicators for advanced management of the aquatic environment. Further study on the simultaneous determination of the occurrence of the candidate indicator pathogens suggested here and in earlier studies[3-5,34,35] should be performed by cost-effective and reliable molecular tools such as multiplex real-time PCR[36,37] to establish an ideal set of hygienic indicators for advanced management of the aquatic environment.

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Abiotic degradation of four phthalic acid esters in aqueous phase under natural sunlight irradiation

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Abstract

Abiotic degradability of four phthalic acid esters (PAEs) in the aquatic phase was evaluated over a wide pH range 5–9. The PAE solutions in glass test tubes were placed either in the dark and under the natural sunlight irradiation for evaluating the degradation rate via hydrolysis or photolysis plus hydrolysis, respectively, at ambient temperature for 140 d from autumn to winter in Osaka, Japan. The efficiency of abiotic degradation of the PAEs with relatively short alkyl chains, such as butylbenzyl phthalate (BBP) and di-*n*-butyl phthalate (DBP), at neutral pH was significantly lower than that in the acidic or alkaline condition. Photolysis was considered to contribute mainly to the total abiotic degradation at all pH. Neither hydrolysis nor photolysis of di-ethylhexyl phthalate (DEHP) proceeded significantly at any pH, especially hydrolysis at neutral pH was negligible. On the other hand, the degradation rate of di-isononyl phthalate (DINP) catalyzed mainly by photolysis was much higher than those of the other PAEs, and was almost completely removed during the experimental period at pH 5 and 9. As a whole, according to the half-life ($t_{1/2}$) obtained in the experiments, the abiotic degradability of the PAEs was in the sequence: DINP (32–140 d) > DBP (50–360 d), BBP (58–480 d) > DEHP (390–1600 d) under sunlight irradiation (via photolysis plus hydrolysis). Although the abiotic degradation rates for BBP, DBP, and DEHP are much lower than the biodegradation rates reported, the photolysis rate for DINP is comparable to its biodegradation rate in the acidic or alkaline condition.

Key words: phthalic acid esters; abiotic degradation; photolysis; hydrolysis; first-order kinetics

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Introduction

In recent years, considerable attention has been paid to toxicity and degradability of phthalic acid esters (PAEs) (Staples *et al.*, 1997), which have been frequently detected throughout aquatic environment (Fromme *et al.*, 2002; Yuan *et al.*, 2002). PAEs undergoing hydrolysis, photolysis, and aerobic/anaerobic biodegradation could be removed from aquatic environment. Among those different degradation processes, relatively abundant data are available on biodegradation of PAEs (Chang *et al.*, 2007; Li *et al.*, 2005; Yuan *et al.*, 2002; Wang *et al.*, 1996, 2000). On the other hand, limited information has been known on the abiotic processes (hydrolysis and photolysis). There are several researchers who have focused on photolysis of PAEs using artificial irradiation sources, such as xenon arc lamps (Bajt *et al.*, 2001), mercury lamps (Mailhot *et al.*, 2002), and ultraviolet light (Lau *et al.*, 2005), to clean up the PAEs-contaminated water. However, those irradiation sources had very different radiation intensities and wavelength distributions from natural sunlight irradiation. As a rare study for describing the abiotic degradation of PAEs under natural aquatic environment, Gledhill *et al.* (1980) have reported that butylbenzyl phthalate (BBP) exposing to sunlight irradiation for 28 d resulted in less than 5% degradation, but limited information is available to date. Wolfe *et al.* (1980) inferred that according to a mathematical model data the photolysis is the primary degradation process of PAEs in oligotrophic lakes. For better understanding of the fate of PAEs in aquatic environment, there is a need to conduct more realistic studies on abiotic degradation with natural solar intensity.

The aim of this study was to assess the contribution of abiotic degradation of PAEs under the sunlight irradiation at ambient temperature over a wide pH range that normally found in natural aquatic environments. Four commercial PAEs (BBP, di-*n*-butyl phthalate (DBP), di-ethylhexyl phthalate (DEHP), and di-isononyl phthalate (DINP)), were subjected to the abiotic degradation tests, and the half-lives of the PAEs by hydrolysis and photolysis were estimated by the first-order degradation kinetics. The role of abiotic degradation in the fate of the PAEs was discussed by comparing the estimated half-lives with those by aerobic/anaerobic biodegradation reported previously.

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