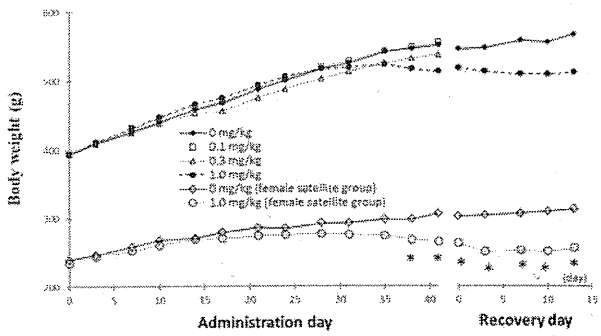

Fig. 1. Body weight of males in main groups and satellite groups for recovery period and females in satellite groups.
*: Significantly different from the control, $p \leqq 0.05$.

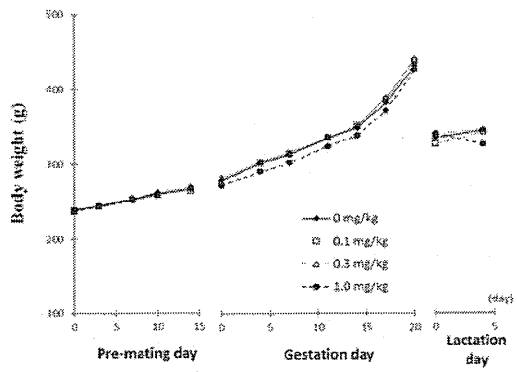

Fig. 2. Body weight of females in main groups.

Organ weights in males and females are shown in Table 3. Relative weight of the liver was increased at 0.3 mg/kg/day in main group males, and absolute and relative weights of the liver were increased in males and females at 1.0 mg/kg/day in main and satellite groups. Absolute and relative weights of the spleen were decreased at 1.0 mg/kg/day in main group males. Enlargement of the liver in two males and a dark red focus in the stomach in three males were observed at 1.0 mg/kg/day in the main group. No other treatment-related findings at necropsy were observed in males and females in main and satellite groups. Histopathological findings are shown in Table 4. Possibly treatment-related changes were observed in the liver and stomach: In the main groups, centrilobular hypertrophy of hepatocytes in males and females were observed at 0.3 mg/kg/day and above, diffuse vacuolation of hepatocytes in males, and minimal focal necrosis in males and females were observed at 1.0 mg/kg/day, and in the satellite groups, minimal diffuse vacuolation of hepatocytes in males, centrilobular hypertrophy/degeneration of hepatocytes in males and females, and Glisson's sheath cell infiltration in females were observed at 1.0 mg/kg/day. In the glandular stomach, minimal erosion was observed in 3/7 males at 1.0 mg/kg/day. Although a similar change was observed in 2/6 control females, the possibility that PFUA treatment affected the stomach in males could not be ruled out. The findings in other organs were considered to be incidental in main and satellite groups, because there was no dose-dependent increase in incidence or severity. On reproductive organs, no treatment-related histopathological changes were found in the epididymides, testis, and uterus in PFUA-treated groups.

Reproductive and developmental findings

There were no significant differences in the mean estrous cycle and in the incidence of females with a normal estrous cycle between the control and PFUA groups either in the main or recovery group (data not shown). The data for reproductive and developmental parameters are shown in Table 5. Reproduction performance of parental rats, delivery and nursing were not significantly different between the control and PFUA-treated groups. Regarding the general appearance of pups, there were no abnormal findings in any groups. The body weights of male and female pups on PNDs 0 and 4 were lowered at 1.0 mg/kg/day. There were no significant differences in the sex ratio of live pups or the viability index on PND 4. At gross pathology in pups on PND 4, thymic remnant in the neck was observed in one male and one female at 0.3 mg/kg/day, and in two females at 1.0 mg/kg/day, and these were considered to be incidental because of the low incidence. There were no other changes in gross internal findings of pups in any PFUA-treated groups.

DISCUSSION

The present study of rats was conducted to examine the possible effects of PFUA on reproduction and development as well as the possible general toxic effects. The dosage of PFUA used in this study was sufficiently high to be expected to induce general toxic effects in parental animals. The following results suggest that the liver is a sensitive target organ. The weight of the liver was increased in males at 0.3 mg/kg/day and above, and in females at 1.0 mg/kg/day, and centrilobular hypertrophy of hepatocytes was observed in both sexes at 0.3 mg/kg/day and above, focal necrosis and/or diffuse vacuolation of hepatocytes were also found in the 1.0

Table 1. Hematological findings

		Main group				Satellite group	
	Group	0 mg/kg/day	0.1 mg/kg/day	0.3 mg/kg/day	1.0 mg/kg/day	0 mg/kg/day	1.0 mg/kg/day
Males							
Number of animals		5	5	5	5	5	5
WBC	(10^2/μl)	121.2 ± 31.4	94.8 ± 21.1	127.6 ± 35.4	129.8 ± 23.5	73.4 ± 26.8	111.6 ± 19.5*
RBC	(10^4/μl)	830 ± 40	846 ± 25	852 ± 20	869 ± 23	894 ± 34	886 ± 47
HGB	(g/dl)	15.6 ± 0.4	15.7 ± 0.6	15.4 ± 0.4	15.6 ± 0.7	16.0 ± 0.4	15.3 ± 0.9
MCV	fl	52.5 ± 1.8	51.4 ± 1.7	50.6 ± 0.7	50.1 ± 1.4*	50.9 ± 1.5	49.4 ± 1.9
MCH	pg	18.8 ± 0.5	18.6 ± 0.8	18.1 ± 0.4	17.9 ± 0.4*	17.9 ± 0.3	17.3 ± 0.7
Platelet	(10^4/μl)	98.7 ± 3.7	121.4 ± 5.2**	109.2 ± 8.8	111.2 ± 8.8*	107.8 ± 12.4	122.7 ± 18.6
APTT	(sec)	22 ± 4.1	19.2 ± 1.9	20.8 ± 4.2	16.6 ± 0.7*	20.4 ± 1.7	17.2 ± 2.6*
Fibrinogen	mg/dl	294 ± 20	273 ± 35	283 ± 31	200 ± 23**	304 ± 35	245 ± 22*
Females							
Number of animals		5	5	5	5	5	5
WBC	(10^2/μl)	143.4 ± 43.8	128.7 ± 25.4	151.8 ± 33.5	159.2 ± 45.1	58.6 ± 14.9	65.1 ± 13.6
RBC	(10^4/μl)	702 ± 46	680 ± 67	692 ± 50	645 ± 51	830 ± 30	846 ± 56
HGB	(g/dl)	13.1 ± 1.0	13.5 ± 1.0	13.5 ± 1.1	13.2 ± 0.8	15.4 ± 0.4	15.4 ± 1.0
MCV	fl	52.7 ± 1.3	56.7 ± 4.5	55.0 ± 1.2	58.0 ± 3.1*	51.4 ± 1.4	50.1 ± 1.2
MCH	pg	18.6 ± 0.5	20.0 ± 1.6	19.5 ± 0.6	20.5 ± 1.1*	18.6 ± 0.6	18.2 ± 0.6
Platelet	(10^4/μl)	159.4 ± 27.4	141.0 ± 22.7	164.8 ± 19.6	161.8 ± 30.9	130.6 ± 13.7	125.7 ± 18.1
APTT	(sec)	17.6 ± 1.8	17.5 ± 2.4	17.9 ± 2.3	15.2 ± 3.3	17.9 ± 2.3	17 ± 2.9
Fibrinogen	mg/dl	335 ± 53	319 ± 95	282 ± 49	228 ± 42*	207 ± 10	176 ± 31

Values are given as the mean ± S.D.
*: Significantly different from the control, $p \leqq 0.05$. **: Significantly different from the control, $p \leqq 0.01$.

mg/kg/day group. In rodents, it is clear that the hepatic response to exposure to many perfluoroalkyl compounds is initiated by the activation of the nuclear hormone receptor, PPARα (ATSDR, 2009), and PFUA activates mouse PPARα *in vitro* (Wolf *et al.*, 2012). The hepatic proliferative responses, including an increase in the liver weight and centrilobular hypertrophy of hepatocytes, observed in the present study might have been initiated by the activation of PPARα, although there is a scientific consensus that compounds which are peroxisome proliferators in rodents have little or no effect on human liver (IARC, 1995). Regarding the toxicity of PFAAs, the involvement of mechanisms other than PPARα has been suggested (Peters and Gonzalez, 2011), so further research on the toxicity mechanism of PFUA is desired.

Effects on the body weight of adult males/females and pups were observed only at 1.0 mg/kg/day. In adult animals, suppression of body weight gain was observed in males/females in the administration and/or recovery periods, although not in females in the premating and gestation periods. It is considered that these body weight changes were a direct effect of PFUA because they were not related to food consumption. There is a possibility of maternal-fetal/infant transfer of PFUA, because maternal-fetal transfer and maternal-infant transfer of PFOA through breast milk have been observed in rats (Hinderliter *et al.*, 2005). Because there was no difference in the length of the gestation period in dams dosed at 1.0 mg/kg/day compared to the controls, and because sup-

Table 2. Blood biochemical findings

		Main group				Satellite group	
	Group	0 mg/kg /day	0.1 mg/kg/day	0.3 mg/kg/day	1.0 mg/kg/day	0 mg/kg/day	1.0 mg/kg/day
Males							
Number of animals		5	5	5	5	5	5
AST	(IU/l)	67 ± 9	70 ± 4	73 ± 17	77 ± 6	62 ± 9	73 ± 12
ALT	(IU/l)	31 ± 3	32 ± 3	34 ± 3	39 ± 7*	31 ± 5	37 ± 5
ALP	(IU/l)	427 ± 12.6	461 ± 85	514 ± 96	1021 ± 179**	379 ± 95	707 ± 152**
Total cholesterol	(mg/dl)	56 ± 14	47 ± 8	34 ± 6**	46 ± 11	55 ± 18	53 ± 13
Triglyceride	(mg/dl)	48 ± 10	70 ± 42	41 ± 9	46 ± 16	52 ± 17	45 ± 27
Phospholipid	(mg/dl)	90 ± 13	82 ± 14	65 ± 9*	87 ± 11	87 ± 19	92 ± 21
BUN	(mg/dl)	13 ± 2	14 ± 3	15 ± 1	21 ± 4**	17 ± 2	23 ± 5*
Na	(mmol/l)	147 ± 2	146 ± 2	147 ± 1	145 ± 1	145 ± 1	143 ± 1**
Cl	(mmol/l)	108 ± 2	108 ± 1	109 ± 1	109 ± 3	107 ± 1	108 ± 1
Ca	(mg/dl)	10.1 ± 0.2	10.0 ± 0.3	10.0 ± 0.3	9.7 ± 0.2*	9.9 ± 0.3	9.5 ± 0.3
Total protein	(g/dl)	6.2 ± 0.2	6.0 ± 0.3	6.1 ± 0.1	5.5 ± 0.3**	6.3 ± 0.1	5.8 ± 0.5
Albumin	(g/dl)	2.8 ± 0.1	2.8 ± 0.1	2.9 ± 0.0	2.6 ± 0.1*	2.7 ± 0.1	2.8 ± 0.2
A/G		0.80 ± 0.07	0.86 ± 0.03	0.93 ± 0.05**	0.88 ± 0.06	0.77 ± 0.04	0.93 ± 0.09**
Females							
Number of animals		5	5	5	5	5	5
AST	(IU/l)	84 ± 21	92 ± 12	86 ± 15	81 ± 12	59 ± 4	68 ± 11
ALT	(IU/l)	53 ± 9	55 ± 12	50 ± 18	49 ± 1	26 ± 4	28 ± 4
ALP	(IU/l)	219 ± 72	242 ± 42	286 ± 176	263 ± 18	158 ± 28	289 ± 54**
Total cholesterol	(mg/dl)	60 ± 11	52 ± 13	41 ± 13*	49 ± 8	78 ± 16	64 ± 14
Triglyceride	(mg/dl)	54 ± 11	38 ± 12	41 ± 18	60 ± 25	28 ± 11	20 ± 3
Phospholipid	(mg/dl)	112 ± 13	94 ± 18	80 ± 20*	98 ± 11	141 ± 20	108 ± 15*
BUN	(mg/dl)	13 ± 2	13 ± 4	16 ± 3	19 ± 2**	20 ± 3	29 ± 7*
Na	(mmol/l)	141 ± 1	141 ± 2	143 ± 1	142 ± 1	143 ± 1	143 ± 1
Cl	(mmol/l)	106 ± 1	107 ± 2	108 ± 2	108 ± 2*	109 ± 1	112 ± 2
Ca	(mg/dl)	10.3 ± 0.2	10.2 ± 0.4	10.3 ± 0.1	10.0 ± 0.3	10.2 ± 0.3	9.9 ± 0.2
Total protein	(g/dl)	6.2 ± 0.2	5.8 ± 0.3*	6.0 ± 0.1	5.6 ± 0.2**	6.7 ± 0.2	5.8 ± 0.3**
Albumin	(g/dl)	2.8 ± 0.2	2.8 ± 0.2	2.8 ± 0.1	2.7 ± 0.2	3.1 ± 0.2	2.9 ± 0.3
A/G		0.85 ± 0.05	0.92 ± 0.05	0.89 ± 0.07	0.91 ± 0.11	0.87 ± 0.04	1.01 ± 0.09*

Values are given as the mean ± S.D.
*: Significantly different from the control, $p \leqq 0.05$. **: Significantly different from the control, $p \leqq 0.01$.

pression of body weight gain in females during pregnancy was not observed, the lowered body weight on PND 0 was considered a direct effect of PFUA due to intrauterine exposure. Also in other PFCAs, low values of body weight of pups at birth without effects on the body weight of dams in the gestation period were observed (Butenhoff *et al.*, 2004; Loveless *et al.*, 2009). The lowered body weight on PND 4 was considered to be a direct effect of PFUA by ingestion of breast milk, as well as a secondary effect of PFUA caused by the lowered body weight in

Table 3. Organ weights

		Main Group				Satellite Group	
Dose (mg/kg/day)		0 (control)	0.1	0.3	1.0	0 (control)	1.0
Males							
No. of animals examined		5	5	5	5	5	5
Brain	(g)	2.18 ± 0.08	2.18 ± 0.09	2.15 ± 0.08	2.17 ± 0.08	2.09 ± 0.04	2.14 ± 0.13
	(%)[a]	0.42 ± 0.03	0.4 ± 0.03	0.41 ± 0.01	0.44 ± 0.05	0.39 ± 0.03	0.44 ± 0.03**
Throid[b]	(mg)	22.4 ± 1.8	25.7 ± 2.5	21.1 ± 2.9	22.3 ± 3.5	23.5 ± 3.8	18.7 ± 2.9
	(%)[a]	4.3 ± 0.4	4.7 ± 0.4	4.1 ± 0.6	4.5 ± 0.7	4.4 ± 0.5	3.8 ± 0.4
Thymus	(mg)	297 ± 90	432 ± 173	342 ± 106	260 ± 61	250 ± 80	251 ± 67
	(%)[a]	57 ± 19	79 ± 27	66 ± 21	53 ± 16	47 ± 17	51 ± 11
Heart	(g)	1.52 ± 0.1	1.5 ± 0.2	1.51 ± 0.03	1.38 ± 0.17	1.46 ± 0.17	1.29 ± 0.19
	(%)[a]	0.29 ± 0.02	0.28 ± 0.04	0.29 ± 0	0.28 ± 0.02	0.28 ± 0.02	0.27 ± 0.02
Liver	(g)	15.12 ± 2.14	16.45 ± 2.06	17.54 ± 0.73	20.95 ± 2.56**	14.19 ± 1.56	19.85 ± 3.03**
	(%)[a]	2.88 ± 0.27	3.02 ± 0.19	3.39 ± 0.16**	4.18 ± 0.19**	2.67 ± 0.22	4.07 ± 0.36**
Spleen	(g)	0.84 ± 0.16	0.76 ± 0.09	0.79 ± 0.05	0.65 ± 0.09*	0.72 ± 0.11	0.72 ± 0.04
	(%)[a]	0.16 ± 0.03	0.14 ± 0.01	0.15 ± 0.01	0.13 ± 0.01*	0.14 ± 0.02	0.15 ± 0.01
Kidney[b]	(g)	3.43 ± 0.31	3.44 ± 0.38	3.51 ± 0.08	3.4 ± 0.17	3.51 ± 0.31	3.32 ± 0.43
	(%)[a]	0.65 ± 0.06	0.63 ± 0.04	0.68 ± 0.03	0.68 ± 0.06	0.66 ± 0.03	0.68 ± 0.04
Adrenal[b]	(mg)	64 ± 13	70 ± 8	68 ± 3	58 ± 9	61 ± 9	46 ± 8*
	(%)[a]	12 ± 2	13 ± 1	13 ± 1	12 ± 1	12 ± 2	9 ± 1
Testis[b,c]	(g)	3.34 ± 0.21	3.57 ± 0.26	3.48 ± 0.28	2.98 ± 0.86	3.49 ± 0.26	3.57 ± 0.35
	(%)[a]	0.63 ± 0.07	0.67 ± 0.05	0.68 ± 0.06	0.62 ± 0.17	0.66 ± 0.03	0.74 ± 0.07*
Epididymis[b,c]	(mg)	1339 ± 84	1420 ± 112	1368 ± 199	1578 ± 950	1337 ± 51	1388 ± 87
	(%)[a]	252 ± 21	265 ± 25	268 ± 36	335 ± 220	252 ± 11	288 ± 34
Females							
No. of animals examined		5	5	5	5	5	5
Brain	(g)	1.99 ± 0.05	1.97 ± 0.08	1.98 ± 0.09	2 ± 0.04	1.96 ± 0.09	1.86 ± 0.06
	(%)[a]	0.64 ± 0.04	0.66 ± 0.03	0.65 ± 0.05	0.67 ± 0.06	0.68 ± 0.08	0.78 ± 0.02**
Throid[b]	(mg)	17.2 ± 1.8	19.2 ± 3.2	17.5 ± 3	16.9 ± 0.7	17.2 ± 2.7	14.7 ± 1
	(%)[a]	5.5 ± 0.8	6.5 ± 1.1	5.8 ± 1.1	5.6 ± 0.6	6 ± 1	6.2 ± 0.3
Thymus	(mg)	192 ± 16	170 ± 102	243 ± 82	249 ± 58	245 ± 98	147 ± 59
	(%)[a]	61 ± 4	56 ± 32	79 ± 24	82 ± 14	85 ± 39	62 ± 23
Heart	(g)	1.02 ± 0.08	0.96 ± 0.06	0.92 ± 0.04	0.94 ± 0.11	0.86 ± 0.05	0.73 ± 0.03**
	(%)[a]	0.33 ± 0.03	0.32 ± 0.01	0.3 ± 0.01	0.31 ± 0.02	0.29 ± 0.02	0.31 ± 0.01
Liver	(g)	10.56 ± 0.68	10.61 ± 0.48	10.55 ± 1.48	12.76 ± 1.00**	7.22 ± 0.38	8.63 ± 1.04*
	(%)[a]	3.37 ± 0.12	3.57 ± 0.13	3.46 ± 0.36	4.21 ± 0.15**	2.48 ± 0.14	3.64 ± 0.47**
Spleen	(g)	0.62 ± 0.06	0.65 ± 0.16	0.65 ± 0.1	0.66 ± 0.15	0.49 ± 0.05	0.43 ± 0.05
	(%)[a]	0.2 ± 0.02	0.22 ± 0.05	0.22 ± 0.02	0.21 ± 0.03	0.17 ± 0.02	0.18 ± 0.01
Kidney[b]	(g)	2.24 ± 0.42	1.96 ± 0.18	2.06 ± 0.19	2.05 ± 0.09	1.89 ± 0.14	1.93 ± 0.17
	(%)[a]	0.72 ± 0.14	0.66 ± 0.06	0.68 ± 0.07	0.68 ± 0.04	0.64 ± 0.02	0.81 ± 0.07**
Adrenal[b]	(mg)	82 ± 4	84 ± 10	89 ± 14	80 ± 13	70 ± 8	49 ± 5**
	(%)[a]	26 ± 2	28 ± 5	30 ± 5	26 ± 3	25 ± 5	21 ± 1

Values are given as the mean ± S.D.

[a]: Ratio of organ weight to body weight (relative organ weight). [b]: Values are represented as the total weights of the organs on both sides. [c]: Organ weight was measured for all animals (number of examined animals: 7 at 0 and 1.0 mg/kg/day and 12 at 0.1 and 0.3 mg/kg/day in the main group, and 5 at 0 and 1.0 mg/kg/day in the recovery group.)

*: Significantly different from the control, $p \leqq 0.05$. **: Significantly different from the control, $p \leqq 0.01$.

Table 4. Histopathological findings

	Males						Females					
	Main				Satellite		Main				Satellite	
Dose (mg/kg/day)	0	0.1	0.3	1.0	0	1.0	0	0.1	0.3	1.0	0	1.0
Heart												
Number examined	5	0	0	5			5	0	0	5		
Cardiomyopathy (minimal)	1			1			0			0		
Kidney												
Number examined	5	0	1	5			5	0	0	5		1
Dilatation, pelvic	0		1	0			1			0		1
(minimal)			1									1
(moderate)							1					
Regeneration, tubular	4		1	1			1			1		0
(minimal)	3		1	1			1			1		
(mild)	1											
Liver												
Number examined	7	12	12	7	5	5	12	12	12	12	5	5
Vacuolation, hepatocytes, diffuse	0	0	0	3	0	1	0	0	0	0	0	0
(minimal)				2		1						
(mild)				1								
Necrosis, focal (minimal)	0	0	0	2	0	0	0	0	0	2	0	0
Cell infiltration, Glisson's sheath (mild)	0	0	0	0	0	0	0	0	0	0	0	2
Microgranuloma	4	3	1	2	3	3	1	1	0	2	4	4
(minimal)	4	3	1	2	3	3	1	1		2	4	2
(mild)												2
Degeneration, hepatocytes, centrilobular (minimal)	0	0	0	0	0	3	0	0	0	0	0	3
Hypertrophy, hepatocytes, centrilobular	0	0	3	7	0	5	0	0	1	11	0	5
(minimal)			2						1	8		
(mild)			1	2		3				3		2
(moderate)				5		2						3
Spleen												
Number examined	5	0	0	5			5	0	0	5		
Hematopoiesis, extramedullary (minimal)	4			1			5			4		
Stomach												
Number examined	7	12	12	7	5	5	6	0	0	5		
Erosion, glandular stomach (minimal)	0	0	0	3	0	0	2			0		
Thymus												
Number examined	5	0	0	5			5	1	0	5		
Atrophy, lymphoid (mild)	0			0			0	1		0		
Thyroid												
Number examined	5	0	0	5			5	0	0	5		
Ectopic thymus (minimal)	0			0			0			1		
Cyst, ultimobranchial (minimal)	1			2			2			0		
Testis												
Number examined	5	0	0	5								
Not remarkable	5			5								
Epididymis												
Number examined	5	1	0	5								
Granuloma, spermatic	1	1		1								
(minimal)		1		1								
(mild)	1											
Uterus												
Number examined							5	1	0	5		
Dilatation, lumina (minimal)							0	1		0		

Table 5. Reproductive and developmental parameters

	0 mg/kg/day	0.1 mg/kg/day	0.3 mg/kg/day	1.0 mg/kg/day
Number of animals (males/females)	12/12	12/12	12/12	12/12
Copulation index (males/females) (%)	100/100	100/100	100/100	100/100
Fertility index (%)	100	91.7	100	100
Gestation index (%)	100	100	100	100
Number of pregnant animals	12	11	12	12
Gestation length (days)	22.0 ± 0.3	22.1 ± 0.5	22.1 ± 0.5	21.7 ± 0.2
Number of corpora lutea	15.8 ± 1.9	16.8 ± 1.8	16.2 ± 1.9	16:2 ± 1.5
Number of implantation sites	14.6 ± 2.0	15.5 ± 3.3	15.0 ± 1.9	15.3 ± 1.6
Implantation index (%)	92.0 ± 5.5	91.0 ± 15.4	92.8 ± 6.0	94.8 ± 4.4
Number of litters	12	11	12	12
Number of live pups on PND 0	13.9 ± 2.2	14.5 ± 3.4	13.1 ± 3.1	13.5 ± 2.2
Live birth index (%)	98.9 ± 2.6	97.3 ± 5.3	93.2 ± 18.2	97.9 ± 4.1
Sex ratio	0.51	0.47	0.55	0.52
Number of live pups on PND 4	13.7 ± 1.9	14.0 ± 3.2	12.8 ± 3.1	13.4 ± 2.2
Viability index (%)	98.5 ± 2.8	97.1 ± 3.3	97.7 ± 5.9	99.4 ± 2.2
Body weight of male pups (g)				
on PND 0	6.7 ± 0.3	6.7 ± 0.6	6.4 ± 0.5	5.8 ± 0.3**
on PND 4	10.5 ± 0.5	10.1 ± 1.8	10.2 ± 1.2	8.5 ± 0.7**
Body weight of female pups (g)				
on PND 0	6.4 ± 0.4	6.3 ± 0.6	6.1 ± 0.6	5.6 ± 0.2**
on PND 4	9.9 ± 0.6	9.7 ± 1.7	9.5 ± 0.8	8.3 ± 0.7**

Values are given as the mean ± S.D.
**: Significantly different from the control, $p \leqq 0.01$.

dams. In the PFOA oral dose study (Abbott *et al.*, 2007), the reduction of postnatal weight gain appeared to depend on PPARα expression.

The elimination rate of PFOA in female rats is approximately 40 times faster than in male rats (ATSDR, 2009). Organic anion transport proteins play a key role in PFCAs (C4 to C10) renal tubular reabsorption (Han *et al.*, 2012), and the slower elimination of PFOA in male rats compared to female rats has been attributed to sex hormone modulation of organic anion transporters in the kidney (ATSDR, 2009). In the present study, there were slight gender differences in the hepatotoxicity of PFUA: liver weight increased in males at 0.3 mg/kg/day and above and in females at 1.0 mg/kg/day, and histopathological findings observed in the 1.0 mg/kg/day groups were more numerous and severer in males than in females. The gender differences in hepatotoxicity observed in the present study are considered to be attributable to faster elimination in female rats, as with other PFCAs.

Increased liver weight and hepatocellular hypertrophy, induced by activation of PPARα, were generally observed in previous studies on PFAAs. Significant peroxisome proliferative activity seems to require a carbon length more than 7 (ATSDR, 2009). In gavage studies of PFAAs in male rats, which are more sensitive than females, the following results were observed; for PFOA (C8), increased liver weight and hepatocellular hypertrophy at 5 mg/kg/day for 28 days (Cui *et al.*, 2009); for perfluorononanoic acid (C9), increased liver weight at 1 mg/kg/day for 14 days (Fang *et al.*, 2012); for perfluorododecanoic acid (C12), increased liver weight at 0.02 mg/kg/day for 110 days (Ding *et al.*, 2009). In the current study of PFUA (C11), increased liver weight and centrilobular hypertrophy of hepatocytes were observed from 0.3 mg/kg/day for 42 days. In consideration of differences in the administration period or doses in these studies, the intensity of the liver toxicity of PFUA (C11) was estimated to be between C9 and C12, suggesting that the toxic potency of PFAAs (C8-C12) increases by lengthening their carbon chain. This is because hydrophobicity, which increases as carbon length increases, seems to favor biliary enterohepatic recirculation, resulting in more protracted toxicity (ATSDR, 2009). In contrast, 42-day administration of PFOdA (C18) increased liver

weight at 200 mg/kg/day but not at 40 mg/kg/day in male rats (Hirata-Koizumi *et al.*, 2012). In comparison with other PFAAs (C8-C12), including PFUA (C11), PFOdA induced liver toxicity at higher doses, and this may be due to the low absorption of PFOdA into the body.

At 1.0 mg/kg/day in the main group, the following effects on hematological and blood biochemical parameters were observed; a decrease in fibrinogen was observed in males and females, but increases in APTT and PT were not observed, suggesting that there would be no toxicologically significant effects on the blood coagulation system; decreases in fibrinogen, total protein and albumin observed in males and/or females may be due to reduced synthesis in the damaged liver; the increase in BUN observed in males and females could be due to increased hepatic protein catabolism, because urinalysis parameters and the gross and microscopic appearance of the kidneys were not changed; and the increase of ALP in males was related to the histopathological findings in the liver. These effects except for the decrease in fibrinogen in females were observed also at the end of the recovery period, and the increase of ALP was observed in females only after the recovery period. Moreover, in histopathological findings, centrilobular degeneration of hepatocytes in both sexes and Glisson's sheath cell infiltration in females were observed only at the end of the recovery period, and in females, centrilobular hypertrophy of hepatocytes was more serious at the end of the recovery period. These results suggest that the whole body elimination of PFUA in rats, as well as other PFCAs, is slow. There are some reports indicating that PFCAs are secreted in bile and undergo extensive reabsorption from the gastrointestinal tract (Kudo *et al.*, 2001; Vanden Heuvel *et al.*, 1991a, 1991b; reviewed in ATSDR, 2009). In general, PFCAs with longer carbon chains (C4-C10) have a longer half-life (Hirata-Koizumi *et al.*, 2012). Although the elimination half-life of PFUA is unknown, the half-life after intravenous injection of perfluorodecanoic acid (PFDeA, C10) in rats was about 40 to 60 days (Ohmori *et al.*, 2003), It is estimated that the half-life of PFUA is longer than the recovery period, 14 days, and it is reasonable that some effects of PFUA appear after the recovery period. The above findings may be effects of PFUA caused by enterohepatic recirculation, which lasted through the dosing and recovery periods. The decrease in grip strength of the forefoot observed in males and females at 1.0 mg/kg/day in the satellite group was considered a secondary effect related to suppression of body weight gain.

In conclusion, the NOAEL for repeated dose toxicity is considered to be 0.1 mg/kg/day based on the observed centrilobular hypertrophy of hepatocytes in both sexes at 0.3 mg/kg/day, and the NOAEL for reproductive/developmental toxicity is considered to be 0.3 mg/kg/day based on the lowered body weight of pups at birth and body weight gain at 4 days after birth inhibited at 1.0 mg/kg/day.

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Selecting pesticides for inclusion in drinking water quality guidelines on the basis of detection probability and ranking☆

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ABSTRACT

Pesticides released into the environment may pose both ecological and human health risks. Governments set the regulations and guidelines for the allowable levels of active components of pesticides in various exposure sources, including drinking water. Several pesticide risk indicators have been developed using various methodologies, but such indicators are seldom used for the selection of pesticides to be included in national regulations and guidelines. The aim of the current study was to use risk indicators for the selection of pesticides to be included in regulations and guidelines. Twenty-four risk indicators were created, and a detection rate was defined to judge which indicators were the best for selection. The combination of two indicators (local sales of a pesticide for the purposes of either rice farming or other farming, divided by the guideline value and annual precipitation, and amended with the scores from the physical and chemical properties of the pesticide) gave the highest detection rates. In this case study, this procedure was used to evaluate 134 pesticides that are currently unregulated in the Japanese Drinking Water Quality Guidelines, from which 44 were selected as pesticides to be added to the primary group in the guidelines. The detection probability of the 44 pesticides was more than 72%. Among the 102 pesticides currently in the primary group, 17 were selected for withdrawal from the group.

1. Introduction

Pesticides are considered to be an integral part of modern agriculture. The annual global consumption of 900 active chemical ingredients is estimated to be 2.4 billion kilograms, with a market value of US $39 billion (USEPA, 2011; World Resources Institute, 1998). The release of pesticides from agricultural fields and the resulting contamination of the environment may pose both ecological and human health risks (Capri and Karpouzas, 2007). Governments and nongovernmental organizations select certain pesticides and regulate their concentrations in drinking water. For example, the World Health Organization (2011) lists 48 active pesticide ingredients in its Drinking Water Quality Guidelines. In the United States, the Environmental Protection Agency (USEPA) lists 21 pesticides and related products in the National Primary Drinking Water Regulations (USEPA, 2009). "The USEPA uses the Unregulated Contaminant Monitoring program to collect data for contaminants that are suspected to be present in drinking water but for which health-based standards have not been set," and the agency also periodically reviews the contaminants listed in the National Primary Drinking Water Regulations (USEPA, 2009). In Japan, no pesticides are listed in the Drinking Water Quality Standards (DWQS), but pesticides are included in a category referred to as "Complementary Items to Set the Target for Water Quality Management" (hereafter called the Japanese Drinking Water Quality Guidelines, JDWQG), for which analysis is recommended in line with DWQS (MHLWJ, 2003a). The JDWQG adopted the concept of a hazard index (e.g., Reffstrup et al., 2010), otherwise known as the *DI* value, for the purpose of assessing the total risk associated with exposure to multiple pesticides (MHLWJ, 2003a). The *DI* value is defined as

$$DI = \sum_i \frac{DV_i}{GV_i} \quad (1)$$

where DV_i is the observed concentration of pesticide i, and GV_i is the reference concentration of pesticide i, which is determined in the JDWQG based on the acceptable daily intake (ADI) of the pesticide. Pesticide monitoring should be conducted with the minimum detection limit equal to 1% of each GV_i value, the summation should include monitored pesticides, and the *DI* should be 1.0 or less. For inclusion in the primary group of pesticides regulated by the JDWQG, the Ministry of Health, Labour and Welfare selected 102 pesticides from approximately

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550 registered pesticides (MHLWJ, 2003a). The selection was based on the annual sales and the ADI values of pesticides because actual data on their presence in drinking water sources were limited at the time of the selection, particularly for pesticides that were unregulated at that time. The selected 102 pesticides were suspected to be present in water sources at concentrations greater than 1% of each GV_i value, but the reasoning behind this was scarce. Every a few years, pesticides are newly developed and the pesticides applied to fields are steadily changing. Therefore, regulatory authorities collect data for pesticides that are suspected to be present in drinking water in order to update the list of regulated pesticides. Monitoring authorities must determine which pesticides are likely to be present in a given water supply.

The European Union Drinking Water Directive (1998) specifies acceptable concentrations of pesticides (and related products) both separately (0.1 μg/L) and in total (0.5 μg/L). However, the target pesticides are not defined by the directive; instead, the monitoring authority must determine which pesticides are likely to be present in a given water supply. Under these circumstances, a rationale and methodology for reviewing unregulated/regulated pesticides and monitoring pesticides based on available but limited data are needed. Several pesticide risk indicators have been developed through various methodologies and with various objectives (Finizio et al., 2001; Gramatica and Guardo, 2002; Reus et al., 2002; Verro et al., 2009a, 2009b). The objectives include the assessment of toxicity to a particular organism and the assessment of human health risks associated with occupational exposure and exposure to contaminated water or food. Ranking and comparing the relative risks of pesticides according to risk indicator scores is expected to serve as a tool in decision making and policy formulation, such as the identification of more environmentally friendly pesticides and application practices (Juraske et al., 2007; Reus and Leendertse, 2000; Trevisan et al., 2009).

The score values for some pesticide risk indicators are directly related to the potential for surface water contamination, pesticide concentration in surface water, or the ratio between concentration and toxicity. The score values are then used to assist in the prioritization or selection of pesticides to be targeted in monitoring programs in local catchment areas (Kookana et al., 2005; Papa et al., 2004; Tani et al., 2012). The results of the pesticide ranking approach have been validated against measured concentrations (Kookana et al., 2005; Peterson, 2006; Tani et al., 2012). However, ranking and scoring methods have not yet been used to select pesticides to be regulated in national drinking water guidelines or standards, partly because ranking methods represent a relative risk rating for which the cutoff value for selection is rather arbitrary. Simulation by means of a hydrological diffuse pollution model may directly predict pesticide concentrations and provide absolute risks (Holvoet et al., 2007; Yang and Wang, 2010); however, such simulation requires the input of precise data sets, and the application of such a method is limited to the catchment scale (Matsui et al., 2007).

In the current study, our aim was to develop a procedure for selecting suspected pesticides to be included in regulation and to screen out the inessential pesticides from the regulation by applying a ranking method involving score values for pesticide risk indicators. While the procedure was applied to pesticide selection in the revision of the primary group of pesticides in the JDWQG, the concept and the fundamental structure of the procedure can be applied to other situations.

2. Materials and methods

2.1. Risk indicators

We created and tested 24 risk indicators for pesticides in this study (Table 1). We tested the indicator A1 on the assumption that the occurrence of a pesticide in environmental waters is related to its annual application rate. We also used indicator A2, which is A1 divided by the guideline value (here, the GV_i value, MHLWJ, 2003a) so the probability of detection would be taken into consideration. For the pesticides that are not assigned official GV_i values, GV_i values were calculated from their ADI value using the normal procedure, with the assumption of a water consumption of 2 L/day, a body weight of 50-kg, and a 10% allocation factor (MHLWJ, 2003b).

The pesticides applied for rice farming enter river water at high rates because of the large amount of natural freshwater required during the cropping season (Matsui et al., 2002). As shown in Fig. 1S (supplementary data), the current study also confirmed that the concentrations of pesticides used in rice farming are higher than the concentrations of pesticides applied to upland fields, although the pesticides applied to upland fields are, nevertheless, detected in river water. Pesticides applied to rice paddies may therefore have a greater potential to contaminate river water than pesticides applied to upland fields. To account for these tendencies, we also used indicators A3 and A4, which are upland-field modifications of A1 and A2, respectively. Indicators A5 and A6 are rice-specific modifications of A1 and A2, respectively.

Runoff of a pesticide to surface water is affected by the properties of the pesticide. In a previous study (Tani et al., 2010), we used the diffuse pollution hydrologic model to conduct sensitivity analyses for the purpose of evaluating the influence of various pesticide properties on runoff, and our results indicated that pesticide adsorption and degradation in soil are the most influential properties and that water solubility also affects pesticide runoff to a certain extent. In a subsequent sensitivity analysis (Tani et al., 2012), we quantitatively evaluated the influence of three pesticide properties (the soil adsorption coefficient normalized by the organic-carbon content of the soil (K_{oc}), the half-life in soil and half-life in water) on the concentrations of rice-farming pesticides in river water. Using the results of the analyses, we systematically designed score tables for the pesticide properties in such a way that the sum of the scores for a particular pesticide was proportional to the logarithm of the predicted concentration of that pesticide in river water. Scores for soil adsorption and soil degradation, defined as Score Y, are given in a matrix table as a function of log K_{oc} and half-life with respect to degradation in soil (Table 1S, supplementary data). Scores for degradation in water, defined as Score Z, are given in a table as a function of half-life with respect to degradation in water.

Indicators A7 and A8 correspond to A5 and A6, respectively, modified by incorporation of scores to account for the effects of soil adsorption and half-life. Because score tables have not yet been developed for upland-field pesticides, indicators that reflect the effects of pesticide properties cannot be used for upland-field pesticides.

Pesticide use varies regionally. For example, approximately 43% of the fenobucarb sold in Japan is sold in the Kyushu region in Japan, and 34% and 23% of phenthoate are sold in the Hokkaido and Tohoku regions, respectively (Fig. 2S, supplementary data). Therefore, these pesticides can be expected to be detected at high concentrations in the surface waters of these regions, even if the national sales quantities are not large. Indicators A1–A8 do not reflect the possible regional differences. Therefore, we divided Japan into 10 geographical regions and used indicators similar to A1–A8 for each region. For example, B1 is the regional version of A1 and is the maximum value of (quantity of sales)/(regional area) among the values for the 10 regions. Indicators C1–C8 are modifications of B1–B8, respectively, in which regional land area is replaced by regional precipitation, in order to account for possible dilution effects.

2.2. Pesticides

In 2011, the number of registered pesticides in Japan was approximately 530 (FAMIC, 2011). The primary group of JDWQG consisted of 102 pesticides. The secondary and tertiary groups had 26 and 77 pesticides, respectively (Table 2). In addition to the currently listed pesticides, we selected 31 pesticides from among the following three categories: (1) pesticides listed in the "Provisional guidance relating to prevention of water contamination with pesticides used on golf

Table 1
Indicators used for pesticide selection.

Indicator	Definition	Unit
A1	(Quantity of sales)/(national land area)	(ton year^{-1}) km^{-2}
A2	[(Quantity of sales)/GV_i]/(national land area)	(ton year^{-1}) (μg L^{-1})$^{-1}$ km^{-2}
A3	(Quantity of sales for upland field)/(national land area)	(ton year^{-1}) km^{-2}
A4	[(Quantity of sales for upland field)/GV_i]/(national land area)	(ton year^{-1}) (μg L^{-1})$^{-1}$ km^{-2}
A5	(Quantity of sales for rice-farming)/(national land area)	(ton year^{-1}) km^{-2}
A6	[(Quantity of sales for rice-farming)/GV_i]/(national land area)	(ton year^{-1}) (μg L^{-1})$^{-1}$ km^{-2}
A7	(Quantity of sales for rice-farming) × $10^{(\text{Score Y} + \text{Score Z} - 6)}$/(national land area)	(ton year^{-1}) km^{-2}
A8	[(Quantity of sales for rice-farming) × $10^{(\text{Score Y} + \text{Score Z} - 6)}$/$GV_i$]/(national land area)	(ton year^{-1}) (μg L^{-1})$^{-1}$ km^{-2}
B1	Max [(Quantity of sales)/(regional land area)]$_i$	(ton year^{-1}) km^{-2}
B2	Max {[(Quantity of sales)/GV_i/](regional land area)}$_i$	(ton year^{-1}) (μg L^{-1})$^{-1}$ km^{-2}
B3	Max [(Quantity of sales for upland field)/(regional land area)]$_i$	(ton year^{-1}) km^{-2}
B4	Max {[(Quantity of sales for upland field)/GV_i]/(regional land area)}$_i$	(ton year^{-1}) (μg L^{-1})$^{-1}$ km^{-2}
B5	Max [(Quantity of sales for rice-farming)/(regional land area)]$_i$	(ton year^{-1}) km^{-2}
B6	Max {[(Quantity of sales for rice-farming)/GV_i]/(regional land area)}$_i$	(ton year^{-1}) (μg L^{-1})$^{-1}$ km^{-2}
B7	Max [(Quantity of sales for rice-farming) × $10^{(\text{Score Y} + \text{Score Z} - 6)}$/(regional land area)]$_i$	(ton year^{-1}) km^{-2}
B8	Max {[(Quantity of sales for rice-farming) × $10^{(\text{Score Y} + \text{Score Z} - 6)}$/$GV_i$]/(regional land area)}$_i$	(ton year^{-1}) (μg L^{-1})$^{-1}$ km^{-2}
C1	Max [(Quantity of sales)/(regional precipitation)]$_i$	(ton year^{-1}) (km^3 year^{-1})$^{-1}$
C2	Max {[(Quantity of sales)/GV_i/(regional precipitation)]}$_i$	(ton year^{-1}) (μg L^{-1})$^{-1}$ (km^3 year^{-1})$^{-1}$
C3	Max [(Quantity of sales for upland field)/(regional precipitation)]$_i$	(ton year^{-1}) (km^3 year^{-1})$^{-1}$
C4	Max {[(Quantity of sales for upland field)/GV_i]/(regional precipitation)}$_i$	(ton year^{-1}) (μg L^{-1})$^{-1}$ (km^3 year^{-1})$^{-1}$
C5	Max [(Quantity of sales for rice-farming)/(regional precipitation)]$_i$	(ton year^{-1}) (km^3 year^{-1})$^{-1}$
C6	Max {[(Quantity of sales for rice-farming)/GV_i]/(regional precipitation)}$_i$	(ton year^{-1}) (μg L^{-1})$^{-1}$ (km^3 year^{-1})$^{-1}$
C7	Max [(Quantity of sales for rice-farming) × $10^{(\text{Score Y} + \text{Score Z} - 6)}$/(regional precipitation)]$_i$	(ton year^{-1}) (km^3 year^{-1})$^{-1}$
C8	Max {[(Quantity of sales for rice-farming) × $10^{(\text{Score Y} + \text{Score Z} - 6)}$/$GV_i$]/(regional precipitation)}$_i$	(ton year^{-1}) (μg L^{-1})$^{-1}$ (km^3 year^{-1})$^{-1}$

courses" (MOEJ, 2010), (2) pesticides studied in survey research in Japan (Matsui, 2011), and (3) the top 30 herbicides, 30 insecticides, and 30 fungicides in terms of sales (FAMIC, 2011; JPPA, 2008–2011) and sales/ADI. The total number of pesticides included in the study was 236. Pesticide concentrations in raw water samples from water treatment plants were obtained from *Statistics on Water Supply* for fiscal years 2007–2010 (JWWA, 2009–2012). Each monitoring authority measured the concentration of each pesticide on average two times per year. Therefore, if we had judged detection/no-detection of a pesticide by using data from a single year, we would likely have missed pesticide concentrations that exceeded the detection level and would have incorrectly judged the detection/no-detection of some pesticides. Therefore, for each pesticide, we pooled the data for the 4 years from 2007 to 2010 into a single data point and used that data point to judge detection/no-detection (Table 2). Any clerical mistakes in the data were corrected after email inquiries regarding data that were deemed critical for determining whether the pesticides were detected or not detected. Additionally, inquiries were made for critical data that were deemed suspect, that is, data for pesticides that were detected at a few water authorities but at measured concentrations exactly equal to the reference concentration or the minimum detection limit. Data that were not included in these statistics were obtained directly from nine water supply authorities that conducted frequent measurements (see Acknowledgments). The concentrations of the 102 pesticides in the primary group of JDWQG were measured by 404 water authorities in Japan. Among the 102 pesticides, 78 were detected at concentrations of >1% of the corresponding GV_i value. The remaining 24 pesticides were either not detected within the minimum detection limit or were detected at a concentration of <1% of the GV_i value. Because the quantification of a pesticide concentration >1% of the GV_i value is recommended for evaluation of the *DI* value in JDWQG (MHLWJ, 2003b), the 24 pesticides were treated as pesticides that were not detected at significant concentrations (hereafter referred to as undetected pesticides). Nine water authorities also measured the concentrations of some of the pesticides in the secondary and tertiary groups and reported the detection of three pesticides from these groups. Finally, among the 236 pesticides for which we collected concentration data, 81 pesticides (hereafter referred as detected pesticides) were recorded as detected. The detected pesticides plus undetected pesticides were 105, and these were used as the index pesticides. For the remaining 131 pesticides (unmeasured pesticides), sufficient measurement data were not available.

2.3. Data for calculating risk indicator values

Annual prefectural pesticide sales of commercial product bases were obtained from pesticide sales data books (JPPA, 2008–2011) and were averaged for the years 2007–2010. Because detection/no-detection of each pesticide was judged on the basis of pooled data for the years 2007–2010, the pesticide sales for those years were also treated as a single data point for each pesticide. From these data, the quantities of pesticides sold specifically for rice farming were estimated by referring to the uses indicated on the product labels. For pesticides that can be used for several crops, the percentages used for rice farming were estimated from data for planted areas of crops, including rice (MAFFJ, 2011), and recommended pesticide application rates (FAMIC, 2011; Kamata et al., 2008; Matsui et al., 2006); and then the quantities of pesticides applied for rice farming were calculated. The quantities of a

Table 2
Pesticides included in this study.

Designation	Category of the current JDWQG	No. in primary group	No. in secondary group	No. in tertiary group	No. of others	Total no.	
Detected pesticides	Detected in the 4 years (2007–2010)	78	1	2	0	81	105 (used as index pesticides)
Undetected pesticides	Measured but not detected in the 4 years (2007–2010)	24	0	0	0	24	
Unmeasured pesticides	No data or insufficient data	0	25	75	31	131	
Total no.		102	26	77	31	236	

pesticide applied in upland fields were calculated by subtracting the quantities of the pesticide applied for rice farming from the total sale of the pesticide. Quantities of pesticides in terms of the amounts of active chemical ingredients were calculated from the amounts of the chemical ingredient in the product bases (FAMIC, 2011). The ADI values were obtained from a pesticide ADI database (NIHS, 2011; Sugita et al., 2006). For annual precipitation, we used the average values for the period, from were years 1976 to 2005 (MLITJ, 2011); the latest data including the target years of 2007–2010 was not available. K_{oc} values and half-lives of pesticides for evaluation of the score values (Tani et al., 2012) were obtained from the literature (FSC, 2011; MOEJ, 2011; Tomlin, 2006).

3. Results and discussion

3.1. Detection rates of pesticide indicators

We scored the 236 pesticides by using each indicator, and then we ranked the pesticides according to the scores. A good indicator is one in which the detected pesticides are scored with high values but the undetected pesticides are scored with low values, so that the pesticides that will actually be detected can be selected. We used 105 pesticides (81 detected, 24 undetected, see Table 2) as index pesticides. To judge whether indicators were good for pesticide selection, we used a detection rate for each indicator, which was defined as

$$\text{Detection rate} = \frac{\text{The number of detected pesticides in the selected pesticides}}{\text{The number of index pesticides in the selected pesticides}}. \quad (2)$$

A good indicator was one that gave a high detection rate. When the 236 pesticides were ranked according to indicator A1 and the pesticides with the 50 highest A1 values were selected, the number of index pesticides in the selection was 35 and the number of detected pesticides in the selection was 28 (selectively rate = 28/35 = 80%). As the number of selected pesticides increased, the selectively rate slightly decreased, although there were some ups and downs (dashed gray line, Fig. 1). Detection rates by A2 were higher than those by A1, but the results are not surprising because the detection/no-detection of pesticides is dependent on the GV_i value of the pesticide in consideration.

Indicators A5–A8 (and B5–B8, etc.) consider only rice-farming pesticides, which have a high tendency to run off into surface water. Moreover, A7 and A8 (and B7 and B8, etc.) account for pesticide properties that could affect runoff rates. Therefore, A5–A8 can be expected to show better detection rates than A1 and A2. However, the former cannot be used to select for upland-field pesticides. Therefore, we used A5–A8 in combination with A1–A4. For example, to use the combination of A4 and A6 (hereafter referred to as A4A6) to select 50 pesticides, we first selected a certain number of pesticides by using A6 (regarding paddy-field pesticides) and then selected the remaining pesticides by using A4 (regarding upland-field pesticides). The detection rate depended on the numbers in the first and second selections. In the case of selecting 50 pesticides by using A4A6, for example, the detection rate was maximized when 49 pesticides were selected with A6 and 1 pesticide was selected with A4. We defined this type of high detection rate as the single unique detection rate for each combination of indicators at a given total number of selected pesticides (e.g., 50), and this high detection rate is hereafter referred to simply as the detection rate. The detection rates for A4A6 depended on the number of selected pesticides (solid gray line, Fig. 1). The detection rates were 100% when selected pesticides were less than 82, and the rate gradually decreased as the number of selected pesticides was increased. Overall, the detection rates by A4A6 were higher than those by A2, proving that dividing pesticides according to rice-paddy and upland-field applications and then using the combination of two indicators was successful.

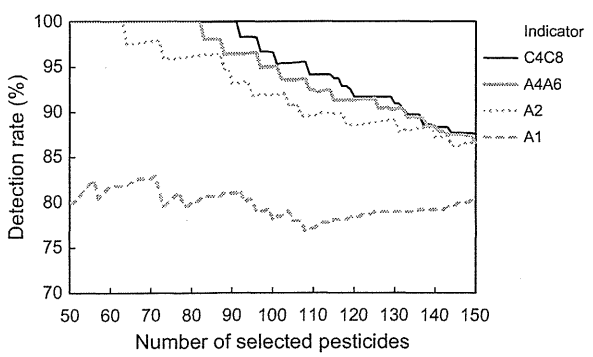

Fig. 1. Variation of detection rate with number of selected pesticides.

Among the four indicators shown in Fig. 1, C4C8 yielded the highest detection rate most of the time when the number of selected pesticides ranged from 50 to 150. The detection rate was 100% until 91 pesticides were selected, at which point the rate began to decrease gradually with increasing pesticide selections.

We actually tested 48 indicator combinations: 16 combinations of indicators based on national pesticide usage [(A1–A4) × (A5–A8)], 16 combinations of indicators based on regional pesticide usage [(B1–B4) × (B5–B8)], and 16 combinations of indicators based on regional pesticide usage and precipitation [(C1–C4) × (C5–C8)] (Table 3). Among these 48 combinations, C4C8 gave the highest detection rate 82 times when the number of selected pesticides ranged from 50 to 150. B4B8 gave the highest rate 78 times. Overall, the indicators in the B series and the C series showed better detection rates than those in the A series. This result indicates the importance of regional differences in pesticide applications: pesticides applied regionally and pesticides applied locally but intensively are detected more often than pesticides applied nationwide. The indicators in the C series were slightly better overall than the B series; this result suggests that precipitation would have a dilution effect on pesticide concentrations, rather than triggering pesticide runoff. Somewhat better detection rates were achieved by the series of "8" (e.g., A8) relative to those achieved by the series of "6" (e.g., A6), proving that the incorporation of pesticide properties, such as soil-adsorbability, in the indicator of the 8-series plays a certain role in better selecting pesticides. On the basis of these results, we used C4C8 to select pesticides in the case study.

3.2. Selecting pesticides for addition to the primary group

When the number of pesticides selected with C4C8 was ≤ 91, the detection rate was 100% (Fig. 1). The 91 pesticides included 56 index pesticides, all of which were detected pesticides. This cutoff level was designated as the first selection level. We changed the selection level stepwise for each by adding approximately 10 index pesticides and then calculating the detection rates. When 108 pesticides (including 11 additional index pesticides) were selected, the detection rate decreased to 95.5%. This cutoff level was designated as the second selection level. Of the 11 additional index pesticides, eight were detected pesticides (detection rate 72.7% for the 11 additional index pesticides, Fig. 2). When 143 pesticides were selected (including 10 additional index

Table 3
Number of times maximum (including ties) detection rate was recorded.

A series	A5	A6	A7	A8	B series	B5	B6	B7	B8	C series	C5	C6	C7	C8
A1	0	7	0	16	B1	0	12	0	3	C1	0	12	0	9
A2	24	42	23	48	B2	39	67	36	67	C2	40	63	34	74
A3	0	8	0	17	B3	0	13	0	4	C3	0	13	0	10
A4	4	45	7	53	B4	19	76	20	78	C4	20	76	20	82

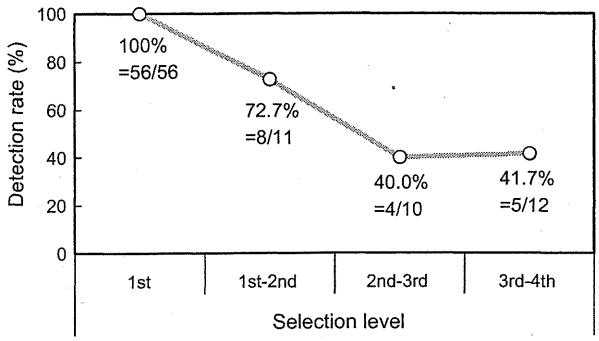

Fig. 2. Variation of detection rate with selection level.

pesticides; third selection level), only four of the 10 index pesticides were detected pesticides (detection rate 40%). That is, there was a substantial decrease in the detection rate below the second selection level. When 12 index pesticides were added (fourth selection level), the detection rate remained low (41.7%). On the basis of these results, we determined that the second selection level was a reasonable cutoff level.

A scatter plot of C8 versus C4 for the index pesticides (Fig. 3) clearly indicated that the first selection level screened out all the undetected pesticides, the second selection level screened out all but three of the undetected pesticides, and the undetected pesticides clustered at the lower left of the plot. A scatter plot of C8 versus C4 for the unmeasured pesticides showed that 35 unmeasured pesticides fell on the right or upper side of the area to the right of or above the first selection level (Fig. 4). Because the index pesticides in this area were all detected, these 35 unmeasured pesticides in the same area would likely be detected if they were measured. Nine unmeasured pesticides were added between the first and second selection levels. Because the detection rate at the second level was 72.7%, these nine unmeasured pesticides would likely be detected at a similarly high percentage if they were measured. Thus, our results suggest that 44 (=35 + 9) among the 131 unmeasured pesticides should be included in the primary group being revised. Collecting data on whether these 44 pesticides are detected or not is of high importance, but collecting those data will take time. In the meantime, standard methods for the determination of these pesticides should be developed. Once the primary group of JDWQG has been revised, water supply authorities will be officially directed to monitor these pesticides.

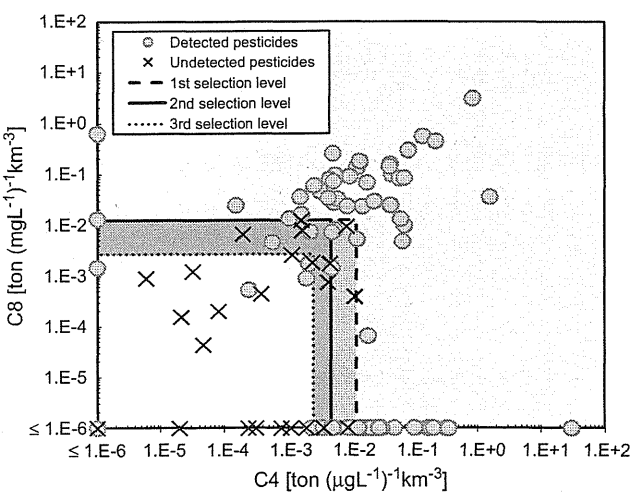

Fig. 3. Scatter plot of C8 versus C4 for the index pesticides.

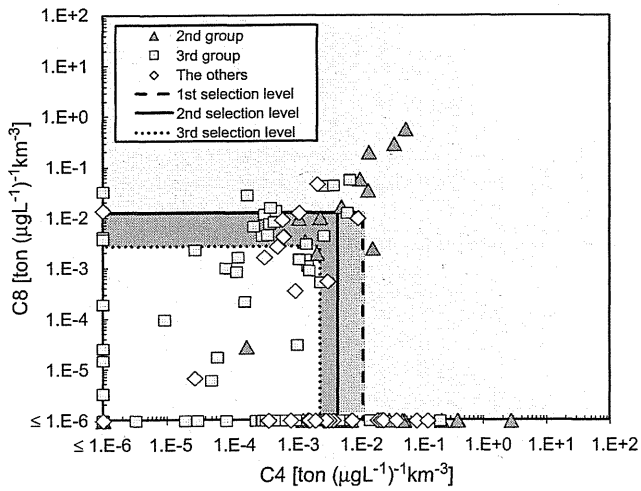

Fig. 4. Scatter plot of C8 versus C4 for the unmeasured pesticides.

3.3. Selecting pesticides to be withdrawn from the primary group

C4C8 was chosen because this combination maximized the detection rate, which is equivalent to minimizing the detection rate for undetected pesticides and in turn is equivalent to maximizing the undetection rate, which is defined as

$$\text{Undetection rate} = \frac{\text{Number of undetected pesticides in the unselected pesticides}}{\text{The number of index pesticides in the unselected pesticides}}. \quad (3)$$

We therefore used C4C8 to choose pesticides that should be withdrawn from the current primary group. For eight detected pesticides among the 105 index pesticides in the group (Table 2), no sales records were available; these are pesticides for which pesticide registration was canceled. The reason that these pesticides were detected is unknown; the statistics may be incorrect, or these pesticides may be extremely persistent in the environment. Our selection method, which is based on pesticide sales, cannot be used to evaluate the probability of detection of these eight pesticides. When they were omitted from the calculation, the undetection rate was 100% (Fig. 5) for the left side of the dashed line (designated as the first withdrawal level; Fig. 6). The 11 pesticides in the first withdrawal level were all undetected pesticides. Withdrawal levels were changed stepwise for each by adding approximately 10 withdrawal candidate pesticides. Until the second withdrawal level, another nine pesticides were identified as candidates for withdrawal, and six out of the nine were undetected pesticides (undetection rate 66.7%). When the cutoff level was further relaxed

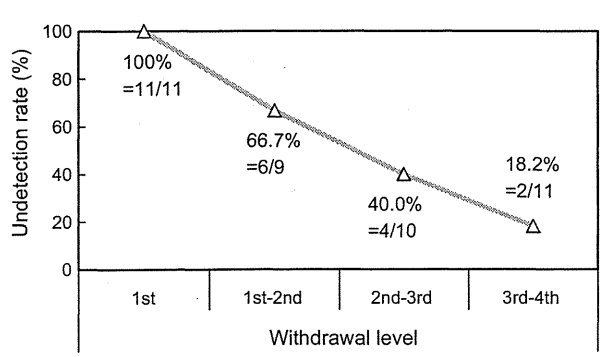

Fig. 5. Variation of undetection rate with withdrawal level.

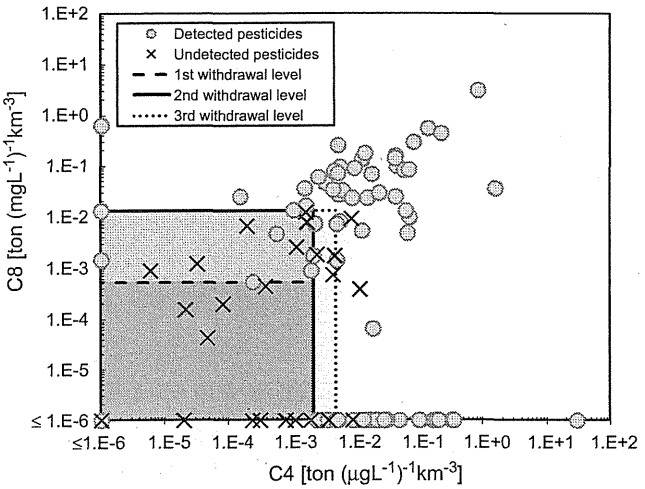

Fig. 6. Scatter plot of C8 versus C4 for the pesticides in the primary group.

(third withdrawal level), the undetection rate decreased to 40.0%. The undetected and detected pesticides were mixed between the second and third withdrawal levels. If we take the second withdrawal level as the cutoff level, 17 undetected pesticides should be withdrawn from the primary group because of the very low probability of their detection and the fact that there are no records of their detection.

4. Conclusions and future work

Twenty-four indicators were created (Table 1) and tested using the detection rate defined by Eq. (1) in order to efficiently select pesticides that would likely be detected if monitored. The combination of indicators C4 and C8 maximized the detection rate, suggesting that this combination was the best for selecting the pesticides of probable detection. This result reflected the importance of local pesticide consumption according to rice-farming/upland-field application, guideline value, degradation and adsorption properties as quantified by score values, and annual precipitation.

The application of the indicators suggests that the primary group of JDWQG should be amended with the addition of 44 pesticides, as well as the removal of 17 pesticides. The probability of detection of the 44 pesticides was more than 72%. Whether these 44 pesticides can actually be detected is an important question, and a long-term, follow-up study is needed to answer this question. Before nationwide monitoring of these pesticides can be implemented, several tasks will have to be completed, including the establishment of standard analytical methods and official revision of the primary group. Furthermore, our results suggest that local variations in pesticide use are an important aspect of predicting the probability of pesticide detection. Additional studies may allow the prediction of pesticide detection locations. In this study, we used binary statistical data: pesticides were either detected or not detected. However, the probability of detection or no-detection could also be predicted from quantitative data for measured pesticide concentrations. Further study will provide additional data for the selection of regulated pesticides.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2013.10.019.

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Improved virus removal by high-basicity polyaluminum coagulants compared to commercially available aluminum-based coagulants

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ABSTRACT

We investigated the effects of basicity, sulfate content, and aluminum hydrolyte species on the ability of polyaluminum chloride (PACl) coagulants to remove F-specific RNA bacteriophages from river water at a pH range of 6–8. An increase in PACl basicity from 1.5 to 2.1 and the absence of sulfate led to a reduction of the amount of monomeric aluminum species (i.e., an increase of the total amount of polymeric aluminum and colloidal aluminum species) in the PACl, to an increase in the colloid charge density of the PACl, or to both and, as a result, to high virus removal efficiency. The efficiency of virus removal at around pH 8 observed with PACl-2.1c, a nonsulfated high-basicity PACl (basicity 2.1–2.2) with a high colloidal aluminum content, was larger than that observed with PACl-2.1b, a nonsulfated high-basicity PACl (basicity 2.1–2.2) with a high polymeric aluminum content. In contrast, although extremely high basicity PACls (e.g., PACl-2.7ns, basicity 2.7) effectively removed turbidity and UV260-absorbing natural organic matter and resulted in a very low residual aluminum concentration, the virus removal ratio with PACl-2.7ns was smaller than the ratio with PACl-2.1c at around pH 8, possibly as a result of a reduction of the colloid charge density of the PACl as the basicity was increased from 2.1 to 2.7. Liquid ^{27}Al NMR analysis revealed that PACl-2.1c contained Al_{30} species, which was not the case for PACl-2.1b or PACl-2.7ns. This result suggests that Al_{30} species probably played a major role in virus removal during the coagulation process. In summary, PACl-2.1c, which has high colloidal aluminum content, contains Al_{30} species, and has a high colloid charge density, removed viruses more efficiently (>4 $\log_{10}$ for infectious viruses) than the other aluminum-based coagulants—including commercially available PACls (basicity 1.5–1.8), alum, and PACl-2.7ns—over the entire tested pH (6–8) and coagulant dosage (0.54–5.4 mg-Al/L) ranges.

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1. Introduction

Aluminum-based coagulants such as polyaluminum chloride (PACl) and alum are commonly used in coagulation processes to destabilize suspended and dissolved materials in water and combine them into large flocs that are easily separated from the water by subsequent sedimentation or filtration. Waterborne enteric viruses, which do not settle from suspension under the influence of gravity, can also be removed with aluminum-based coagulants. For example, Nasser et al. (1995) reported that 88.4% and 47% of hepatitis A virus and poliovirus, respectively, can be removed by coagulation with 30 mg/L of alum. We have reported that a coagulation process with PACl or alum effectively removes bacteriophages, which are viruses that infect bacteria and may be indicators for waterborne enteric viruses (Matsushita et al., 2011).

The efficiency of microorganism removal by coagulation processes is strongly influenced by several factors, including the nature and dosages of the coagulant used, pH, temperature, and mixing method (Hijnen and Medema, 2010). In particular, pH control during the coagulation process is essential for optimal coagulation (Bratby, 2006). Guo and Hu (2011) reported that coagulation with alum at pH 8 does not result in significant virus removal, whereas coagulation at pH 6 and 7 does. Worldwide, the pH of various surface drinking water sources is changing from neutral to alkaline because of the excessive growth of algae (Hu et al., 2006; Matsukawa et al., 2006), and this change can be expected to reduce coagulation efficiency and thus virus removal performance if commercially available PACl or alum is used without pH adjustment. Reducing the pH of drinking water sources with acid or increasing the coagulant dosage is sometimes required to improve coagulation efficiency (Hu et al., 2006; Yan et al., 2008a). However, both of these methods have some disadvantages, such as increasing the residual aluminum concentration in treated water (Matsukawa et al., 2006) and increasing the treatment cost (Yan et al., 2008a). Therefore, the development of new coagulation processes that effectively remove suspended and dissolved materials, including viruses, from both neutral and alkaline drinking water sources without the need for pH optimization is highly desired.

For the improvement of coagulation efficiency, PACl coagulants with various aluminum hydrolysis ratios (basicity = $[OH^-]/[Al^{3+}]$) have been produced, and the influence of PACl basicity on coagulation processes has been investigated (Wang et al., 2002; Yan et al., 2008a,b; Yang et al., 2011; Zhang et al., 2008). For example, Wang et al. (2002) reported that turbidity removal at alkaline pH is improved by increases in PACl basicity. Zhang et al. (2008) reported that the coagulation efficiency of PACl increases with increasing basicity: specifically, PACl with a basicity of 2.4 exhibits higher humic acid removal efficiency and lower residual aluminum concentration at a broader pH range and a wider PACl dosage range compared to PACls with basicities of 1.2 and 1.8. In previous work, we compared PACls with basicities of 2.1 and 1.5 in the pH range of 6.8–7.8 and found that the former, which contains a smaller percentage of monomeric aluminum species and a larger percentage of colloidal aluminum species than the latter, removes dissolved organic carbon more efficiently and with a lower residual aluminum concentration (Kimura et al., 2013). Moreover, PACls with basicities of >2.6 yield a very low residual aluminum concentration (<0.02 mg/L), even at a wide pH range (6.5–8.5; Kimura et al., 2013). High-basicity PACls are expected to effectively remove viruses not only at neutral pH but also at weakly alkaline pH; however, virus removal during coagulation processes with high-basicity PACls has not been fully investigated. In addition, little information is available about how the small amount of sulfate (e.g. 3% w/w) that is present in commercially available PACls to improve flocculation and sedimentation efficiency (Pernitsky and Edzwald, 2003) affects virus removal during coagulation processes.

Here, we conducted batch coagulation experiments to investigate the effect of PACl basicity on virus removal by comparing a wide variety of PACls with different basicities, including commercially available PACls (basicity 1.5–1.8) and extremely high basicity PACls (basicity 2.7). In addition, we investigated the effect of sulfate in the PACls on virus removal by comparing sulfated and nonsulfated PACls. Moreover, we experimentally evaluated the aluminum species distributions and colloid charge densities of the tested coagulants to determine what caused the differences in virus removal performance.

2. Materials and methods

2.1. Source water and coagulants

River water was sampled from the Toyohira River in Sapporo, Japan, on 1 October 2010, 24 June 2011, and 4 December 2012 (water quality data are shown in Table S1, Supplementary Information).

We conducted three sets of coagulation experiments on the river water samples. For the first set of experiments, we used five aluminum-based coagulants (Table S2). Two commercially available PACls (PACl-1.5s and PACl-1.8s, where 1.5 and 1.8 are the basicity values, and "s" stands for "sulfated"; Taki Chemical Co., Kakogawa, Japan). A trial high-basicity PACl (PACl-2.1s, which is now commercially available) was also supplied by the same company. For comparison with the commercially available PACls, we evaluated an $AlCl_3$ solution prepared by dilution of reagent-grade aluminum(III) chloride hexahydrate ($AlCl_3 \cdot 6H_2O$, Wako Pure Chemical Industries, Osaka, Japan) in Milli-Q water (Milli-Q Advantage, Millipore Corp., Billerica, MA, USA), and we also evaluated a commercially available alum (Taki Chemical Co.).

After the first set of experiments was completed, we conducted a second set of experiments with eight aluminum-based coagulants (Table S2). In addition to two of the sulfated PACls described above, we evaluated a trial nonsulfated PACl (PACl-1.5ns, where "ns" stands for "nonsulfated"), a high-basicity nonsulfated PACl (PACl-2.1ns), and an extremely high basicity nonsulfated PACl (PACl-2.7ns), all provided by Taki Chemical Co., to further investigate the effects of basicity and sulfate on virus removal. We also evaluated three PACls (PACl-2.1b, PACl-2.1c, and PACl-2.7, where "b" and "c" indicate high Al_b and Al_c content, as measured by a ferron method, described below) prepared by a base titration

method in our laboratory, as described previously (Kimura et al., 2013).

Finally, we conducted a third set of experiments with eight aluminum-based coagulants (Table S2), which were provided by Taki Chemical Co. or prepared in our laboratory by the base titration method.

All the laboratory-made PACls (PACl-0.9, PACl-1.5, PACl-2.1b, PACl-2.1c, and PACl-2.7) were nonsulfated, and they are distinguished from the company-made PACls in that "ns" is not included in the name.

All the coagulants were used in batch coagulation experiments immediately after dilution with Milli-Q water.

2.2. Characterization of coagulants

2.2.1. Ferron method

The aluminum hydrolyte species in the coagulants were analyzed by means of a ferron method (Wang et al., 2004) after dilution with Milli-Q water to a concentration of 2.7 g-Al/L, i.e., 0.1 M-Al (analytical pH condition was approximately 4–5). On the basis of the kinetic differences between the reactions of the aluminum species and the ferron reagent (8-hydroxy-7-iodoquinoline-5-sulfonic acid, Wako Pure Chemical Industries), aluminum hydrolyte species were categorized as monomeric species, fast-reacting polymeric species, or slow-reacting colloidal species, denoted as Al_a, Al_b, and Al_c, respectively (Wang et al., 2004). After addition of the ferron reagent to the diluted coagulant, the mixture was immediately stirred magnetically for 10 s at 400 rpm, and then the absorbance at 366 nm was measured with a UV-1700 Pharma Spec spectrophotometer (Shimadzu Corp., Kyoto, Japan) at predetermined reaction times. The aluminum hydrolyte species were operationally divided into the three categories as follows: Al_a, species that reacted with ferron within 30 s; Al_b, species that reacted with ferron within 120 min (absorbance at 120 s minus the absorbance due to Al_a); and Al_c, species that did not react with ferron ($Al_c = Al_t - [Al_a + Al_b]$, where Al_t = total Al). To obtain Al_t, we adjusted the pH of the diluted coagulant to approximately 0.5 with ultrapure nitric acid (Kanto Chemical Co., Tokyo, Japan), heated it for 3 h at 85 °C in a muffle furnace, cooled it to room temperature, and then analyzed it by the ferron method as described for Al_a.

2.2.2. Liquid ^{27}Al nuclear magnetic resonance analysis

In addition to the ferron method, ^{27}Al nuclear magnetic resonance (NMR) spectrometry was also used to characterize the aluminum hydrolyte species in the coagulants after dilution with Milli-Q water to a concentration of 2.7 g-Al/L, i.e., 0.1 M-Al (analytical pH condition was approximately 4–5). On the basis of chemical shift differences, aluminum hydrolyte species were categorized into four groups: monomeric species (Al_m), dimeric and trimeric species, tridecameric species (Al_{13}), and Al_{30} species (Chen et al., 2006, 2007; Gao et al., 2005). After addition of deuterium oxide (75% v/v, Wako Pure Chemical Industries) to the diluted coagulant, the solution was placed in a 5-mm NMR tube. A 3-mm coaxial capillary filled with diluted sodium aluminate (Wako Pure Chemical Industries) solution, which was diluted with Milli-Q water to 0.01 M-Al and then added the deuterium oxide (75% v/v). The coaxial capillary was used as an internal standard for Al content and as the deuterium lock (Chen et al., 2007; Gao et al., 2005). The NMR spectra were measured with a JEOL JNM-ECA 600 spectrometer (JEOL, Tokyo, Japan) by means of a single-pulse method (field strength 14.09 T, resonance frequency 156.39 MHz, pulse width 5.0 μs, repetition time 1.13 s, scans 8000, X-sweep 78.25 kHz). The reference chemical shift (0 ppm) was adjusted with $AlCl_3$ solution prepared by the procedure described above.

2.2.3. Colloid titration analysis

The positive colloid charges of the coagulants were determined by colloid titration with a COM-555 Potentiometric Titrator (Hiranuma Sangyo Co., Mito, Japan). Each coagulant was diluted with Milli-Q water to 1–2 mg-Al/L (analytical pH condition was approximately 4–5), and then 150 mL of diluted coagulant was transferred to a titration vessel. After addition of 0.3 mL of toluidine blue indicator (Wako Pure Chemical Industries) to the vessel, the solution was titrated by means of a pump with 0.001 N potassium polyvinyl sulfate (a standard negative colloid, Wako Pure Chemical Industries) at a constant rate of 10 mL/min. The vessel contents were magnetically stirred during the titration, and the absorbance at 630 nm was recorded continuously until little change in the absorbance (i.e., subtle change in the color of the indicator from light blue to bluish-purple) was observed. The positive colloid charge was determined from the volume of potassium polyvinyl sulfate that corresponded to the half height of the descending slope of the recorded absorbance curve.

2.3. Bacteriophages

F-specific RNA bacteriophages Qβ (NBRC 20012) and MS2 (NBRC 102619) were obtained from the NITE Biological Research Center (Kisarazu, Japan). Qβ (Boudaud et al., 2012; Matsui et al., 2003; Matsushita et al., 2011; Shirasaki et al., 2009a,b) and MS2 (Boudaud et al., 2012; Fiksdal and Leiknes, 2006; Guo and Hu, 2011; Matsushita et al., 2011; Nasser et al., 1995; Shirasaki et al., 2009a,b; Zhu et al., 2005) are widely used as surrogates for waterborne enteric viruses in coagulation processes because these bacteriophages are morphologically similar to hepatitis A viruses and polioviruses, removal of which during drinking water treatment is important. Qβ is the prototype member of the genus *Allolevivirus* in the virus family Leviviridae, and MS2 is the prototype member of the genus *Levivirus* in the Leviviridae family. The genomes of these two bacteriophages contain a single molecule of linear, positive-sense, single-stranded RNA, which is encapsulated in an icosahedral protein capsid with a diameter of 24–26 nm (Fauquet et al., 2005). Each bacteriophage was propagated and purified prior to the preparation of a bacteriophage stock solution as described in our previous report (Shirasaki et al., 2010).

2.4. Coagulation experiments with bacteriophage-spiked river water

Batch coagulation experiments were conducted with 1000 mL of bacteriophage-spiked river water in square plastic beakers at 20 °C. The bacteriophage stock solution (see Section 2.3) was added to the river water in a beaker at approximately 10^8

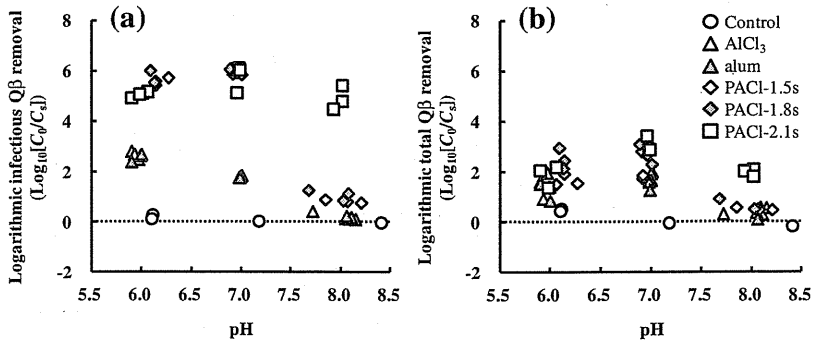

Fig. 1 – Effect of coagulant type on removal of infectious Qβ as evaluated by the PFU method (a) and on total Qβ removal as evaluated by the PCR method (b) after settling during the coagulation process. The source water was river water 1, and the coagulant dosage was 2.16 mg-Al/L.

plaque forming unit (PFU)/mL (C_0), and the spiked water was mixed with an impeller stirrer. After enough HCl or NaOH was added to the water to bring the final pH to a target value of at 6, 7, or 8, coagulant was injected into the water at a dosage of 0.54, 1.08, 1.89, 2.16, or 5.4 mg-Al/L. The water was stirred rapidly for 1 min (G = 200 s^{-1}, 136 rpm) and then slowly for 10 min (G = 20 s^{-1}, 29 rpm). The water was left at rest for 60 min to allow the generated aluminum floc particles to settle. Then the supernatant was sampled from the beaker for quantification of the bacteriophage concentrations (C_s) and turbidity. A portion of each supernatant was filtered through a membrane filter (first and second sets of experiments, nominal pore size 0.4 μm, polycarbonate, Isopore, Millipore; third set of experiments, nominal pore size 0.45 μm, polytetrafluoroethylene, Dismic-25HP, Toyo Roshi Kaisha, Tokyo, Japan) for quantification of the ultraviolet absorbance at 260 nm (an indication of natural organic matter [NOM] concentration) and for measurement of the aluminum concentration. Turbidity and UV260-absorbing NOM were quantified with a 2100AN turbidity meter (Hach Company, Loveland, CO, USA) and a UV-1700 Pharma Spec spectrophotometer, respectively. After ultrapure nitric acid (1% v/v, Kanto Chemical Co.) was added to the membrane permeate, the aluminum concentration was determined by means of inductively coupled plasma–mass spectrometry (Agilent 7700 series, Agilent Technologies, Inc., Santa Clara, CA, USA).

2.5. *Bacteriophage assay*

The infectious bacteriophages were quantified by determination of the number of PFUs according to the double-layer method (Adams, 1959) with *Escherichia coli* (NITE Biological Research Center 13965) as the bacterial host. The average of the plaque counts of triplicate plates prepared from one sample was considered as the infectious bacteriophage concentration for that sample.

Bacteriophage RNA was quantified by a real-time reverse transcription-polymerase chain reaction (RT-PCR) method, which detects all bacteriophages regardless of their infectivity and the existence of aggregates. The details of the real-time RT-PCR method are described in Supplementary Information.

3. Results and discussion

3.1. *First set of experiments*

3.1.1. *Effect of coagulant type on bacteriophage removal*

The effect of coagulant type on the infectious Qβ removal ratio ($\log_{10}[C_0/C_s]$) during the coagulation process was evaluated by the PFU method after settling (Fig. 1a). Because Qβ is small and was stably dispersed in the river water (because of electrical repulsion), no removal (<0.3-$\log_{10}$) of infectious Qβ was observed in the absence of coagulant at any pH. In contrast, the coagulation process removed infectious Qβ at a pH range of 6–7 no matter what type of coagulant was used. This result indicates that the Qβ stably monodispersed in the river water was destabilized by the addition of coagulant and became adsorbed on or entrapped in the aluminum floc particles generated during the coagulation process and that the aluminum floc particles along with the destabilized Qβ then settled out from the suspension under the influence of gravity during the settling process. The efficiency of infectious Qβ removal depended on coagulant type: whereas coagulation with $AlCl_3$ and alum resulted in approximately 2-$\log_{10}$ removal at a pH range of 6–7, approximately 6-$\log_{10}$ removal was achieved with all the PACls, regardless of their basicity. Matsushita et al. (2011) also reported that the infectious Qβ removal ratio during the coagulation process with PACl is larger than that with alum at neutral pH. Moreover, we previously reported that PACl is more effective than alum for removing norovirus particles (Shirasaki et al., 2010).

The virus removal performances of $AlCl_3$, alum, PACl-1.5s, and PACl-1.8s markedly decreased when the pH of the treated water was increased from 7 to 8 (Fig. 1a). Hu et al. (2006) reported that the aluminum species distributions of $AlCl_3$ and commercially available PACl during coagulation process were greatly changed depending on the pH: although the aluminum species distributions of those coagulants were almost same in the pH range from 6 to 7, monomeric aluminum species were increased while polymeric and colloidal aluminum species were decreased when the pH of the treated water was increased from 7 to 8. Therefore, difference in the aluminum species distributions of the $AlCl_3$, alum, PACl-1.5s and PACl-

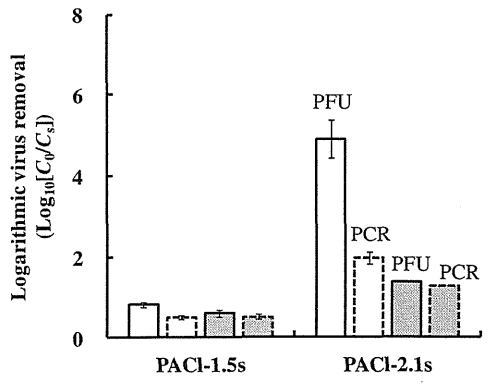

Fig. 2 – Comparison of Qβ (white) and MS2 (gray) removal ratios from treated water at around pH 8 after settling during the coagulation process. The source water was river water 1, and the coagulant dosage was 2.16 mg-Al/L. Values are means (n = 2–3), and the error bars indicate standard deviations.

1.8s probably contribute to the difference in the virus removal performances between pH range from 6 to 7 and pH 8. In contrast, PACl-2.1s retained its high virus removal performance (~5-$\log_{10}$ removal) even at weakly alkaline pH. This result indicates that PACl basicity affected virus removal performance during the coagulation process and that a high-basicity PACl (PACl-2.1s) effectively removed the virus not only under weakly acidic and neutral pH conditions but also at weakly alkaline pH. The total Qβ removal ratios evaluated by the PCR method were also observed to be somewhat larger with PACl-2.1s than the ratios with $AlCl_3$, alum, PACl-1.5s, and PACl-1.8s, especially at around pH 8 (Fig. 1b). In addition, the coagulation process with PACl-2.1s removed turbidity and UV260-absorbing NOM more efficiently and resulted in a lower residual aluminum concentration than did $AlCl_3$, alum, PACl-1.5s, and PACl-1.8s, especially at weakly alkaline pH (Fig. S1).

The Qβ removal ratios determined by the PFU and PCR methods differed markedly: the infectious Qβ removal ratios (Fig. 1a) were larger than the total Qβ removal ratios (Fig. 1b). This difference between the PFU and PCR methods could be explained by the formation of aggregates consisting of several infectious Qβ particles, the inactivation of Qβ during the coagulation process, or both. Matsushita et al. (2011) reported that Qβ loses its infectivity after being mixed with aluminum hydrolyte species during the coagulation process with PACl-1.5s, as indicated by a combination of filtration and particle size measurements at neutral pH. This result suggests that the virucidal activity of the aluminum-based coagulants contributed to the efficiency of infectious Qβ removal during the coagulation process.

3.1.2. *Comparison of Qβ and MS2 removal ratios during the coagulation process*

As described above, PACl-2.1s removed Qβ more efficiently than did other aluminum-based coagulants used in the present study, especially at weakly alkaline pH. To confirm that PACl-2.1s actually removed viruses more effectively than PACl-1.5s, we also evaluated the MS2 removal ratio, because MS2 is less sensitive than Qβ to the virucidal activity of PACl (Matsushita et al., 2011; Shirasaki et al., 2009a). We evaluated the MS2 removal efficiency by means of the PFU and PCR methods after settling during the coagulation process, and then compared the results with those for Qβ (Fig. 2). For both bacteriophages, the removal ratios observed with PACl-2.1s were larger than those with PACl-1.5s at around pH 8. This result means that compared to coagulation with PACl-1.5s, coagulation with PACl-2.1s more effectively removed not only a virus that is highly sensitive to the virucidal activity of the aluminum-based coagulants but also a virus that is less sensitive.

The infectious Qβ removal ratio of PACl-2.1s was approximately 3-$\log_{10}$ larger than the infectious MS2 removal ratio, partly because of the different sensitivities of Qβ and MS2 to the virucidal activity of PACl-2.1s. Because Qβ is more sensitive than MS2, the infectious Qβ concentration after settling during the coagulation process may have been less than the quantification limit of the PFU method when the other high-basicity PACl was applied. Therefore, we used MS2 in our second and third sets of experiments.

3.2. *Second set of experiments*

3.2.1. *Effects of coagulant basicity and sulfate content on bacteriophage removal*

To further investigate the effective virus removal observed with PACl-2.1s, we conducted batch coagulation experiments

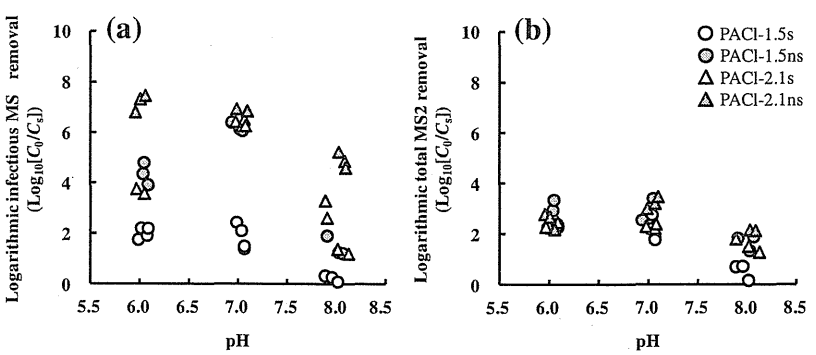

Fig. 3 – Effects of coagulant basicity and sulfate content on infectious MS2 removal as evaluated by the PFU method (a) and on total MS2 removal as evaluated by the PCR method (b) after settling during the coagulation process. The source water was river water 2, and the coagulant dosage was 1.89 mg-Al/L.