Regulatory Toxicology and Pharmacology 65 (2013) 108–114

Contents lists available at SciVerse ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Safety assessment of boron by application of new uncertainty factors and their subdivision

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ARTICLE INFO

Article history:
Received 12 June 2012
Available online 5 November 2012

Keywords:
Derivation of boron TDI
Chemical risk assessment
Application of new uncertainty factor (UF)
Subdivision of new uncertainty factor (UF)

ABSTRACT

The available toxicity information for boron was reevaluated and four appropriate toxicity studies were selected in order to derive a tolerable daily intake (TDI) using newly proposed uncertainty factors (UFs) presented in Hasegawa et al. (2010). No observed adverse effect levels (NOAELs) of 17.5 and 8.8 mg B/kg/day for the critical effect of testicular toxicity were found in 2-year rat and dog feeding studies. Also, the 95% lower confidence limit of the benchmark doses for 5% reduction of fetal body weight ($BMDL_{05}$) was calculated as 44.9 and 10.3 mg B/kg/day in mouse and rat developmental toxicity studies, respectively. Measured values available for differences in boron clearance between rats and humans and variability in the glomerular filtration rate (GFR) in pregnant women were used to derive chemical specific UFs. For the remaining uncertainty, newly proposed default UFs, which were derived from the latest applicable information with a probabilistic approach, and their subdivided factors for toxicokinetic and toxicodynamic variability were applied. Finally, overall UFs were calculated as 68 for rat testicular toxicity, 40 for dog testicular toxicity, 247 for mouse developmental toxicity and 78 for rat developmental toxicity. It is concluded that 0.13 mg B/kg/day is the most appropriate TDI for boron, based on rat developmental toxicity.

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1. Introduction

To ensure drinking water safety, a variety of toxicity information on environmental pollutant chemicals is collected and evaluated in order to derive a tolerable daily intake (TDI). A TDI is derived by dividing the no observed adverse effect level (NOAEL) for the selected critical effect (identified from key toxicity studies such as repeated dose toxicity, reproductive and developmental toxicity, and carcinogenicity) by an appropriate composite uncertainty factor (UF). A default composite UF of 100, consisting of 10 for interspecies differences (UF_a) and 10 for human variability (UF_h), has been commonly used in the derivation of TDIs in Japan and some international organizations. WHO (2005) took the approach further by determining that each component UF can be subdivided into toxicokinetics (TK), disposition of substance (generally measured as species differences in blood concentration at the same dose), and toxicodynamics (TD), toxic intensity of substances (generally measured as species differences in toxicity level at the same blood concentration). Appropriate measured or estimated TK and/or TD values can be incorporated into the safety assessment process by replacing the default component UFs.

To date, this subdivision approach has been applied in several situations in Health Canada (Meek et al., 1994) and the United States (US EPA, 2004), but the approach is limited internationally. The WHO drinking water quality guidelines used the approach for boron, where measured data on the human glomerular filtration rate (GFR) was used to determine the chemical specific UF used in the TDI calculation (WHO, 2009). However, an even newer approach for UF selection has been derived from the latest data related to interspecies differences and human variability with a probabilistic approach to the TK and TD subdivisions (Hasegawa et al., 2010). Therefore, in this article, we apply the new UF probabilistic subdivisions during the UF selection process in order to derive a TDI for boron.

2. Concept of current uncertainty factor

An UF of 100 (Lehman and Fitzhugh, 1954) was proposed for boron without substantial reasons, as was common practice, and has been widely used around the world until recently. Dourson and Stara (1983) justified using an UF of 100 ($UF_a = 10$, $UF_h = 10$) in risk calculations by gathering and organizing supporting

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http://dx.doi.org/10.1016/j.yrtph.2012.10.013

information. Analysis of interspecies differences (Freireich et al., 1966) demonstrated a good relationship between the body surface area and the maximum tolerated dose of 18 anti-cancer drugs after repeated administration in humans and various experimental animals. The body surface area can be presented as:

$$BW^{2/3} \times K \times 10^{-4}\ m^2$$

the body surface area per body weight becomes:

$$BW^{2/3} \times K \times 10^{-4} \times BW^{-1} = K \times 10^{-4} \times BW^{-1/3}$$

where BW is the body weight (g) and K is an adjustment factor.

As K ranges from 9 to 11 in various experimental animals as well as humans, the body surface ratio of animal/human is:

$$K_a \times 10^{-4} \times BW_a^{-1/3}/K_h \times 10^{-4} \times BW_h^{-1/3} = BW_h^{1/3}/BW_a^{1/3} = (BW_h/BW_a)^{1/3}.$$

The body surface correction factor becomes 5.6 for rats and 11.4 for mice when the body weight is 60 kg for humans, 350 g for rats and 40 g for mice. Based on these data, a default UF_a of 10 is considered to be an appropriate numerical value, since it lies between these two values. When logarithmic dose/probit slopes were calculated for acute rat toxicity data on 490 chemicals, 92% of the slopes were 3 or greater, suggesting that a 10-fold decrease in dose would yield a 3 probit reduction in risk. This supports the use of an UF_h of 10 for within species variability.

Renwick (1993) proposed that the UF_a and UF_h can each be subdivided into a TK and TD component. Renwick analyzed toxicokinetic parameter data, such as clearance rate and area under the concentration time curve (AUC) in plasma or tissue for TK and *in vitro* dose–response or *in vivo* toxicodynamic data were analyzed for TD to support the UF division. IPCS and WHO (IPCS, 1994; WHO, 2005) determined that the distribution of the TK:TD ratio is 60:40 for UF_a and 50:50 for UF_h:

$$UF_a = (TK) \times (TD) = 10^{0.6} \times 10^{0.4} = 4\ (TK) \times 2.5\ (TD)$$

$$UF_h = (TK) \times (TD) = 10^{0.5} \times 10^{0.5} = 3.2\ (TK) \times 3.2\ (TD).$$

The default value of 4 for UF_a (TK) is consistent with the differences in fundamental physiological parameters, for example, the heart output volume of rats is approximately 4-fold higher than in humans. The equal subdivision of UF_h is supported by the analysis of kinetic parameters for 60 chemicals and toxicity dose–response data for 49 chemicals.

3. Toxicity-related information on boron

Boron has almost complete absorption via the gastrointestinal tract and is excreted via the urine in both humans and experimental animals. The average clearance rate for boron is 163 mL/h/kg (2.72 mL/min/kg) in rats and 41 mL/h/kg (0.68 mL/min/kg) in humans, the rat clearance value is approximately 4-fold higher than the human value (Dourson et al., 1998). Boron clearance in pregnant women averages at 1.02 mL/min/kg (66.1 mL/min/person) (Pahl et al., 2001) and the rate in pregnant rats is 3.3 mL/min/kg (1.0 mL/min/rat) (Vaziri et al., 2001), indicating that boron clearance rates increase during pregnancy by 50% in humans and 21% in rats.

Evidence of human male reproductive toxicity was not observed in the epidemiological studies of men exposed to high levels of boron (Sayli, 2001, 2003; Whorton et al., 1994; Yazbeck et al., 2005; Robbins et al., 2010; Duydu et al., 2011). However, testicular and developmental toxicity were observed in multiple experimental animal toxicity studies. Boron was neither genotoxic nor carcinogenic in cancer bioassays (NTP, 1987).

Nine repeat dose toxicity studies and five reproductive/developmental toxicity studies for boric acid were evaluated in order to derive a TDI for boron. Brief study details and NOAELs for selected target organs or endpoints are shown in Table 1. NOAELs are expressed as mg B (boron)/kg (body weight)/day, which are converted to mg of boron by multiplying by the ratio of the molecular weight of boron to the molecular weight of boric acid (10.81/61.84 = 0.1748).

4. Derivation of boron TDI in WHO and US

4.1. Drinking water quality guideline in WHO (2009)

The critical endpoint of interest for boron was determined to be fetal body weight changes and skeleton malformations (high incidence of short rib XIII and wavy ribs) observed in two rat developmental toxicity studies (Heindel et al., 1992; Price et al., 1996b). The 95% lower confidence limit of the benchmark dose for 5% reduction of fetal body weight ($BMDL_{05}$ = 10.3 mg B/kg/day) (Allen et al., 1996) was adopted as the point of departure (POD) for this evaluation.

The available boron data was not sufficient to derive a chemical specific interspecies UF, thus the default UF of 10 was used. The UF for human variability was subdivided into TK and TD components according to the WHO methodology (IPCS, 1994; WHO, 2005). TK data from pregnant women were analyzed as a sensitive subpopulation to determine the TK portion of the UF_h. Given that boron is essentially not metabolized and is mostly excreted via the urine, the GFR in pregnant women is used in place of the default TK UF_h. Dourson et al. (1998) combined data from multiple studies obtaining a GFR of 144 ± 32 mL/min for healthy pregnant women in their last trimester. In order to account for 95% of the population, the average GFR_A (144 mL/min) was divided by the GFR_{2SD} at two standard deviations below the average (GFR_A–GFR_{2SD} = 144 − 2 × 32 = 80 mL/min), resulting in a human TK variability UF_h of 1.8 (144/80 = 1.8) (Dourson et al., 1998). There were no data on TD variation in pregnant women, therefore the default TD UF_h of 3.2 was used. The resulting human variability UF is approximately 6; derived by multiplying the TK and TD values together (1.8 × 3.2 = 5.7).

Finally, a TDI of 0.20 mg B/kg/day was derived by applying the composite UF of 60 ($UF_a \times UF_h = 10 \times 6$) to the $BMDL_{05}$ of 10.3 mg B/kg/day for rat developmental toxicity.

$$\frac{10.3\ \text{mg B/kg/day}}{60} = 0.2\ \text{mg B/kg/day}$$

4.2. Toxicological review by US EPA (2004)

As with WHO, combined data on fetal body weight changes, rib XIII effects and variations of the first lumbar rib from two rat developmental toxicity studies (Heindel et al., 1992; Price et al., 1996b) were selected as the critical endpoints, and the $BMDL_{05}$ of 10.3 mg B/kg/day calculated for reduction of fetal body weight by Allen et al. (1996) was selected as the POD.

In a slightly different approach US EPA subdivided the default UF of 10 for UF_a and UF_h into 3.16 for TK variability and 3.16 for TD variability in animals and humans. As there was no TD data for interspecies differences and human variability, only TK data were analyzed. TK analysis was conducted for differences between pregnant rats and women (species difference) and for variations in pregnant women (human variability). Boron is easily absorbed after oral administration in both humans and animals, but is not metabolized in the body. More than 90% of the absorbed boron was excreted in a short period via the urine. In humans, 92–94%

Table 1
Summary of repeat dose, developmental toxicity, and generation studies for boric acid.

Species	Route	Period	Target	NOAEL[a]	References
Repeated dose toxicity study					
Rats	Feeding	28 d	Testes	61[b]	Treinen and Chapin (1991)
Rats	Feeding	30–60 d	Testes	25	Lee et al. (1978)
Rats	Feeding	9 w	Testes	26[b]	Ku et al. (1991)
Mice	Feeding	13 w	Testes	70	Dieter (1994)
Rats	Feeding	90 d	Testes	38	Weir and Fisher (1972)
Dogs	Feeding	90 d	Testes	3.9	Weir and Fisher (1972)
Mice	Feeding	2 y	Testes	48	Dieter (1994)
Rats	Feeding	2 y	Testes	17.5	Weir and Fisher (1972)
Dogs	Feeding	2 y	Testes	8.8	Weir and Fisher (1972)
Developmental or generation toxicity study					
Mice	Feeding	gd 0–17	Fetal bw	43	Heindel et al. (1992)
Rats	Feeding	gd 0–20	Fetal bw	14[b]	Heindel et al. (1992)
Rats	Feeding	gd 0–20	Fetal tox	9.6	Price et al. (1996b)
Rats	Feeding	3 gen	Reproduction	17.5	Weir and Fisher (1972)
Rabbits	Gavage	gd 6–19	Fetal tox	22	Price et al. (1996a)

d: Day; w: week; y: year; gd: gestational day; bw: body weight; gen: generation; tox: toxicity.
[a] mg B/kg/day.
[b] Lowest observed adverse effect level.

of boron was excreted as the unchanged form in urine after 96 h of digestion. In rats, boron was completely absorbed within 24 h, the absorbed boron was uniformly distributed throughout the body and the 95% was excreted in urine over 3 days. Thus, it is considered that the disposition of boron is the same in humans and animals.

To assess interspecies TK differences, UF_a (TK), boron clearance was compared between pregnant rats and pregnant women. The study assessed on pregnant rats given 0.3, 3.0 or 30 mg B/kg orally showed the dose-independent value of boron clearance as 1.00 mL/min (Vaziri et al., 2001; US Borax, 2000). For human assessment, after 15 pregnant women had eaten fruits and vegetables containing a high content of boron, the concentration of boron in blood and urine was determined for the first two hours, resulting in a 66.1 mL/min boron clearance (Pahl et al., 2001; US Borax, 2000). The steady state blood concentration (Css) of boron in the 2-compartment model can be expressed as follows:

$$Css = \frac{Dose \times f \times BW}{Cl}$$

where f is the absorption rate, BW is body weight, and Cl is clearance. When pregnant rats and women have equal blood concentrations of boron, differences in the administered dose can be adopted as the UF_a (TK). Therefore, using the boron absorption rate of humans (f_h) – 0.92 (Schou et al., 1984), the rate of rats (f_a) – 0.95 (Vanderpool et al., 1994), the body weight of pregnant women – 67.6 kg (Pahl et al., 2001) and that of pregnant rats – 0.303 kg (Vaziri et al., 2001), the UF_a (TK) was calculated as follows:

$$\frac{Dose_a}{Dose_h} = UF_a(TK) = \frac{Cl_a \times f_h \times BW_h}{Cl_h \times f_a \times BW_a} = \frac{1.00 \times 0.92 \times 67.6}{66.1 \times 0.95 \times 0.303} = \frac{62.192}{19.027} = 3.3$$

An UF_a of 10.4 results from $TK_a \times TD_a$ (3.3 × 3.16).

Adoption of boron clearance data of pregnant women used for the interspecies TK differences (Pahl et al., 2001) was considered for the human variability UF. However, US EPA determined that the Pahl et al. (2001) study could not be used because the number of subjects was inadequate and the boron content of the food was not controlled. Therefore, GFR data from healthy pregnant women (Dunlop, 1981; Krutzen et al., 1992; Sturgiss et al., 1996) were used as a surrogate for boron clearance. In order to account for pregnant women with very low GFR, particularly when preeclampsia is present, the average GFR_A was divided by the GFR_{3SD} at three standard deviations below the average (GFR_A–GFR_{3SD}) to calculate UF_h (TK) (Dourson et al., 1998) (Table 2). An UF_h of 6.3 results from $TK_h \times TD_h$ (2.0 × 3.16).

Finally, an oral RfD of 0.20 mg B/kg/day was derived by applying the composite UF of 66 (3.3 × 3.16 × 2.0 × 3.16) to the $BMDL_{05}$ of 10.3 mg B/kg/day for rat developmental toxicity.

$$\frac{10.3 \text{ mg B/kg/day}}{66} = 0.2 \text{ mg B/kg/day}$$

5. Concept of new uncertainty factors

Recently, the metabolic rate or caloric demand correction was demonstrated to be more appropriate than body surface correction when determining interspecies differences for UFs (Schneider et al., 2004). Body surface area is proportional to 2/3 power of body weight, while metabolic rate or caloric demand is proportional to 3/4 power of body weight.

$$\text{Caloric demand} = f \times BW^{3/4}$$

$$\text{Caloric demand/body weight} = f \times BW^{3/4} \times BW^{-1} = f \times BW^{-1/4}$$

Then, the animal/human ratio for metabolic rate or caloric demand per body weight is:

$$\frac{\text{Caloric demand of animal/body weight}}{\text{Caloric demand of human/body weight}} = \frac{f_a \times BW_a^{-1/4}}{f_h \times BW_h^{-1/4}} = \frac{BW_h^{1/4}}{BW_a^{1/4}} = \left(\frac{BW_h}{BW_a}\right)^{1/4}$$

Table 2
Variation of glomerular filtration rate in healthy pregnant women.

$GFR_A \pm SD$ (mL/min)	GFR_A–GFR_{3SD}	UF_h (TK)	References
150.5 ± 17.6[a]	97.7	1.54	Dunlop (1981)
195 ± 32[b]	99	1.97	Krutzen et al. (1992)
138.9 ± 26.1[c]	60.6	2.29	Sturgiss et al. (1996)
	Average of above data	1.93	

[a] Combined data of individual average of GFRs at 12, 26 and 36 weeks of pregnancy in 25 healthy women.
[b] Combined data of 13 healthy pregnant women in the last trimester.
[c] Combined data of individual average of GFRs in early and late pregnancy in 21 healthy women.

where f is the absorption rate and BW is body weight. When the body weights of humans, rats and mice are 60 kg, 350 g and 40 g, respectively, the caloric demand correction factor is 3.6 for rats and 6.2 for mice, which are less than the default UF_a of 10. However, it should be noted that these correction values are derived using median values for animals and humans, thus do not sufficiently cover the possible range of variation. Schneider et al. (2004) compared human and various experimental animal maximum tolerated dose data for 63 anti-cancer drugs, this analysis demonstrates the appropriateness of using the geometric standard deviation along with caloric demand correction values as medians to cover 95% of the variation. When the 95th percentile of caloric demand correction is calculated for animals to humans, the correction is 38 for mice and 27 for rats, indicating that an UF_a of 10 is not sufficient. Falk-Filipsson et al. (2007) agreed with the appropriateness of this caloric demand correction for interspecies differences.

The difference between NOAELs in the standard human population and the sensitive subpopulation are considered the most appropriate data for determining human variability. However, this type of human data is limited, generally making the use of experimental animal data inevitable. Among sensitive subpopulations, the effects on pregnant women and the elderly are evaluated by reproductive/developmental and chronic toxicity studies, respectively. Newborn animals are another sensitive subpopulation but toxicity studies are not usually conducted for them because there are no officially agreed upon testing guidelines. Recently, a comparative analysis of NOAELs in newborn and young rats from repeated dose toxicity studies of 18 chemicals was conducted (Hasegawa et al., 2007). As a result, the median ratio for the NOAELs (young/newborn rats) was 3 with a geometric standard deviation of 1.38 (Hasegawa et al., 2010).

Based on these considerations, new UFs for each animal, which were calculated by multiplying two log-normal distribution data for interspecies differences and human variability with a probabilistic approach, were proposed as shown in Table 3 (Hasegawa et al., 2010).

Based on the information found in Table 3, new subdivision default values for each animal were proposed, as shown in Table 4. Consistent with the information found in Table 3, the contribution ratio of interspecies differences depends on animal size. For example, the new proposed UF of 100 for hamsters and rats approximates either 111 or 88.7. Using the contribution ratio, the default 100 factor can be subdivided as either 25 and 4 for UF_a and UF_h, respectively. Furthermore, for subdivision of the default UF_a and UF_h, the ratio distribution used by WHO was applied, 60:40 for TK and TD for interspecies differences (UF_a) and 50:50 for TK and TD for human variability (UF_h). As a result, the default UF_a of 25 is subdivided into 7.0 (TK) and 3.6 (TD), and the default UF_h of 4.0 is subdivided into 2.0 for both TK and TD. When appropriate measured data or PBPK data are available, that data can be used instead of the subdivision default values; otherwise, the given default subdivision values should be used.

Table 3
Proposal of new uncertainty factors for each animal.

Species	UF_a	UF_h	$UF_a \times UF_h$	Proposal
Mice	48.2	5.09	155	150
Hamsters	34.4	5.09	111	100
Rats	27.5	5.09	88.7	
Rabbits	13.8	5.09	44.3	40
Monkeys	11.7	5.09	37.7	
Dogs	9.63	5.09	31.0	

UF_a: 95th percentile of interspecies differences between each animal and human based on caloric demand correction and geometric standard deviation of 3.23.
UF_h: 95th percentile of human variability based on median of 3.0 and geometric standard deviation of 1.38.

Table 4
Proposal of subdivision default for new uncertainty factors.

Species	Proposal of New UF	Default UF_a Default UF_h	Subdivision default TK × TD
Mice	150	UF_a: 38 UF_h: 4.0	9.0 × 4.3 2.0 × 2.0
Hamsters Rats	100	UF_a: 25 UF_h: 4.0	7.0 × 3.6 2.0 × 2.0
Rabbits Monkeys Dogs	40	UF_a: 10 UF_h: 4.0	4.0 × 2.5 2.0 × 2.0

6. Application of new uncertainty factors and their subdivision

We reviewed the available data for critical endpoints to calculate a TDI using the proposed new UFs with the subdivision default values described in Table 4. From the 14 toxicity endpoints shown in Table 1, two repeat dose studies and two developmental studies were selected as the appropriate data sets to derive a TDI. For each endpoint, the NOAEL/BMDL is obtained, followed by application of newly proposed UFs with subdivisions in order to derive a TDI.

In a 2-year rat feeding study (Weir and Fisher, 1972), testicular toxicity was found at 1170 ppm (58.5 mg B/kg/day), with 350 ppm (17.5 mg B/kg/day) as the NOAEL. Since toxicity was found only at the highest dose of 1170 ppm, application of the BMD approach was not appropriate.

The UF assessment for the testicular toxicity endpoint in male rats, UF_a (TK), was estimated on the basis of boron clearance in male rats and adult men. Boron clearance in male rats reported by Usuda et al. (1998) was 0.359 mL/min/100 g BW and in male volunteers reported by Jansen et al. (1984) was 54.6 mL/min/1.73m^2 (equivalent to 72 kg body weight). Using the US EPA UF formula the UF_a (TK) becomes 4.6 as follows:

$$\frac{\text{Dose}_a}{\text{Dose}_h} = \text{UF}_a(\text{TK}) = \frac{\text{Cl}_a \times f_h \times \text{BW}_h}{\text{Cl}_h \times f_a \times \text{BW}_a} = \frac{0.359 \times 0.92 \times 72}{54.6 \times 0.95 \times 0.10} = \frac{24}{5.2} = 4.6$$

where f is the absorption rate, BW is body weight, and Cl is clearance. TD data is lacking, therefore, the UF_a is 17 resulting from 4.6 × 3.6, the latter value from Table 4.

UF_h is generally the difference in NOAELs between the standard human population and the sensitive subpopulation. Male children should especially be considered as an appropriate target population because they are usually sensitive to the endpoint of interest (testicular toxicity). However, information on boron clearance or GFR in male children is not available. Furthermore, no reports of testicular abnormalities were found for fetuses exposed in utero, pups exposed in multiple generation studies, or male children exposed to boron. Therefore, a default factor of 4.0 for UF_h from Table 4 was applied.

By applying a UF of 70, resulting from $UF_a \times UF_h = 17 \times 4.0 = 68$ (rounded to 70), the TDI becomes 0.26 mg B/kg/day, based on a NOAEL of 17.5 mg B/kg/day for testicular toxicity in rats.

In a 2-year dog feeding study (Weir and Fisher, 1972), no toxic effects were observed at the highest dose of 350 ppm (8.8 mg B/kg/day), reported as the NOAEL. In a 90-day dog feeding study, testicular toxicity clearly appeared at 1750 ppm (30.4 mg B/kg/day) and toxicity at 1,170 ppm (29 mg B/kg/day) was also observed in a 26-week dog feeding study (Weir and Fisher, 1972). Therefore, it is deduced that testicular toxicity in dogs seems to not develop further in association with longer exposure.

No data are available on interspecies TK and TD differences for testicular toxicity in male dogs. As there are also no available data on human variability, as mentioned above, the integrated default UF of 40 from Table 4 was used. In the targeted dog study, the exposure period was 2 years, not lifetime exposure; however, an additional UF was not considered necessary because testicular toxicity appearing after 90-day exposure was not enhanced by significantly longer exposure (i.e. 26 weeks). Therefore, a TDI based on testicular toxicity in dogs is 0.22 mg B/kg/day, resulting from dividing the NOAEL of 8.8 mg B/kg/day by the UF of 40.

The incidence of malformation (especially shortening of rib XIII) was increased in mice given a diet containing 0.4% boric acid on gestation days 0–17 (Heindel et al., 1992); fetal body weight was also decreased at dietary concentrations of more than 0.2%. The NOAEL was 0.1% (43 mg B/kg/day) based on reduced fetal body weight. The BMD approach resulted in 44.9 mg B/kg/day of $BMDL_{05}$ for fetal body weight reduction.

For the assessment of developmental toxicity in mice, the default UF_a of 38 was applied to the $BMDL_{05}$, as there are no available data on interspecies differences.

The UF_h (TK) was evaluated by reviewing boron clearance variation data in pregnant women (Pahl et al., 2001). However, this data was judged to be inappropriate because the available study was not designed to evaluate human variability (low number of subjects and uncontrolled dietary intake of boron). Therefore, three GFR data in healthy pregnant women used by EPA and additional GFR data in pregnant subjects suffering from hypertension, preeclampsia and diabetes reported by Krutzen et al. (1992) (Table 5) were examined in order to evaluate human TK. In contrast to the US EPA approach, the difference between the average value in the standard population and the lower 95th percentile GFR in the sensitive subpopulation was considered to be more appropriate for human variability. In this case, average GFR in healthy pregnant women was used as the average in the standard population and the two Standard deviation below the average (GFR_A–GFR_{2SD}) in pregnant subjects suffering from preeclampsia, who have the lowest GFR among diseased pregnant subjects, was used as the value for the most sensitive pregnant subjects. Since the three GFR measurements in healthy pregnant women were quite different from each other, the one reported by Krutzen et al. (1992) was selected because the GFR appears to have been obtained according to the same protocol as GFR of preeclampsia subjects. Therefore, an UF_h (TK) of 3.23 from 195/60.3 was determined and the UF_h of 6.5 was developed by multiplying by the default UF_h (TD) of 2.0 due to lack of available data.

Therefore, the overall UF is 247 calculated from 38 × 6.5. TDI based on mouse developmental toxicity was 0.18 mg B/kg/day calculated by dividing the $BMDL_{05}$ of 44.9 mg B/kg/day by the UF of 247.

In a rat developmental study, boric acid was given via the diet on gestation days 0–20, and reduction of body weight and skeleton malformation of the fetus were observed at dietary concentrations of more than 0.1% (12.9 mg B/kg/day) (Heindel et al., 1992). The same study protocol was used for a lower dose range from 0.025%, resulting in a good dose–response outcome (Price et al., 1996b). Allen et al. (1996) analyzed the combined data from these two studies using the BMD approach, resulting in a $BMDL_{05}$ of 10.3 mg B/kg/day for reduced fetal body weight.

In order to assess the developmental toxicity in rats, boron clearance data were obtained for pregnant rats (Vaziri et al., 2001) and pregnant women (Pahl et al., 2001). Based on these data, the UF_a (TK) was 3.3 using the same US EPA method, as shown above. Due to the lack of available interspecies TD data, the UF_a is 12, calculated from 3.3 (default) × 3.6.

The UF_h (TK) of 3.23 was derived from average the GFR in healthy pregnant women and the lower 95th percentile GFR in preeclampsia pregnant subjects reported by Krutzen et al. (1992), as shown above. Due to the lack of available data for UF_h (TD), the UF_h is 6.5, calculated by 3.23 × 2.

Accordingly, by applying an UF of 78, resulting from UF_a × UF_h = 12 × 6.5 = 78, to the $BMDL_{05}$ of 10.3 mg B/kg/day, the TDI based on rat developmental toxicity was 0.13 mg B/kg/day.

The four resultant TDI calculations are summarized in Table 6. The lowest TDI is obtained from the $BMDL_{05}$ of 10.3 mg B/kg/day in the rat developmental study, by applying the evidence-based UF_a(TK) and UF_h(TK) with the default TDs.

Table 5
Glomerular filtration rate in healthy and diseased pregnant subjects.

Subjects	No.	GFR_A ± SD (mL/min)	GFR_A–GFR_{2SD}
Healthy[a]	25	150.5 ± 17.6	115.3
Healthy[b]	21	138.9 ± 26.1	86.7
Healthy[c]	13	195 ± 32	131
Hypertension[c]	8	198.9 ± 57.9	83.1
Preeclampsia[c]	12	128.1 ± 33.9	60.3
Diabetic[c]	20	169 ± 34.7	99.6

[a] Dunlop (1981).
[b] Sturgiss et al. (1996).
[c] Krutzen et al. (1992).

7. Discussion and conclusion

A default UF of 10 for interspecies differences has commonly been used for all experimental animals even though there are marked size differences (e.g., approximately 500-fold from mice to dogs). For over 20 years, US EPA and ICH (Connelly et al., 1997) have used body surface correction, but it has been suggested that the metabolic rate or caloric demand correction may be more

Table 6
TDI derivation trial using available TK data.

Toxicity endpoints NOAEL/$BMDL_{05}$	Subdivision of UF_a	Subdivision of UF_h	TDI
Rats: testes NOAEL = 17.5 mg B/kg/day	TK = 4.58 TD = 3.6 (default) →**17**	UF_h = 4.0 (default)	UF = 68 0.26 mg B/kg/day
Dogs: testes NOAEL = 8.8 mg B/kg/day	UF_a = 10 (default)	UF_h = 4.0 (default)	UF = 40 (default) 0.22 mg B/kg/day
Mice: development $BMDL_{05}$ = 44.9 mg B/kg/day	UF_a = 38 (default)	TK = 3.23 TD = 2.0 (default) →6.5	UF = 247 0.18 mg B/kg/day
Rats: development $BMDL_{05}$ = 10.3 mg B/kg/day	TK = 3.3 TD = 3.6 (default) →12	TK = 3.23 TD = 2.0 (default) →6.5	UF = 78 0.13 mg B/kg/day

appropriate (Schneider et al., 2004; Falk-Filipsson et al., 2007). Recently, an integrated UF method has been proposed, incorporating new information on human variability (Hasegawa et al., 2010). However, TK and TD subdivision have only been applied for cases of boron in the drinking water quality guidelines in WHO (WHO, 2009) and US EPA (US EPA, 2004). The subdivision has been not applied in Japan, even though the application potential has been discussed. In this article, under these circumstances, we apply the new UFs and subdivisions for the safety assessment of boron.

Borate or borax is easily absorbed via the gastrointestinal tract and excreted in both humans and experimental animals. Borax becomes boric acid during absorption, boric acid is neither metabolized nor accumulated in the body and is excreted relatively quickly by the kidney; therefore, boron clearance is considered to approximate the boron blood concentration. Boron clearance in humans and rats, calculated from the boron blood concentration and excretion in urine for a certain period after oral administration of boric acid, was the TK data used for the UF_a. Boron clearance studies in rats, two reports in adult males (Usuda et al., 1998) and non-pregnant and pregnant females (Vaziri et al., 2001) were used to determine the clearance rate. There are two human studies of adult men (Jansen et al., 1984) and non-pregnant and pregnant women (Pahl et al., 2001). The former human study was reported from Denmark and the latter from the USA. There is no mention of race, but likely they were people of western decent. As there are no reports on boron clearance in Japan, it is necessary to consider whether the above human data can apply to people of East Asian decent including Japanese. Comparing the above two human data, there were no sex differences in boron clearance: 54.6 ± 8.0 mL/min/1.73m^2 in adult men versus 54.31 ± 19.35 mL/min/1.73m^2 in nonpregnant women. In pregnancy, clearance increased by approximately 25% (68.30 ± 35.00 mL/min/1.73m^2). Pahl et al. (2001) determined creatinine clearance (123.0 ± 23.8 mL/min/1.73m^2) along with boron clearance in non-pregnant women, which is about double that of boron clearance, the difference indicating that boron is reabsorbed by human renal tubular cells. In Japan, the normal creatinine clearance range is 70–130 mL/min, and abnormal values ranging from 50 to 70 mL/min, 30 to 50 mL/min, and <30 mL/min; these values suggest slight, moderate and severe kidney damage, respectively. Creatinine clearance is expressed as mL/min in Japan, but this actually means mL/min/1.73m^2. Therefore, Japanese creatinine clearance is considered to be almost equal to that in the western people, and boron clearance data obtained from western people is considered able to be directly applied to Japanese.

As for UF_a (TK), appropriate boron clearance data for rats and humans were obtained and the calculation formula given by (US EPA, 2004) was applied. On the other hand, to consider TK variability in pregnant women, WHO and US EPA adopted GFR variation data in healthy pregnant women to adequately cover pregnant women with very low GFR. WHO used UF_h (TK) of 1.8 on the basis of $GFR_A/(GFR_A-GFR_{2SD})$ from the data of late pregnant women in three reports (Dunlop, 1981; Krutzen et al., 1992; Sturgiss et al., 1996), and US EPA used UF_h (TK) of 2.0 given as an average of three values; $GFR_A/(GFR_A-GFR_{3SD})$ for early to late pregnant women in the same three reports. However, we understand that human variability means how much the values differ between the standard population and sensitive subpopulation. In this case, it should be considered that a standard population is healthy pregnant women and a sensitive subpopulation is pregnant subjects suffering from the most concerned disease, preeclampsia. There is only one report on preeclampsia, which clearly indicates lower GFR than in other pregnant women. We therefore decided to use the lower 95th percentile GFR in preeclampsia subjects and average GFR in healthy pregnant women in the same report (Krutzen et al., 1992). These data and the UF_h (TK) calculation method are considered to be preferable from the aspect of sufficient safety.

Other groups have developed tolerable upper intake limits, which are similar to the TDIs described here. For example, an Upper Limit (UL) for boron was developed by the US Institute of Medicine (US IOM, 2001) based on the same animal study but using a NOAEL for the same endpoint rather than BMDL. The NOAEL of 9.6 mg B/kg/day was divided by an UF of 30, resulting in an UL of 0.3 mg B/kg/day. For interspecies differences, the usual default value of 10 was selected but an UF of 3 was chosen for intraspecies variability, in view of the expected similarity in toxicokinetics among humans, leading to yield a UF of 30. Subsequently, the European Food Safety Authority (2004) developed an UL based on the same NOAEL of 9.6 mg B/kg/day but divided by an UF of 60 to allow for variability between rats and humans and between-person variability in humans. The resulting UL was 0.16 mg B/kg/day (EFSA, 2004). In a review of information developed for the Pesticide Management Regulatory Authority (PMRA) of Health Canada by an independent scientific panel, it was concluded that a BMDL of 14 mg B/kg/day could be developed from testicular effects in dogs and that a combined UF of approximately 160 would be appropriate. This UF consisted of a 3-fold factor for database concerns, a 6.4 factor for intraspecies variability, and a 8.3 factor for interspecies variability (Chapin et al., Submitted for publication).

This article presents a new concept for UFs and the subsequent subdivision led to new TDIs, based on the appropriate NOAEL or $BMDL_{05}$ derived from the reevaluation of available toxicity studies, and measured boron clearance and GFR used to derive them (Table 6). TDIs calculated from rat or dog studies were very similar, specifically, 0.26 and 0.22 mg B/kg/day, respectively. TDIs for developmental toxicity in mice and rats were also very similar, specifically 0.18 and 0.13 mg B/kg/day, and lower than TDIs for testicular toxicity. At this moment, it is considered that this is the best approach based on the latest scientific concept of uncertainty factors. In conclusion, the overall TDI of boron is 0.13 mg B/kg/day, based on a $BMDL_{05}$ from rat developmental toxicity and the scientifically developed uncertainty factors.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by a Health and Labour Sciences Research Grant (H22-Kenki-Ippan-006) from the Ministry of Health, Labour and Welfare, Japan.

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ウイルス処理に有効な新規アルミニウム系凝集剤の開発

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本研究では，凝集剤の塩基度，凝集剤中の硫酸およびアルミニウム形態がウイルスの処理性に与える影響を詳細に評価し，ウイルス処理に有効な新規アルミニウム系凝集剤を開発した．開発した新規凝集剤を凝集沈澱処理に用いた場合，弱酸性および中性のpH領域のみならず，弱アルカリ性のpH領域においても，約6 logの高い除去率が得られ，従来のアルミニウム系凝集剤を用いた場合に比べ，除去率が飛躍的に向上した．また，ESI-FT-MS法および^{27}Al-NMR法による分析の結果，新規凝集剤には，アルミニウム13量体や30量体が含まれていることが明らかとなったことから，これらのアルミニウム種がウイルスの処理性の向上に大きく影響している可能性が示唆された．

Key Words : *aluminum species, basicity, novel aluminum-based coagulant, sulfate ion, virus removal*

1. はじめに

分子生物学的なウイルス検出法および水中からのウイルス回収・濃縮法の発展に伴い，水環境におけるウイルスの実態調査が世界的に広く行われるようになり，我が国においても，水道水源と成り得る水環境中にノロウイルスに代表される水系感染症を引き起こすウイルス（水系感染症ウイルス）が存在していることは周知の事実となってきている[1-4]．従って，水系感染症ウイルスに汚染された環境水を水道水源として取水する場合，仮に浄水処理が不十分であれば，水道水を媒体としたウイルスによる大規模な水系感染症が突発的に発生する可能性は十分に考えられる．実際に，水道水を媒体としたウイルスによる水系感染症の報告もなされている[5-7]．また，気候変動や人口増加に伴う世界的な水不足の顕在化により，これまで使用されてこなかったウイルス汚染レベルの高い低水質の環境水や排水をも水道水源として利用（再利用）する必要が生じてきている[8]．その一方で，トリハロメタンに代表される消毒副生成物による発癌性等の健康影響が指摘され[9]，水系感染症制御のために塩素等の消毒剤の注入量を容易に増加させることが困難な状況となってきている．このような状況から，消毒副生成物の生成を最小限に抑え，ウイルスを含む広範な原水水質に柔軟に対応可能な，低コスト・省エネルギー型の新たな浄水処理技術の開発が求められている[10]．

一方，現行の浄水処理においては，水道水源中に含まれる懸濁質やコロイド粒子，溶解性有機物の除去を目的として，凝集剤の添加による凝集沈澱処理が広く用いられている．凝集沈澱処理におけるウイルスの処理性評価はこれまでに数多くなされており，最適処理条件下では，ノロウイルス，A型肝炎ウイルス，ポリオウイルス等の効果的な除去も期待できることが示されている[11-13]．従って，凝集沈澱処理は，水系感染症発生リスク低減のためのマルチバリアの一つとしても位置付けられている[10]．また，凝集剤の多量注入とpH制御による強化凝集沈澱処理を実施することにより，水系感染症ウイルスのみならず，消毒副生成物の前駆物質である自然由来有機物（NOM）を含む溶解性有機物を効果的に除去できることが報告されている[14,15]．従って，水系感染症ウイルスおよび消毒副生成物による健康被害を低減化する観点から，凝集沈澱処理の重要性が再認識されてきている．

凝集沈澱処理に用いられる凝集剤としては，ポリ塩化アルミニウム（PACl）や硫酸バンドといったアルミニウム系凝集剤が一般的であり，国内/国外を問わず広く普及している．しかしながら，近年の水道水源の富栄養化に伴うpH上昇により，従来のアルミニウム系凝集剤では最適pH条件下（中性付近）での処理が困難な状況が生じてきている．このような弱アルカリ性の原水にお

表-1. プライマーとプローブの塩基配列

Coliphage		Oligonucleotide sequences	Positions	Reference
MS2	Forward primer	5'-GTC GCG GTA ATT GGC GC-3'	632-648	22)
	Reverse primer	5'-GGC CAC GTG TTT TGA TCG A-3'	690-708	
	TaqMan probe	5'-AGG CGC TCC GCT ACC TTG CCC T-3'	650-671	

いては，ウイルスの処理性も著しく低下することが知られており[16]，最適pH条件下での処理のために凝集剤の多量注入や酸注入によるpH制御を実施せざるを得ない場合も多く，結果として，薬品の大量消費，注入設備の増設，管理の煩雑性，更には処理水中の残留アルミニウム濃度の増加といった問題が生じている[17,18]．これに対し，PACIの塩基度（アルミニウムと結合したOHの当量数/アルミニウムの当量数）を高めた高塩基度PACI（塩基度70%）を凝集沈澱処理に用いた場合，従来のPACI（塩基度50%）や硫酸バンドに比べ，中性付近の原水のみならず，弱アルカリ性の原水においても溶解性有機物を効果的に除去可能であり，また，処理水中の残留アルミニウム濃度をも低減可能であることが報告されている[19]．

そこで，本研究では，組成の異なる複数のアルミニウム系凝集剤を実験に使用し，凝集剤の塩基度がウイルスの処理性に与える影響を評価した．また，従来のPACIには，フロック形成速度の促進等を目的に，加水分解しない範囲であらかじめ硫酸が適当量混合されるが，凝集剤中の硫酸がウイルスの処理性に与える影響についてはこれまでに知見が得られていないことから，凝集剤に混合される硫酸がウイルスの処理性に与える影響についても評価した．加えて，凝集剤中のアルミニウム形態等の特性を詳細に分析し，これらの結果を踏まえた上で，ウイルス処理に有効な新規アルミニウム系凝集剤を開発した．

これまで，凝集沈澱処理におけるpHや凝集剤添加濃度等の凝集条件を最適化することによりウイルスの処理性を向上させようとした例は数多くあるが[13-15]，凝集剤自体を根本から開発し，ウイルスの処理性を向上させようとした例はない．従って，本研究は，ウイルス処理に影響を与え得る凝集剤の特性を詳細に把握した上で，ウイルス処理に有効な新規アルミニウム系凝集剤の開発を目指した世界で初めての試みである．

2. 実験方法

(1) 使用したウイルスの培養，精製，定量法

本研究では，（独）製品評価技術基盤機構（NITE）バイオテクノロジー分野 生物遺伝資源部門（NBRC）から分譲された大腸菌ファージMS2（NBRC 102619）を使用した．レビウイルス科に属する大腸菌ファージMS2は，直径約24 nmの正20面体構造を有しており，一本鎖RNAを遺伝子として持つ．この構造がA型肝炎ウイルスやポリオウイルスと類似しているため，水系感染症ウイルスの代替指標ウイルスとして広く用いられている[12-15,20]．

MS2は，F繊毛大腸菌（NBRC 13965）を用いて37℃のシェイキングバス内にて22-24時間振とう培養した後，2,000 × gにて10分間遠心分離し，上清をメンブレンフィルター（膜孔径 0.45 μm，酢酸セルロース，Advantec）にて滅菌ろ過することにより高濃度保存液を得た．得られた高濃度保存液中の有機物の持ち込みを低減させるため，実験に先立ち，遠心式フィルターユニットAmicon Ultra-15（分画分子量 100,000，再生セルロース，Millipore）を用いて，12 mLの高濃度保存液を5,000 × gにて20分間遠心濃縮し，得られた約100 μLの濃縮液に12 mLのMilli-Q水を加えることでバッファー置換した．

MS2の定量には，プラック形成法およびリアルタイム定量RT-PCR法を用いた．なお，プラック形成法は，Adams[21]の方法に従って行った．一方，リアルタイム定量RT-PCR法においては，QIAamp MinElute Virus Spin Kit（Qiagen）を用いてMS2のRNAを抽出し，これをHigh Capacity cDNA Reverse Transcription Kit with RNase Inhibitor（Applied Biosystems）を用いて逆転写させ，cDNAを合成した．このcDNAをTaqMan Universal PCR Master Mix with UNG（Applied Biosystems），プライマー（最終濃度 400 nM，タカラバイオ），プローブ（最終濃度 250 nM，Applied Biosystems），Distilled waterと混合した後，リアルタイム定量PCR装置（Applied Biosystems 7,300，Applied Biosystems）に供した．本研究で使用したプライマーおよびプローブの塩基配列を表-1に示す．なお，PCR反応は，50℃で2分間および95℃で10分間の加熱を行った後，95℃で15秒間と60℃で1分間から成るサイクルを40回繰り返した．

(2) 使用した凝集剤

a) ポリ塩化アルミニウム（PACI）

本研究では，我が国の浄水処理場で従来から広く用いられているPACI（PACI-B50s: 塩基度 50%，硫酸 2-3%，多木化学）に加え，凝集剤の塩基度がウイルスの処理性に与える影響を評価するため，PACIの塩基度を高めた高塩基度PACI（PACI-B70s: 塩基度 70%，硫酸 2-3%，多木化

学）を実験に使用した．また，凝集剤中の硫酸がウイルスの処理性に与える影響を評価するため，硫酸無添加の高塩基度PACl（PACl-B70ns: 塩基度 70%，硫酸 0%，多木化学）を実験に使用した．なお，各凝集剤は，使用まで4℃にて冷蔵保存した．

b) 高塩基性塩化アルミニウム（HPA）

本研究では，凝集剤中のアルミニウム形態の大きく異なる複数のHPAを作製し，実験に使用した．HPAは，加熱撹拌下で塩化アルミニウム溶液にNaOHを添加し，室温下で静置させることにより作製した．なお，塩基度，塩化アルミニウム溶液濃度，NaOH濃度，加熱撹拌時間を変化させることにより，凝集剤中のアルミニウム形態の大きく異なる複数のHPAを作製した．なお，各凝集剤は，作製後3日以上常温で静置することにより安定化させた後，使用まで4℃にて冷蔵保存した．

(3) 使用した凝集剤の分析

a) フェロン法

各凝集剤を0.1 mol-Al/Lになるようにに希釈した後，フェロン（8-ヒドロキシ-7-ヨードキノリン-5-スルホン酸，$C_9H_6INO_4S$，和光純薬工業），酢酸ナトリウム，HClおよびMilli-Q水により作製したフェロン混合溶液25 mLに20 μL添加した．これをマグネティックスターラーを用いて10秒間撹拌した後，凝集剤添加1分後および120分後の吸光度（波長366 nm）を分光光度計（UV-1700，島津製作所）にて測定した（分析時のpHは4-5程度）．フェロン法は，フェロンと凝集剤に含まれるアルミニウム種の反応速度の違いから，アルミニウム種の形態存在割合を分析する手法であることから[23,24]，フェロンと1分以内に反応したアルミニウム種をモノマー状のアルミニウム種（Alモノマー），1分から120分の間に反応したアルミニウム種をポリマー状態のアルミニウム種（Alポリマー），120分以降もフェロンと反応しなかったアルミニウム種をコロイド状のアルミニウム種（Alコロイド）とした．なお，希釈した0.1 mol-Al/Lの各凝集剤のpHを0.5になるように硝酸にて調整し，これをドライオーブンを用いて85℃にて3時間加熱することにより，凝集剤に含まれるAlポリマーおよびAlコロイドをAlモノマーまで分解させた後，上述した手順で凝集剤添加1分後の吸光度を測定したものを全アルミニウムとし，凝集剤中のアルミニウム種の形態存在割合を計算した．

b) ESI-FT-MS法

各凝集剤を20 mg-Al/Lになるようにに希釈したものを試料とし，ESI-FT-MS装置（Exactive，Thermo Scientific）に供した．分析はシリンジポンプを用いたインフュージョン法で実施し，ポジティブイオンモード，分解能 50,000，質量範囲 50-1,000 m/z，スプレーボルテージ 3.0 kV，キャピラリーボルテージ 25 V，チューブレンズボルテージ 55 V，スキマーボルテージ 15 V，流速 200 μL/minとした（分析時のpHは4-5程度）．分析により得られたマススペクトルから，凝集剤中のアルミニウム種を特定した．

c) ^{27}Al-NMR法

各凝集剤を0.1 mol-Al/Lになるようにに希釈した後，重水を75%（v/v）となるよう添加したものを試料とした．これをNMR分析用石英製サンプルチューブ（外径 5.0 mm，内径 4.2 mm，シゲミ）に注入した．また，アルミン酸ナトリウムを0.01 mol-Al/Lになるようにに希釈した後，重水を75%（v/v）となるよう添加したものを内部標準物質として使用した．内部標準物質は，NMR分析用ガラス製細型チューブ（外径 3.0 mm，内径 2.5 mm，シゲミ）に注入した後，試料を含むサンプルチューブ内に挿入し，二重管構造とした．この二重管にチューブキャップ（ポリエチレン，和光純薬工業）をした後，NMR装置（ECA-600，JEOL）に供した．なお，塩化アルミニウムを上述した手順で調整したものを基準物質とし，試料の化学シフトを計算した．分析はシングルパルス法で実施し，磁場強度 14.09 T，共鳴周波数 156.39 MHz，パルス幅 5.0 μs，パルス繰り返し時間 1.13 s，観測範囲 78,247，ポイント数 65,536，積算回数 8,000回とした（分析時のpHは4-5程度）．分析により得られたNMRスペクトルから，凝集剤中のアルミニウム種を特定した．

d) コロイド滴定法

Milli-Q水150 mLに滴定終点検出指示薬としてトルイジンブルー指示薬溶液（$C_{15}H_{16}ClN_3S$，和光純薬工業）を300 μL添加し，マグネティックスターラーを用いて撹拌した．ここに，凝集剤を1.0，1.5，1.6，2.0 mg-Al/Lになるように添加した後，直ちにアニオンポリマーであるポリビニル硫酸カリウムによる滴定を開始し，トルイジンブルーの色調が変化した点を滴定終点とした．滴定操作および滴定終点の検出は，自動滴定装置（COM-555，平沼産業）を用いて行い，ポリビニル硫酸カリウムの滴定量から凝集剤のコロイド荷電量を計算した．

(4) 凝集沈澱処理実験

本研究では，回分式凝集沈澱処理による大腸菌ファージMS2の処理性を評価した．精製したMS2を10^8 PFU/mLになるように添加した北海道札幌市豊平川河川水（札幌市水道局藻岩浄水場原水: pH 7.4，DOC 0.8 mg/L，UV260

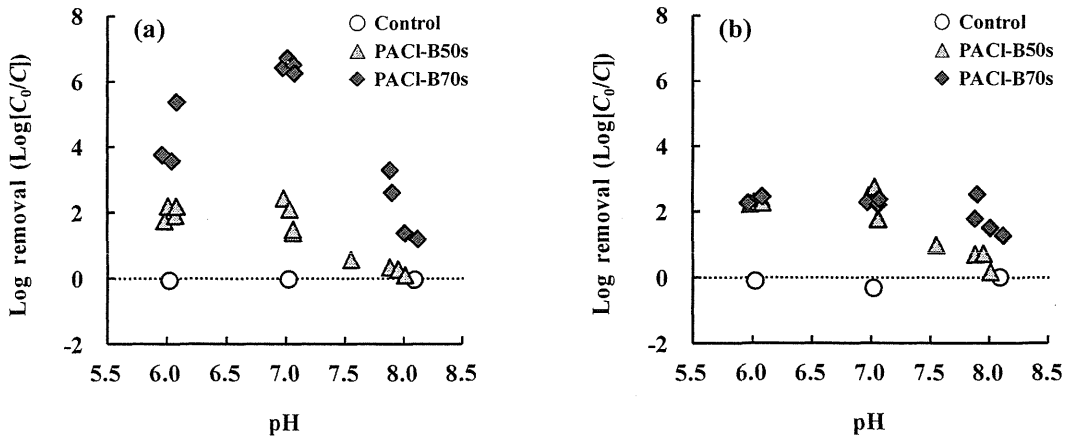

図-1. 凝集剤の塩基度がMS2の処理性に与える影響：MS2濃度はプラック形成法（a）およびリアルタイム定量RT-PCR法（b）にて定量

0.029 cm^{-1}，濁度 7.2 NTU，アルカリ度 17.1 mg-$CaCO_3$/L）を原水とし，角型ビーカーに1,000 mL添加した．ここに，凝集剤を1.89 mg-Al/L（河川水採水時の藻岩浄水場における凝集剤添加濃度）になるように添加し，直ちにHClあるいはNaOHにてpHを6，7あるいは8に調整した．これを攪拌翼を用いてG値200 s^{-1}にて1分間急速攪拌，20 s^{-1}にて10分間緩速攪拌し，60分間静置した．原水および静置後の上澄水約600 mLを採取し，それぞれのMS2濃度をプラック形成法およびリアルタイム定量RT-PCR法にて定量することにより，MS2の凝集沈澱処理性を評価した．また，原水および上澄水をメンブレンフルター（膜孔径 0.45 μm，PTFE，Advantec）にてろ過し，それぞれの試料の吸光度（波長260 nm）を分光光度計（UV-1700，島津製作所）にて測定することにより，NOMの処理性を評価した．加えて，ろ過後の試料に硝酸を1%（v/v）となるよう添加した後，ICP-MS（Agilent 7,700，Agilent Technologies）にてアルミニウム濃度を測定することにより，凝集剤の残留アルミニウム性を評価した．

(5) 粒径分布および電気移動度測定

河川水中における大腸菌ファージMS2の粒径および表面電位特性を把握するために，粒径分布および電気移動度の測定を行った．分画分子量100,000のUF膜（YM-100，再生セルロース，Millipore）にてろ過した豊平川河川水を，HClを用いてpH 7に調整した後，精製したMS2を10^{10} PFU/mLになるように添加した．この試料の粒径分布および電気移動度をゼータ電位・粒子径・分子量測定装置（Zetasizer Nano ZS，Malvern）にて測定した．

3. 結果と考察

(1) 凝集剤の塩基度がウイルスの処理性に与える影響

従来PACl（PACl-B50s）とPAClの塩基度を高めた高塩基度PACl（PACl-B70s）を用いた場合の凝集沈澱処理後（静置後）のMS2の除去率を図-1に示す．なお，図の縦軸はLog[C_0/C]（C_0: 原水のMS2濃度，C: 処理水のMS2濃度）にて表記し，MS2濃度はプラック形成法（a）およびリアルタイム定量RT-PCR法（b）にて定量した．図より，凝集剤を添加しない場合は，いずれのpH領域においてもMS2は全く除去されなかった．河川水中におけるMS2の粒径分布を測定したところ，MS2の粒径は，20-30 nmの範囲となり，電子顕微鏡観察により確認された直径と同程度であった[25]．また，河川水中におけるMS2の電気移動度を測定したところ，-1.734 (μm/s)/(V/cm)となり，負に帯電していることが明らかとなった．従って，本研究で使用した河川水中においては，負に帯電した粒子間の電気的反発力により，MS2が凝集塊を形成せず，安定的に単分散した状態で存在していたことから，凝集剤を添加しない場合は，いずれのpH領域においてもMS2は全く除去されなかったものと考えられた．これに対し，PACl-B50sを用いた場合，pH 6付近の弱酸性領域およびpH 7付近の中性領域において約2 logの除去率が得られた．これは，凝集剤の添加により，負に帯電したMS2および共存する懸濁質の表面電位が中和され，MS2間あるいはMS2と懸濁質間の引力（ファンデルワールス力）が電気的反発力に比べて大きくなることにより，自重沈降可能な大きさまで凝集粗大化し，静置により水相から沈澱除去されたためであると考えられた．なお，Hijnenらは，既往の凝集沈澱処理によるウイルス除去研究を実験スケール等を考慮した上でReviewしており，凝集沈澱処理により1.8 logの除去率が得られると推定している[26]．このことから，従来PAClであるPACl-B50sを用いた場合に得られた約2 logの除去率は，妥当な値であると考えられる．一方，pH 8付近の弱アルカリ性領域では，弱酸性および中性領域に比べて除去率が著しく低下した．

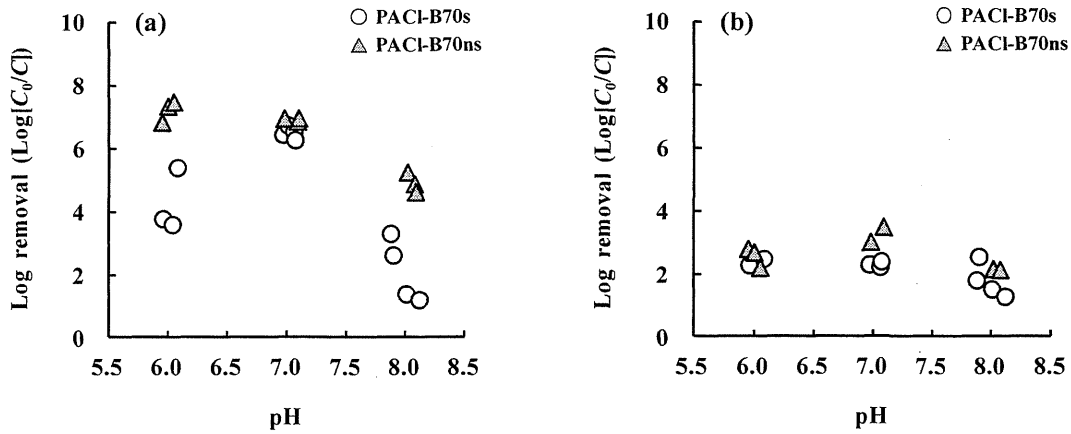

図-2. 凝集剤中の硫酸がMS2の処理性に与える影響：MS2濃度はプラック形成法（a）およびリアルタイム定量RT-PCR法（b）にて定量

従って，従来PAClを用いた凝集沈澱処理においては，弱アルカリ性のpH領域ではウイルスの除去率がほとんど期待できないことが明らかとなった．

これに対し，PAClの塩基度を高めたPACl-B70sを用いた場合，プラック形成法にて評価した除去率は，pH 6付近では4-5 log程度，pH 7付近では更に向上し，6-7 log程度となった．また，pH 8付近においても，1-2 log程度の除去率が得られ，いずれのpH領域においても，PACl-B50sを用いた場合に比べて高い除去率が得られた．一方，リアルタイム定量RT-PCR法にて評価した除去率は，pH 6付近およびpH 7付近においては，PACl-B50sを用いた場合と同程度であり，pH 8付近においては，PACl-B50sを用いた場合に比べて高い除去率が得られた．加えて，PACl-B70sを用いた場合，pH 6付近およびpH 7付近においては，プラック形成法にて評価した除去率とリアルタイム定量RT-PCR法にて評価した除去率の間に2-5 log程度の差が見られ，プラック形成法にて評価した除去率の方がリアルタイム定量RT-PCR法にて評価した除去率に比べて高くなった．2つの定量法によって得られた結果に差が生じた原因として，感染力を失ったウイルス，すなわち，不活化したウイルスの存在と，幾つかの感染性のあるウイルスによって形成される凝集塊をプラック形成法にて評価した場合に生じる除去率の過大評価の2つが考えられた．なお，Matsushitaらは，PAClを用いた凝集沈澱処理によってウイルスが除去されるのみならず不活化されることを報告している[27]．このことから，PACl-B70sを用いた凝集沈澱処理においては，ウイルスの物理的な除去のみならず，不活化効果も期待できる可能性が示唆された．以上の結果から，凝集剤の塩基度はウイルスの処理性に影響し，塩基度の高い凝集剤の方がウイルス処理に有効であることが示された．

(2) 凝集剤中の硫酸がウイルスの処理性に与える影響

硫酸無添加の高塩基度PACl（PACl-B70ns）を用いた場合の凝集沈澱処理後のMS2の除去率を図-2に示す．なお，MS2濃度はプラック形成法（a）およびリアルタイム定量RT-PCR法（b）にて定量した．図より，硫酸無添加のPACl-B70nsを用いた場合，プラック形成法にて評価した除去率は，pH 6付近およびpH 7付近において約7 logとなった．また，pH 8付近においても，約5 logの除去率が得られ，いずれのpH領域においても，硫酸を含む高塩基度PACl（PACl-B70s）を用いた場合に比べて高い除去率が得られた．一方，リアルタイム定量RT-PCR法にて評価した除去率は，いずれのpH領域においても，PACl-B70sを用いた場合と同程度であった．なお，PACl-B70nsを用いた場合，pH 6付近およびpH 7付近のみならず，pH 8付近においても，プラック形成法にて評価した除去率とリアルタイム定量RT-PCR法にて評価した除去率の間に約3 logの差が見られたことから，PACl-B70nsを用いた凝集沈澱処理においては，弱酸性および中性のpH領域のみならず，弱アルカリ性のpH領域においてもウイルスの不活化効果が期待できる可能性が示唆された．以上の結果から，凝集剤中の硫酸はウイルスの処理性に影響し，硫酸を含まない凝集剤の方がウイルス処理に有効であることが示された．

(3) フェロン法，コロイド滴定法による凝集剤の分析

フェロン法により得られた各凝集剤中のアルミニウム種の形態存在割合を図-3に示す．図より，ウイルスの処理性が高かったPACl-B70sおよびPACl-B70nsは，硫酸の有無に関わらずPACl-B50sに比べてAlモノマーの存在割合が小さく，Alコロイドの存在割合が大きかった．従って，凝集剤の塩基度がアルミニウム種の形態存在割合に与える影響は大きいことが明らかとなった．既往研究に

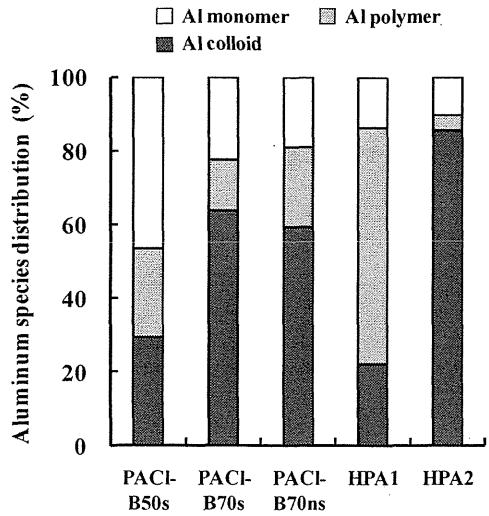

図-3. フェロン法により得られた各凝集剤中のアルミニウム種の形態存在割合

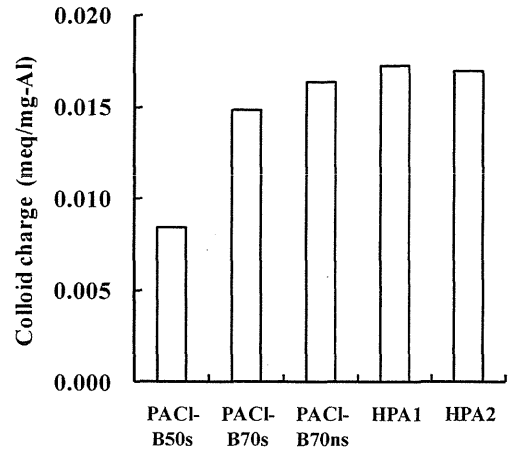

図-4. コロイド滴定法により得られた各凝集剤のコロイド荷電量

おいても，凝集剤の塩基度を高めることにより，凝集剤中のAlモノマーの存在割合が減少し，Alコロイドの存在割合が増加することが報告されている[23,28,29]．一方，高塩基度PACIにおいては，硫酸の有無に関わらず，いずれのアルミニウム種の形態存在割合も同程度であった．従って，凝集剤中の硫酸がアルミニウム種の形態存在割合に与える影響は小さいものと考えられた．

コロイド滴定法により得られた各凝集剤のコロイド荷電量を図-4に示す．PACl-B70sは，PACl-B50sに比べてコロイド荷電量が大きかった．従って，凝集剤の塩基度を高めることにより，凝集剤中のAlコロイドの存在割合が増加し，結果として荷電中和力が増加したことによりウイルスの処理性が向上した可能性が示唆された．また，硫酸無添加のPACl-B70nsは，硫酸を含むPACl-B70sに比べてコロイド荷電量が統計学的に有意に大きかった（t検定，$n = 3$，$P < 0.05$）．PACIに硫酸を混合させることにより，弱酸性のpH領域における濁度の処理性が向上することが報告されているが[28]，コロイド荷電量については減少することが明らかとなった．従って，凝集剤中に硫酸を含まないことにより，荷電中和力が増加し，結果としてウイルスの処理性が向上した可能性が示唆された．

(4) 新規アルミニウム系凝集剤の作製

上述したように，塩基度が高く，硫酸を含まない凝集剤を凝集沈澱処理に用いることにより，弱酸性，中性，弱アルカリ性のいずれのpH領域においても，高いウイルスの処理性が得られることが明らかとなった．また，塩基度の増加に伴う凝集剤中のAlコロイドの存在割合の増加がウイルスの処理性の向上に影響している可能性が考えられた．そこで，凝集剤中のアルミニウム形態がウイルスの処理性に与える影響をより詳細に評価するため，凝集剤中のアルミニウム形態の大きく異なる複数のHPAを作製した．HPAの作製においては，塩基度を高めることによりAlポリマーの存在割合が増加し，塩化アルミニウム溶液濃度およびNaOH濃度を高め，且つ加熱撹拌時間を長くすることによりAlコロイドの存在割合が増加する傾向が見られた．これらの傾向を考慮し，Alポリマーの存在割合が大きいHPA1（塩基度 70%，硫酸 0%）およびAlコロイドの存在割合が大きいHPA2（塩基度 70%，硫酸 0%）を作製した（図-3）．なお，これら2種類のHPAのコロイド荷電量は，凝集剤中のアルミニウム形態が大きく異なるにも関わらず同程度であった（図-4）．

(5) 新規アルミニウム系凝集剤によるウイルスの処理

作製したHPA1およびHPA2を用いた場合の凝集沈澱処理後のMS2の除去率を図-5に示す．なお，MS2濃度はプラック形成法（a）およびリアルタイム定量RT-PCR法（b）にて定量した．図より，Alポリマーの存在割合が大きいHPA1を用いた場合，プラック形成法にて評価した除去率は，pH 6付近およびpH 7付近において，7-8 log程度となった．一方，pH 8付近においては，弱酸性および中性のpH領域に比べて除去率が低下したものの，3-5 log程度の除去率が得られた．これに対し，Alコロイドの存在割合が大きいHPA2を用いた場合，pH 6付近およびpH 7付近においては，HPA1を用いた場合と同程度の除去率であったのに対し，pH 8付近では，HPA1を用いた場合に比べて約2 log高い6-7 log程度の除去率が得られた．また，弱酸性および弱アルカリ性のpH領域において，硫酸無添加のPACl-B70nsを用いた場合に比べて高い

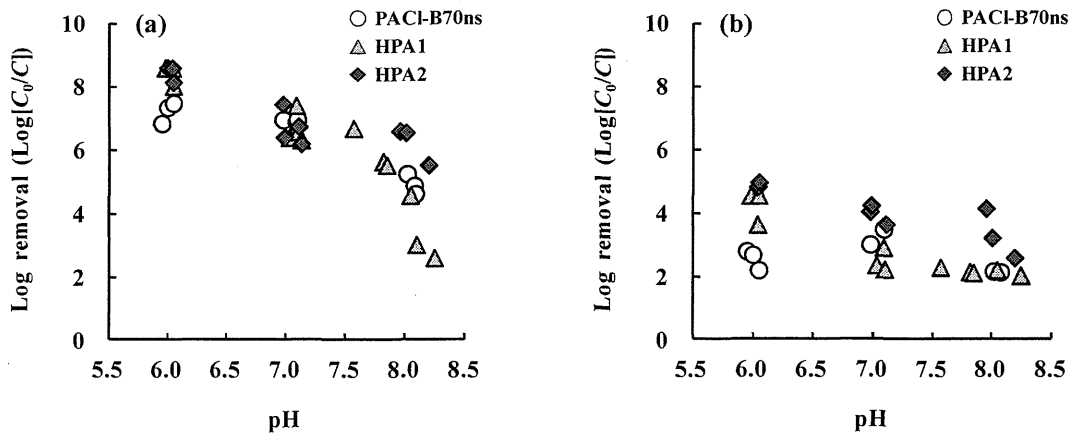

図-5. 新規アルミニウム系凝集剤によるMS2の処理：MS2濃度はプラック形成法（a）およびリアルタイム定量RT-PCR法（b）にて定量

除去率が得られた．リアルタイム定量RT-PCR法にて評価した除去率についても，HPA1およびPACl-B70nsを用いた場合に比べて高い除去率が得られた．以上の結果から，凝集剤中のアルミニウム形態はウイルスの処理性に影響し，Alコロイドの存在割合が大きい凝集剤の方がウイルス処理に有効であることが示された．

(6) ESI-FT-MS法，^{27}Al-NMR法による凝集剤の分析

作製したHPA1とHPA2に含まれるアルミニウム種を特定するために，ESI-FT-MS法および^{27}Al-NMR法による分析を行った．ESI-FT-MS法により得られたHPA1およびHPA2のマススペクトルを図-6に示す．図より，いずれの凝集剤においても，アルミニウム単量体（m/z = 97, $[Al(OH)_2(H_2O)_2]^+$）[30]のピークの相対強度が最も大きく，相対強度80%程度のアルミニウム2量体（m/z = 157, $[Al_2H_7O_6]^+$）[30]のピークも検出された．加えて，HPA1においては，相対強度20%以上において，m/z = 309, 315, 321, 445のピークが，また，HPA2においては，m/z = 315, 321, 436, 445, 454のピークが検出された．これらのピークは，アルミニウム13量体のフラグメントイオンであることが報告されていることから（m/z = 309, $[Al_{13}O_4(OH)_{28}(H_2O)_2]^{3+}$, m/z = 315, $[Al_{13}O_4(OH)_{28}(H_2O)_3]^{3+}$, m/z = 321, $[Al_{13}O_4(OH)_{28}(H_2O)_4]^{3+}$, m/z = 436, $[Al_{13}O_6(OH)_{25}]^{2+}$, m/z = 445, $[Al_{13}O_5(OH)_{27}]^{2+}$, m/z = 454, $[Al_{13}O_4(OH)_{29}]^{2+}$）[31]，作製したHPAにはアルミニウム13量体が含まれていることが明らかとなった．

^{27}Al-NMR法により得られたHPA1およびHPA2のNMRスペクトルを図-7に示す．図より，いずれの凝集剤においても，アルミニウム単量体（δ = 0 ppm），アルミニウム13量体（δ = 63 ppm），内部標準物質として使用したアルミン酸ナトリウム（δ = 80 ppm）[32,33]のピークが検出された．また，HPA1のアルミニウム13量体のピーク

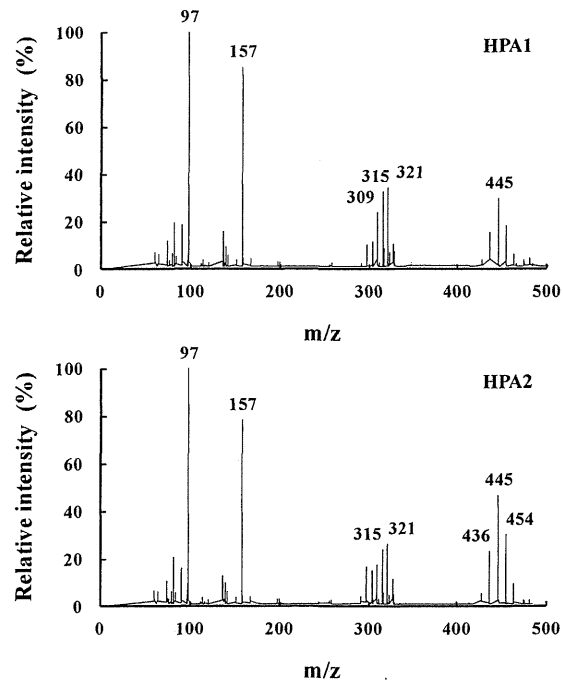

図-6. ESI-FT-MS法により得られたHPA1およびHPA2のマススペクトル

は，HPA2のピークに比べて大きかった．一方，HPA2においては，HPA1には見られなかったδ = 10-12 ppmおよび70 ppmにおいてブロード状のピークが検出された．δ = 10-12 ppmのピークは，アルミニウム13量体およびアルミニウム30量体の外殻部分の存在を，また，δ = 70 ppmのピークは，アルミニウム30量体の核部分の存在を示すことが報告されていることから[32]，作製したHPA2にはアルミニウム13量体のみならずアルミニウム30量体が含まれていることが明らかとなった．これに対し，従来PACIであるPACl-B50sにおいては，ESI-FT-MS法および^{27}Al-NMR法のいずれの分析においても，アルミニウム13

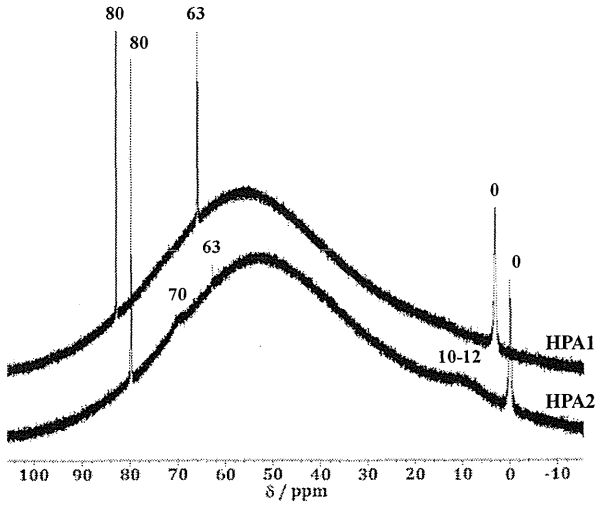

図-7. ^{27}Al-NMR法により得られたHPA1およびHPA2のNMRスペクトル

量体および30量体のピークは検出されなかった．以上の結果から，アルミニウム13量体および30量体の存在がウイルスの処理性に大きく影響し，中でも，アルミニウム30量体を含むAlコロイドを主成分とする凝集剤を凝集沈澱処理に用いることにより，弱酸性，中性，弱アルカリ性のいずれのpH領域においても，従来PACl（PACl-B50s）に比べてウイルスの除去率を飛躍的に向上できる可能性が示唆された．

(7) 開発した新規アルミニウム系凝集剤の適用可能性

本研究で開発した塩基度70%，硫酸無添加のAlコロイドを主成分とする新規アルミニウム系凝集剤（HPA2）を凝集沈澱処理に用いた場合，弱酸性，中性，弱アルカリ性のいずれのpH領域においても，約6 logあるいはそれ以上の高いウイルスの除去率が得られたことから，従来のアルミニウム系凝集剤（PACl-B50s）を用いた場合に比べ，後段の消毒処理への負荷低減が可能であり，凝集剤使用量の削減にも繋がるものと考えられる．また，本研究で使用した凝集剤の中で，消毒副生成物の前駆物質であるNOMの除去率が最も高く（pH 8付近においても60%以上，PACl-B50sを用いた場合は20-30%程度），処理水中の残留アルミニウム濃度も最も低かったことから（pH 8付近においても0.06 mg-Al/L未満，PACl-B50sを用いた場合は0.3-0.4 mg-Al/L程度），水系感染症発生リスクの低減のみならず，消毒副生成物の生成抑制や残留アルミニウム濃度の低減にも繋がるものと考えられる．以上のことから，本研究で開発した新規アルミニウム系凝集剤を凝集沈澱処理に適用することにより，凝集剤の多量注入や酸注入によるpH制御に頼ることなく，広範なpH領域の原水のウイルス処理に対応可能であると考えられる．

4. 結論

本研究では，凝集剤の塩基度，凝集剤中の硫酸およびアルミニウム形態がウイルスの処理性に与える影響を詳細に評価し，これらの結果を踏まえた上で，ウイルス処理に有効な新規アルミニウム系凝集剤を開発した．

本研究で得られた知見を以下にまとめる．

(1) 凝集剤の塩基度および凝集剤中の硫酸はウイルスの処理性に影響し，塩基度が高く，硫酸を含まない凝集剤を用いることにより，従来PAClに比べて高い除去率が得られた．

(2) 凝集剤中のアルミニウム形態はウイルスの処理性に影響し，Alコロイドの存在割合が大きい凝集剤は，Alポリマーの存在割合が大きい凝集剤に比べてウイルス処理に有効であった．

(3) Alコロイドの存在割合が大きい凝集剤には，アルミニウム13量体のみならずアルミニウム30量体が含まれていたことから，これらのアルミニウム種がウイルスの処理性の向上に大きく影響している可能性が示唆された．

(4) 本研究で開発したAlコロイドを主成分とする新規アルミニウム系凝集剤を凝集沈澱処理に用いることにより，弱酸性，中性，弱アルカリ性のいずれのpH領域においても，約6 logあるいはそれ以上の高いウイルス除去率が得られた．

謝辞：本研究は，科学研究費補助金　研究活動スタート支援「ウイルス処理に有効な新規凝集剤の開発」（平成23-24年度，課題番号 23860002，代表　白崎伸隆），科学研究費補助金　基盤研究(B)「VLPsを用いた培養不可能なウイルスの浄水処理性評価」（平成24-26年度，課題番号 24360211，代表　松下拓）ならびに厚生労働科学研究費補助金「水道における水質リスク評価および管理に関する総合研究」（平成22-24年度，主任研究者　松井佳彦）により行われた．記して感謝の意を表す．

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(2012. 5. 25 受付)

Development of Novel Aluminum-Based Coagulant for Effective Virus Removal

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A novel aluminum-based coagulant for effective virus removal was developed in the present study based on the investigation of roles of the basicity, sulfate ion and aluminum speciation in coagulation of virus. The coagulation process with the novel aluminum-based coagulant effectively removed viruses compared with other aluminum-based coagulants, and achieved apporimately 6-log removals not only in the weakly acidic and neutral pH conditions but also weakly alkaline pH conditions. In addition, Al_{13} and Al_{30} polymers were detecteded by electrospray ionization mass spectrometry and ^{27}Al-NMR spectrometry in the novel aluminum-based coagulant. Accordingly, Al_{13} and Al_{30} species are probably dominant species to control the virus removal performance, and that lead effective removals of viruses in the coagulation process with novel aluminum-based coagulant even in the weakly alkaline pH condition.

Difference in behaviors of F-specific DNA and RNA bacteriophages during coagulation–rapid sand filtration and coagulation–microfiltration processes

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ABSTRACT

Difference in behaviors of F-specific DNA and RNA bacteriophages during coagulation–rapid sand filtration and coagulation–microfiltration (MF) processes were investigated by using river water spiked with F-specific DNA bacteriophage f1 and RNA bacteriophage f2. Because the particle characteristics of f1 (filamentous) and f2 (spherical) are quite different and the surface charge of f1 in the river water was slightly more negative than that of f2, the removal ratios of f1 were approximately 1-log lower than the removal ratio of f2 after any treatment process used in the present study. This result indicates that the behaviors of the two bacteriophages during the treatment processes were different, and that the removal of f1 by the combination of coagulation and filtration processes was more difficult than that of f2. The removal ratios for f1 and f2 were approximately 3-log and 4-log, respectively, in the coagulation–rapid sand filtration process, and 6-log and 7-log, respectively, in the coagulation–MF filtration process. Therefore, as expected, the coagulation–MF process appears to be more effective than the coagulation–rapid sand filtration process for the removal of not only spherical viruses but also filamentous viruses.

Key words | coagulation, F-specific DNA bacteriophage, F-specific RNA bacteriophage, microfiltration, rapid sand filtration

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INTRODUCTION

Microbial safety of drinking water has been of primary interest for public health protection. Most waterborne pathogens are introduced into drinking water supplies by human or animal feces (Guillot & Loret 2010). Because fecal coliform bacteria are consistently present and often abundant in human and animal feces (Nappier *et al.* 2006), these bacteria have traditionally been used as surrogates for fecal contamination in source and drinking water. However, some researchers have demonstrated that fecal coliform bacteria may not be appropriate surrogates for waterborne enteric viruses, owing to the differences in their resistance to drinking water treatment processes (Payment *et al.* 1985; Havelaar *et al.* 1993). In other words, fecal coliform bacteria are less resistant than enteric viruses to physicochemical treatments such as filtration and disinfection processes. Hence, other reliable surrogates are required for enteric viruses so as to guarantee the microbial safety of drinking water.

Bacteriophages, which are viruses that infect bacteria, have been proposed as surrogate candidates for enteric viruses. This is based on the greater similarity of bacteriophages to enteric viruses than to fecal coliform bacteria, in terms of their environmental persistence and resistance to drinking water treatment processes as well as lack of pathogenicity to humans (Stetler 1984; Havelaar *et al.* 1993). Among bacteriophages, especially the F-specific bacteriophages, viruses that infect F+ male *Escherichia coli* bacteria through the F sex pilus, are considered to be better surrogates for enteric viruses (World Health Organization 2008).

doi: 10.2166/ws.2012.041

F-specific bacteriophages are categorized into DNA and RNA bacteriophages, and belong to the two families, Inoviridae and Leviviridae (Cole *et al.* 2003). Virions in the Inoviridae family are rods or filaments containing a single molecule of circular, positive-sense, single-stranded DNA, whereas virions in the Leviviridae family are spherical and of icosahedral symmetry and contain a single molecule of linear, positive-sense, single-stranded RNA (Fauquet *et al.* 2005). Because these F-specific DNA and RNA bacteriophages are widely present in fecal waste (Cole *et al.* 2003), the presence, prevalence and population of F-specific DNA and RNA bacteriophages in surface water have been investigated to identify fecal contamination sources (Cole *et al.* 2003; Haramoto *et al.* 2009). In addition, because F-specific RNA bacteriophages are morphologically similar to hepatitis A viruses and polioviruses, the bacteriophages are used worldwide as surrogates for enteric viruses to estimate the removal of enteric viruses during drinking water treatment processes (Shelton & Drewry 1973; Matsushita *et al.* 2005; Zhu *et al.* 2005; Fiksdal & Leiknes 2006; Shirasaki *et al.* 2009; Pierre *et al.* 2010). F-specific DNA bacteriophages are not used as surrogates for enteric viruses because of their morphological differences. However, the F-specific DNA bacteriophage of *Vibrio cholerae* has been implicated in the lysogenic conversion of *V. cholerae* to its toxic form, suggesting that at least some F-specific DNA bacteriophages are indirectly involved in waterborne disease transmission (Waldor & Mekalanos 1996; Redman *et al.* 1999). Accordingly, removal of not only enteric viruses but also F-specific DNA bacteriophages by drinking water treatment processes is important to demonstrate the microbial safety of drinking water. However, removal of F-specific DNA bacteriophages has not been fully investigated in drinking water treatment processes, although the environmental persistence of F-specific DNA bacteriophages has been investigated and compared with that of F-specific RNA bacteriophages (Long & Sobsey 2004).

Our objective in the present study was to investigate the difference in behaviors of F-specific DNA and RNA bacteriophages during the coagulation–rapid sand filtration process, which is commonly used in drinking water treatment facilities, and during the coagulation–microfiltration (MF) process, which is becoming an important technology in this century for drinking water treatment.

MATERIALS AND METHODS

Source water, coagulant, filter media and MF membrane

River water was sampled from the Toyohira River (Sapporo, Japan; water quality shown in Table 1) on 12 October 2007. The coagulant used was a commercial aluminium coagulant, polyaluminium chloride (PACl) (PACl 250A; 10.5% Al_2O_3, relative density 1.2 at 20 °C; Taki Chemical Co., Ltd, Hyogo, Japan). Silica sand (effective size 0.6 mm, uniformity coefficient <3; Nihon Genryo Co., Ltd, Kanagawa, Japan) was used as a filter medium for rapid sand filtration. A flat type of ceramic MF membrane (nominal pore size 0.1 μm, effective filtration area 0.0007 m^2; NGK Insulators, Ltd, Nagoya, Japan), which was installed in an acrylic-resin casing, was used for the MF process.

Bacteriophages

The F-specific DNA bacteriophage, f1 (NBRC 20015), and the F-specific RNA bacteriophage, f2 (NBRC 20011), were obtained from the NITE Biological Research Center (NBRC, Chiba, Japan). f1 is a filamentous particle that has a diameter of 6 nm and length of 800 nm (Dotto *et al.* 1981). In contrast, f2 is an icosahedral particle that has a diameter of 22 nm (Shelton & Drewry 1973). Each bacteriophage was propagated for 22–24 h at 37 °C in *E. coli* (NBRC 13965) obtained from NBRC. The bacteriophage culture solution was centrifuged (2,000×g, 10 min) and then passed through a membrane filter (pore size 0.45 μm, hydrophilic cellulose acetate; Dismic-25cs, Toyo Roshi Kaisha, Ltd, Tokyo, Japan). The filtrate was purified by using a centrifugal filter device (molecular weight cutoff 100,000, regenerated cellulose; Centriplus-100, Millipore Corp.,

Table 1 | Water quality of the Toyohira River

pH	7.5
DOC (mg/L)	0.90
OD260 (cm^{-1})	0.027
Turbidity (NTU)	0.50
Alkalinity (mg-$CaCO_3$/L)	19.1

Billerica, MA, USA) to prepare the bacteriophage stock solution. The concentration of each bacteriophage stock solution was approximately 10^{12} PFU/mL.

Coagulation experiments

Batch coagulation experiments were conducted with 200 mL of bacteriophage-spiked river water in glass beakers at 20 °C. The bacteriophage stock solution (see section on Bacteriophages) was added to the beaker at approximately 10^6 or 10^8 PFU/mL, and the spiked water was mixed with an impeller stirrer. Because around 1 mg-Al/L of PACl is usually dosed for the treatment of Toyohira River water, which is the source water in the present study, in the actual drinking water treatment plant (Moiwa drinking water treatment plant, Sapporo, Japan), PACl was injected into the water at a coagulant dose of 0.54, 1.08 or 1.62 mg-Al/L. The pH of the water was immediately adjusted to, and maintained at, 6.8 with HCl. The water was stirred rapidly for 2 min ($G = 200\ s^{-1}$, 61 rpm) and then slowly for 28 min ($G = 20\ s^{-1}$, 13 rpm). The water was then left at rest for 20 min to settle the generated aluminium floc particles. Samples were taken from the beaker before coagulant dosing (C_{c0}) and after settling (C_{cs}) for quantification of the bacteriophage concentrations.

The suspended aluminium floc particles that had not settled by gravity were separated from the floc mixture by centrifugation (2,000×g, 10 min) to quantify the bacteriophage concentration in the liquid phase of the floc mixture.

Rapid sand filtration experiments

After the coagulation experiments (without centrifugal separation), rapid sand filtration experiments were carried out with a glass column (diameter 0.8 cm, length 20 cm) packed with silica sand washed with Milli-Q water and dried at 105 °C for 1 h. The cleaned silica sand was gradually added into the glass column to achieve a filter depth of 10 cm. This column was connected to another such column to achieve a total filter depth of 20 cm. Subsequently, Milli-Q water was pumped through the column with the help of a peristaltic pump for 15 min to saturate the filter medium, and the excess Milli-Q water was drained from the column just before the filtration experiment. Approximately 170 mL of the supernatant of the settling sample (see section on Coagulation experiments) was withdrawn from the beaker by the peristaltic pump and transferred to another glass beaker to be considered as raw water for rapid sand filtration experiments. During the filtration experiments, the raw water was continuously mixed with a magnetic stirrer at 200 rpm and fed into the column at a constant flow rate (120 m/day) by the peristaltic pump. Samples were taken from the beaker (C_{r0}) and from the first (10 cm) and second (20 cm) column filtrates (C_{rf}) at 5, 15 and 30 min after the initiation of filtration for the quantification of bacteriophage concentrations.

MF experiments

After the coagulation experiments (without centrifugal separation), MF filtration experiments were carried out with a flat type of ceramic MF membrane. Approximately 170 mL of the supernatant of the settling sample (see section on Coagulation experiments) was withdrawn from the beaker by the peristaltic pump and transferred to another glass beaker to be considered as raw water for MF filtration experiments. During the filtration experiment, the raw water was continuously mixed with a magnetic stirrer at 200 rpm and fed into a ceramic MF membrane at a constant flux (83 L/(m^2 h)) by the peristaltic pump. Samples were taken from the beaker (C_{m0}) and from the MF permeate (C_{mf}) at 15, 30, 60 and 120 min after the initiation of filtration for the quantification of bacteriophage concentrations.

Bacteriophage assay

The infectious bacteriophages were enumerated by the determination of the number of plaque-forming units (PFU) according to the double-layer method (Adams 1959) with the bacterial host *E. coli* (NBRC 13965). The average of the plaque counts of triplicate plates prepared from one sample was considered as the infectious bacteriophage concentration for that sample.

Electron micrograph

Negative-stain electron microscopy was used to analyze the morphology of the bacteriophages. Ten microliters of f1 or