表 5-6　模擬試料を用いた最適 AMDIS パラメーターでの同定精度

Sample	Number of spiked chemicals	Detected number	Number of false negative	Number of false positive
Tomato	97	97	0	23
Glebionis coronaria	97	95	2	22
Green peas	97	96	1	20

試料抽出液 1mL に各 1μg を添加

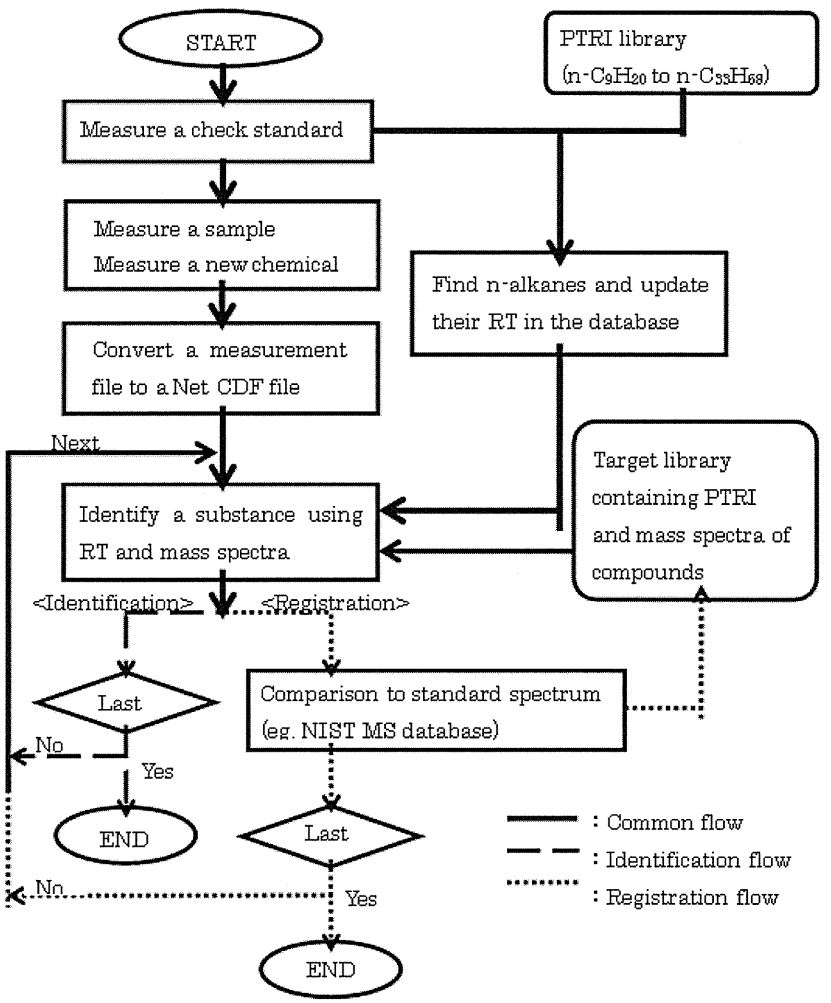

図 5-1　開発システムを用いた物質同定及びデータベースへの物質登録の流れ

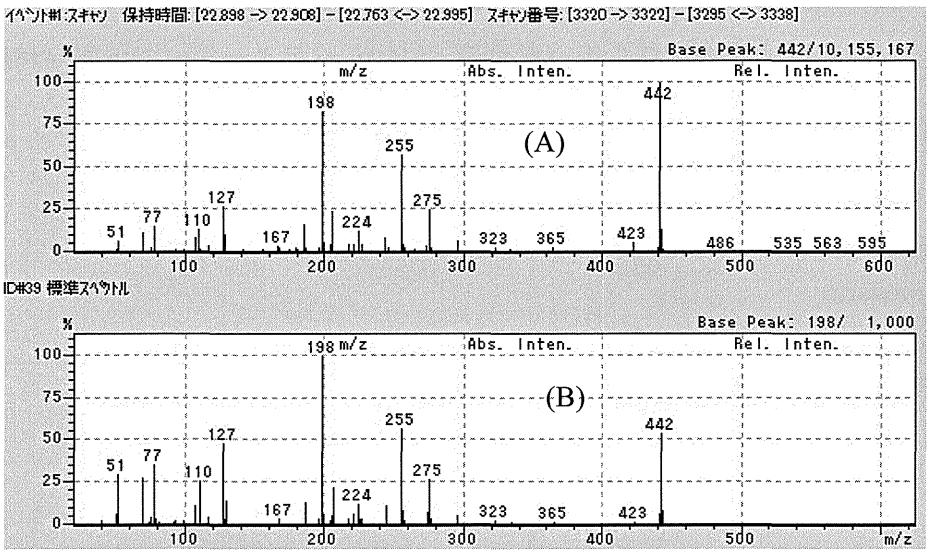

図 5-2　デカフルオロトリフェニルホスフィン (DFTPP)のマススペクトル
A: TSQ Quantum GC 測定結果, B: NIST データベース登録マススペクトル

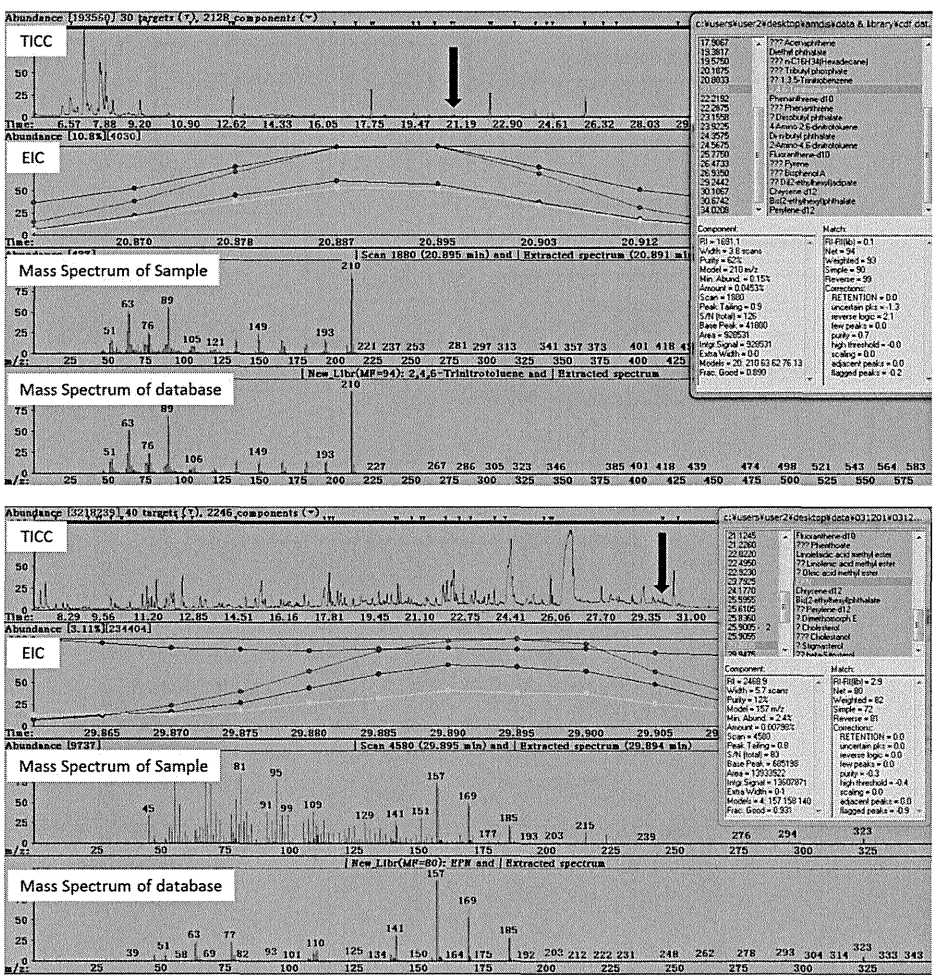

図 5-3　開発同定システムの実試料への適用例
(A) 地下水から TNT とその代謝物を検出，(B) タマネギから農薬の EPN を検出

研究成果の刊行に関する一覧表

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研究成果の刊行物・別刷

Virus removal by an in-line coagulation—ceramic microfiltration process with high-basicity polyaluminum coagulation pretreatment

N. Shirasaki, T. Matsushita, Y. Matsui, T. Urasaki, M. Kimura and K. Ohno

ABSTRACT

The ability of in-line coagulation pretreatment with high-basicity polyaluminum chloride (PACl) coagulants to enhance virus removal by ceramic microfiltration (MF) was examined by comparing virus removal efficiencies from water pretreated with PACl-2.2 (basicity 2.2) and PACl-2.5 (basicity 2.5) versus alum, a synthetic aluminum chloride ($AlCl_3$) solution, and two commercially available PACls, PACl-1.5 and PACl-1.8. The virus removal ratios for $AlCl_3$, alum, PACl-1.5, and PACl-1.8 decreased markedly when the pH of the treated water shifted from 6.8 to 7.8, but was high at both pHs for PACl-2.2 and PACl-2.5. PACl-2.5 contains Al_{13} species and possibly Al_{30} species, and has a high colloid charge density. It removed viruses more efficiently than the other aluminum-based coagulants, not only at neutral pH, but also under weakly alkaline conditions. Moreover, the in-line coagulation—ceramic MF process with PACl-2.5 pretreatment removed not only viruses but also dissolved organic carbon and UV260-absorbing natural organic matter more efficiently and resulted in a lower residual aluminum concentration than did commercially available PACls, especially under weakly alkaline conditions. A combination of coagulation pretreatment with a high-basicity PACl and ceramic MF can provide effective treatment of drinking water over a broader pH range than is possible with commercially available aluminum-based coagulants.

Key words | aluminum hydrolyte species, bacteriophage, ceramic microfiltration, colloid charge density, in-line coagulation

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INTRODUCTION

Low-pressure membrane (LPM) filtration, including microfiltration (MF) and ultrafiltration, is widely used for drinking water treatment because of its ability to produce high-quality water, its small footprint, and its relatively low costs (Huang *et al.* 2009). To improve the filtration efficiency of LPM, integration of pretreatment with LPM filtration has been widely employed in actual drinking water treatment plants. Pretreatment methods include adsorption, coagulation, and oxidation. Among these methods, coagulation is the most successful pretreatment for controlling membrane fouling (Huang *et al.* 2009), which reduces membrane permeability and increases the frequency of hydrodynamic or chemical cleaning. In addition, coagulation pretreatment is also useful for improving the quality of treated water. Enhancement of removal of dissolved organic carbon (DOC) and natural organic matter (NOM) can be expected with a combination of pre-coagulation and membrane filtration (Lee *et al.* 2000). Moreover, effective removal of waterborne enteric viruses, those having diameters of 20–100 nm, is possible by this hybrid process. For example, Tanneru *et al.* (2013) have reported that a coagulation–MF process with a coagulant dosage of more than 20 mg-Al/L produced 5.5–6.0 log reduction of viruses when the pH of the treated water was about 6.4, whereas MF alone with a 0.22-μm-pore-size, hydrophilic polyvinylidene difluoride filter produced only a 0.2-log

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removal of viruses. Our research group has also reported the effectiveness of the coagulation–MF process: an approximately 6-log reduction of viruses was achieved at around pH 6.8 with a combination of in-line coagulation and a 0.1 μm-pore-size ceramic membrane filter (Shirasaki *et al.* 2009). This means that the coagulation–MF process has the potential to effectively mitigate the public health risk posed by virus contamination of drinking water.

Many factors affect the virus removal performance of the coagulation–MF process. Matsushita *et al.* (2005) have reported the effects of coagulant dosage, coagulation time, and MF membrane pore size on virus removal. They concluded that coagulant dosage strongly affected virus removal compared with two other factors when the pH was near 7. In addition, Zhu *et al.* (2005) have investigated the effect of pH on virus removal and reported a significant reduction of virus removal as the pH increased from 6.3 to 8.3 when a solution of ferric chloride ($FeCl_3$) was used as the coagulant. Adjustment of the pH during coagulation pretreatment is therefore one of the important steps that must be taken to control virus removal in the coagulation–MF filtration process. However, an increase in the pH of drinking water sources from neutral to alkaline conditions because of excessive algal growth has been reported throughout the world (Hu *et al.* 2006; Matsukawa *et al.* 2006). Under these circumstances, reducing the pH of the drinking water source with acid or adding more coagulant is sometimes required to improve coagulation efficiency when commercially available aluminum-based coagulants such as polyaluminum chloride (PACl) and alum are used (Hu *et al.* 2006; Yan *et al.* 2008). However, both of these methods have some disadvantages, including an increase of the residual aluminum concentration in treated water (Matsukawa *et al.* 2006) and treatment cost (Yan *et al.* 2008).

An alternative investigated by some researchers has been the effect of adjusting the aluminum hydrolyte ratio (basicity = $[OH^-]/[Al^{3+}]$) in PACl on DOC removal and residual aluminum concentration during the coagulation process. They have reported that high-basicity PACls (basicity 2.1–2.7) yield higher removal of DOC and lower residual aluminum concentrations than commercially available PACls with basicities of 1.5–1.8, especially under weakly alkaline conditions (Yan *et al.* 2008; Kimura *et al.* 2013). Accordingly, effective removal of viruses as well as DOC is possible not only under neutral pH conditions but also under weakly alkaline conditions when the MF process is combined with a coagulation pretreatment with high-basicity PACl instead of commercially available PACl or alum. However, there is no report about the effectiveness of coagulation pretreatment with high-basicity PACl for virus removal during the coagulation–MF process. Our objective in the present study was to investigate the effects of coagulant type and PACl basicity on virus removal during the coagulation–MF process by comparing four PACls with different basicity, a synthetic aluminum chloride ($AlCl_3$) solution, and commercially available alum.

MATERIALS AND METHODS

Source water, coagulants, and MF membrane

On 17 July 2009 and 10 November 2009, river water was sampled from the Toyohira River (Sapporo, Japan), the water quality of which is shown in Table 1. The coagulants used for the coagulation process were six aluminum-based coagulants, the specifications of which are shown in Table 2. Two commercially available PACls with normal basicities of 1.5 (PACl-1.5) and 1.8 (PACl-1.8) were provided by the Taki Chemical Co., Ltd. (Kakogawa, Japan). For experimental purposes, the same company also supplied high-basicity PACls with basicities of 2.2 (PACl-2.2, presently available commercially) and 2.5. To provide a comparison of coagulation efficiency with PACls, a synthetic $AlCl_3$ solution, which was prepared by dilution of reagent-grade aluminum (III) chloride hexahydrate ($AlCl_3 \cdot 6H_2O$, Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in Milli-Q

Table 1 | Water quality of the Toyohira River

	River water 1	River water 2
Sampling date	17-Jul-09	10-Nov-09
pH	7.5	7.7
Turbidity (NTU)	2.0	0.8
DOC (mg/L)	0.9	0.8
UV260 (cm^{-1})	0.031	0.027
Alkalinity (mg-$CaCO_3$/L)	14.5	22.2

Table 2 | Specifications of aluminum-based coagulants used in the present study

Coagulants	Basicity	Aluminum concentration	Sulfate concentration	Relative density at 20 °C	Aluminum species distribution		
					Al_a(%)	Al_b(%)	Al_c(%)
$AlCl_3$	0.0	2.7 g-Al/L	0.0 g/L	1.0	75.8	4.6	19.6
Alum	0.0	8% (w/w) as Al_2O_3	23% (w/w)	1.3	73.3	9.4	17.3
PAC1-1.5	1.5	10% (w/w) as Al_2O_3	3% (w/w)	1.2	46.2	15.5	38.3
PAC1-1.8	1.8	10% (w/w) as Al_2O_3	3% (w/w)	1.2	42.2	11.6	46.3
PAC1-2.1	2.2	10% (w/w) as Al_2O_3	3% (w/w)	1.2	36.4	6.3	57.3
PAC1-2.5	2.5	23% (w/w) as Al_2O_3	0% (w/w)	1.3	24.6	3.8	71.6

water (Milli-Q Advantage, Millipore Corp., Billerica, MA, USA), and commercially available alum (Taki Chemical Co., Ltd.), were used in the present study. The distributions of aluminum species in the coagulants were analyzed by a ferron method (Wang *et al.* 2004) and are shown in Table 2. 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 (Al_a), fast-reacting polymeric species (Al_b), or slow-reacting colloidal species (Al_c) (Wang *et al.* 2004). The details of the ferron method have been described in our previous study (Kimura *et al.* 2013). A monolithic, modular, ceramic MF membrane (55-channel tubular; nominal pore size 0.1 μm; effective filtration area 0.043 m^2; NGK Insulators, Ltd., Nagoya, Japan) installed in a stainless-steel casing was used for the MF process.

Characterization of coagulants

Electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS)

ESI-TOF-MSwas used to analyze the aluminum hydrolyte species in the coagulants. Each coagulant was diluted with Milli-Q water to 2 mg-Al/L, and the diluted coagulant was introduced into an ESI-TOF-MS (model JMS-T100LP; JEOL Ltd., Tokyo, Japan) at a constant flow rate of 30 μL/min by using a syringe pump. Analysis was conducted in a positive-ion mode at a needle voltage of 2,000 V, an orifice 1 voltage of 10–30 V (0–100%), an orifice 2 voltage of 5 V, a ring lens voltage of 10 V, and a mass range m/z of 10–500.

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 homogenized with a magnetic stirrer during the titration, and the absorbance at 630 nm was recorded continuously until there was little change in the absorbance (i.e., subtle change in the color of the indicator from light blue to bluish-purple). The positive colloid charge was determined from the volume of potassium polyvinyl sulfate that corresponded to the half-height of the descending side of the recorded absorbance curve.

Bacteriophage

F-specific RNA bacteriophage Qβ (NITE Biological Research Center (NBRC) 20012) was obtained from the NBRC (Kisarazu, Japan). The bacteriophage Qβ is widely used as a surrogate for waterborne enteric viruses in the membrane filtration process (Matsushita *et al.* 2005; Shirasaki *et al.* 2009) because of its morphological similarities to hepatitis A viruses and polioviruses, which are important to remove during drinking water treatment. The bacteriophage Qβ is the

prototype member of the genus *Allolevivirus* in the virus family Leviviridae. The genome of this bacteriophage contains 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). Bacteriophage was propagated and purified as described in our previous report (Shirasaki *et al.* 2010) prior to the preparation of a bacteriophage stock solution.

In-line coagulation—ceramic MF experiments

The river water, placed in a raw water tank, was spiked with Qβ at approximately 10^8 PFU/mL. Throughout the experiments, the raw water was mixed constantly with an impeller stirrer. The raw water was fed into the experimental system at a constant flow rate (83.3 L/(m^2 h) = 2.0 m/d) by a peristaltic pump. To maintain the MF filtrate at pH 6.8 or 7.8, hydrochloric acid or sodium hydroxide was added to the water before it reached the first in-line static mixer [hydraulic retention time (HRT) 1.8 s; 1/4-N40-172-0, Noritake Co., Ltd., Nagoya, Japan]. Because about 1 mg-Al/L of PACl is usually dosed for the treatment of Toyohira River water (the source water in the present study) in the actual drinking water treatment plant (Moiwa drinking water treatment plant, Sapporo, Japan), coagulant was injected after the first in-line static mixer and before the second in-line static mixer at a constant dose rate (1.08 or 2.16 mg-Al/L). To obtain a coagulation time of 1 min, a combination of the in-line static mixer (G value 260 s^{-1}, HRT 1.8 s) and a subsequent Tygon tube reactor (inside diameter 1.6 mm, total HRT 1 min) was used as the second in-line static mixer. After the coagulant had been admixed with the water, the water was fed into the ceramic MF module in dead-end mode. Filtration was performed for 4 h without any backwashing. Bacteriophage concentrations in the raw water tank (C_0) and in the MF filtrate (C_f) were measured every hour. In addition, DOC concentrations and UV260-absorbing NOM were quantified with a SIEVERS 900 laboratory TOC analyzer (GE Analytical Instruments, Boulder, CO, USA) and a UV-1700 Pharma spectrophotometer (Shimadzu Corp., Kyoto, Japan), respectively. After adding the nitric acid (1% (v/v), ultrapure, Kanto Chemical Co., Inc., Tokyo, Japan) into the MF filtrate, the aluminum concentration was analyzed with an HP4500 inductively coupled plasma–mass spectrometer (Yokogawa Analytical Systems Inc., Tokyo, Japan).

Bacteriophage assay

PFU method

The infectious bacteriophages were enumerated according to the double-layer method (Adams 1959) by using the bacterial host *Escherichia coli* (NBRC 13965). Serially diluted raw water or MF filtrate (1 mL) was poured onto a solid-bottom agar plate followed by 0.3 mL of host *E. coli* culture mixed with 3 mL of molten top agar. The plates were incubated for 16–24 h at 37 °C. To measure the concentrations of infectious bacteriophage in the water samples, we calculated the average plaque counts of triplicate plates prepared from one sample on plates with 30 to 300 PFU, which we considered a countable number of plaques, and determined the number of plaque forming units per milliliter.

For quantification of low infectious bacteriophage concentrations (i.e., <30 PFU/mL) in the MF filtrate, 50 mL of MF filtrate was mixed with 5 mL of bacterial host *E. coli* culture and 50 mL molten agar, and the mixture was then poured into 10 plates (without bottom agar). The plates were incubated for 16–24 h at 37 °C. We calculated the number of plaque forming units per milliliter by dividing the total plaque counts for the 10 plates by the sample volume (50 mL).

Real-time reverse-transcription polymerase chain reaction method (RT-PCR)

The viral RNA of bacteriophages was quantified by the real-time RT-PCR method. This method detects all bacteriophages, regardless of their infectivity and the existence of aggregates. The detailed procedure for the real-time RT-PCR method has been described in our previous study (Shirasaki *et al.* 2010).

RESULTS AND DISCUSSION

Effect of coagulant type on bacteriophage removal

Figure 1(a) shows the effect of coagulant type on the removal of infectious Qβ, assessed by the PFU method, in the in-line coagulation–ceramic MF process (source water was river water 1). Because the diameter of Qβ

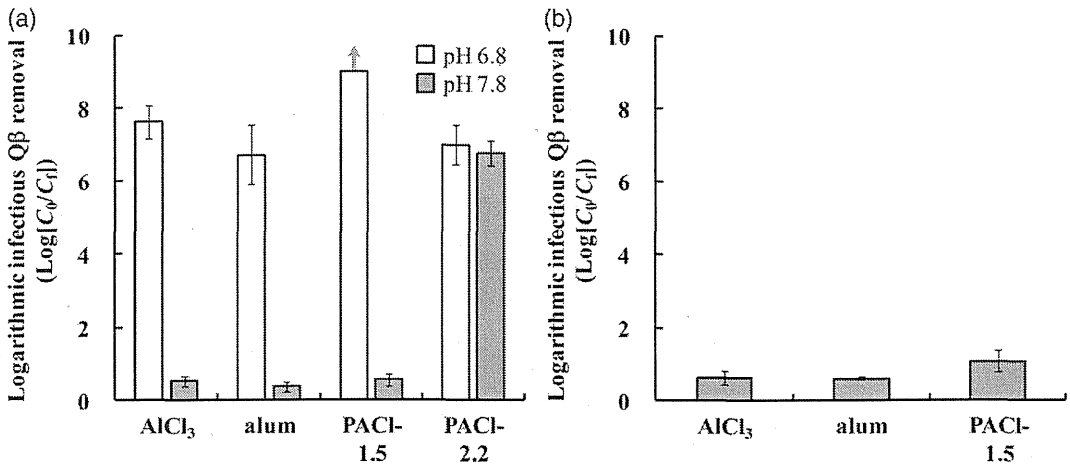

Figure 1 | Effect of coagulant type on removal of infectious Qβ by the in-line coagulation—ceramic MF process as evaluated by the PFU method. Source water was river water 1. Coagulant dosage was 1.08 (a) or 2.16 (b) mg-Al/L. Values are filtration time-averaged removals, and the error bars indicate standard deviations. The absence of a bar indicates that the Qβ concentrations were below the quantification limit in the treated water.

(approximately 25 nm) was smaller than the nominal pore size of the ceramic MF membrane (0.1 μm), no removal of infectious Qβ was observed in the absence of coagulation pretreatment (data not shown). In contrast, in-line coagulation pretreatment dramatically enhanced the removal ratios ($\log[C_0/C_f]$) of infectious Qβ compared with ceramic MF alone at around pH 6.8 of treated water. Greater than 6-log removals were achieved by a combination of in-line coagulation pretreatment and ceramic MF filtration, regardless of the type of coagulant, because during the coagulation pretreatment, Qβ was effectively incorporated into the aluminum flocs, which were larger than the nominal pore size of the ceramic MF membrane, and the flocs with incorporated Qβ were removed by ceramic MF filtration.

The infectious Qβ removal performances of $AlCl_3$, alum, and PACl-1.5 were markedly decreased when the pH of the treated water shifted from 6.8 to 7.8. Even when the coagulant dosage was increased from 1.08 to 2.16 mg-Al/L, <1-log removals were observed for those coagulants (Figure 1(b)). In contrast, PACl-2.2 continued to remove viruses with high efficiency (>6-log removal of infectious Qβ) even under weakly alkaline pH conditions (Figure 1(a)). This result indicates that the type of coagulant affects virus removal performance in the in-line coagulation–ceramic MF process, especially under weakly alkaline pH conditions, and that a high-basicity PACl (e.g., PACl-2.2) can remove viruses more effectively than the other commercially available aluminum-based coagulants used in the present study.

Effect of PACl basicity on bacteriophage removal

As described above, PACl-2.2 removed infectious Qβ more efficiently than did other aluminum-based coagulants, including PACl-1.5, especially under weakly alkaline pH conditions. To precisely quantify the effect of PACl basicity on virus removal, we compared the removal of Qβ by in-line coagulation pretreatment with four PACls with different basicities followed by ceramic MF filtration within the pH range 6.8–7.8 (Figure 2, source water was river water 2). More than 8-log removals of infectious Qβ, assessed by the PFU method, were attained by the in-line coagulation–ceramic MF process with all the PACls when the pH of the treated water was about 6.8, regardless of their basicity (Figure 2(a)). In contrast, the removal efficiencies of infectious Qβ depended on the basicity of the PACls when the pH of the treated water was about 7.8. The in-line coagulation–ceramic MF process with PACl-1.5 and PACl-1.8 achieved approximately a 1-log removal of infectious Qβ, but approximately 6-log and >8-log removals were achieved with PACl-2.2 and PACl-2.5, respectively. A similar trend was observed for removal ratios of total Qβ, assessed by the RT-PCR method. Although high removal efficiencies of total Qβ, at least

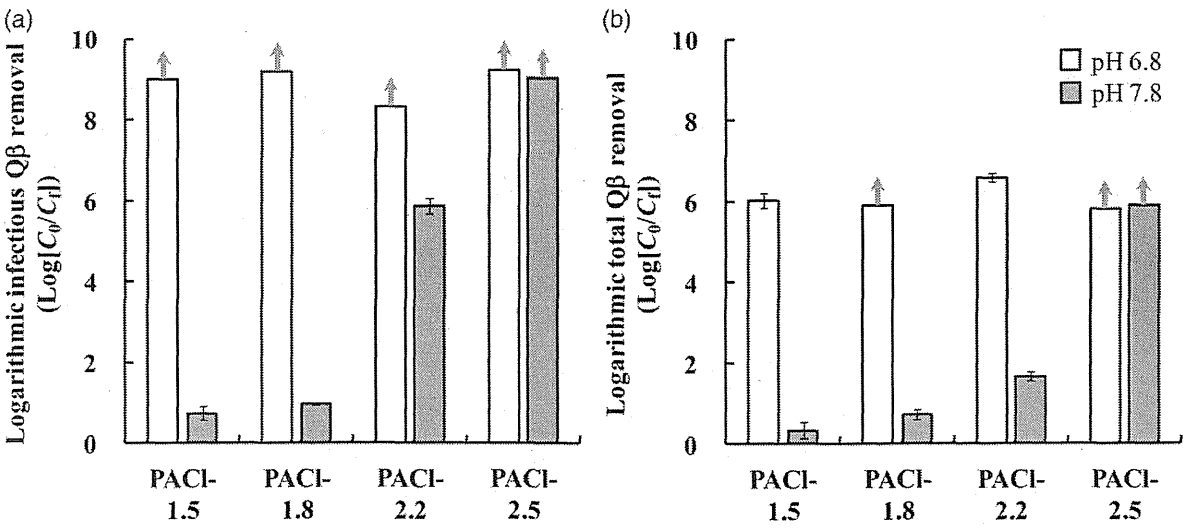

Figure 2 | Effect of PACl basicity on infectious Qβ removal as evaluated by the PFU method (a) and on total Qβ removal as evaluated by the RT-PCR method (b) by the in-line coagulation—ceramic MF process. Source water was river water 2. Coagulant dosage was 1.08 mg-Al/L. Values are filtration time-averaged removals, and the error bars indicate standard deviations. The absence of a bar indicates that the Qβ concentrations were below the quantification limit in the treated water.

6-log removals, were achieved for all PACls at around pH 6.8, marked differences in removal ratios were observed among the four PACls when the pH of the treated water was about 7.8 (Figure 2(b)). These results suggest that the basicity of PACls affects virus removal performance in the in-line coagulation–ceramic MF process, especially under weakly alkaline conditions.

As shown Figure 2, the in-line coagulation–ceramic MF process with PACl-2.5 effectively removed viruses compared to the performance of the other PACls used in the present study. In addition, this process removed DOC and UV260-absorbing NOM more efficiently and resulted in a lower residual aluminum concentration than did the processes with PACl-1.5, PACl-1.8, and PACl-2.2, especially under weakly alkaline conditions (whereas approximately 20–30% and 40–50% removals for DOC and UV260-absorbing NOM, respectively, and 0.08–0.15 mg-Al/L residual aluminum concentrations were observed for PACl-1.5, PACl-1.8, and PACl-2.2 at a pH of about 7.8, approximately 50% and 70% removals for DOC and UV260-absorbing NOM, respectively, and <0.01 mg-Al/L residual aluminum concentrations were attained for PACl-2.5). Accordingly, the combination of coagulation pretreatment with high-basicity PACl and ceramic MF is effective in treating drinking water, including virus removal, over a broader pH range compared to commercially available aluminum-based coagulants.

The Qβ removal ratios determined by the PFU and RT-PCR methods differed markedly, depending on the coagulation conditions. The infectious Qβ removal ratios (Figure 2(a)) were larger than the total Qβ removal ratios (Figure 2(b)). Because our previous study demonstrated that Qβ loses its infectivity after being mixed with aluminum hydrolyte species during coagulation with PACl-1.5 (Matsushita *et al.* 2011), the virucidal activity of the PACls contributed to the removal efficiency of infectious Qβ during the in-line coagulation–ceramic MF process.

Characterization of coagulants

To elucidate why PACl-2.5, which has a higher basicity, was more effective in removing viruses, we used ESI-TOF-MS to analyze the aluminum hydrolyte speciation in PACl-2.5. In the ESI-TOF-MS spectra of all the coagulants (Figure 3), we found the most intensive peak at *m/z* 97, which is assigned to the monomeric aluminum species $[Al(OH)_2(H_2O)_2]^+$ (Urabe *et al.* 2007). In addition, fragment ions of tridecameric species – i.e., Al_{13} species such as $[(Al_{13}O_4(OH)_{24}(H_2O)_{12})]^{7+}$ with peaks at *m/z* 303 $[Al_{13}O_4(OH)_{28}(H_2O)]^{3+}$ and 454 $[Al_{13}O_4(OH)_{29}]^{2+}$ (Stewart *et al.* 2009) – were also observed for

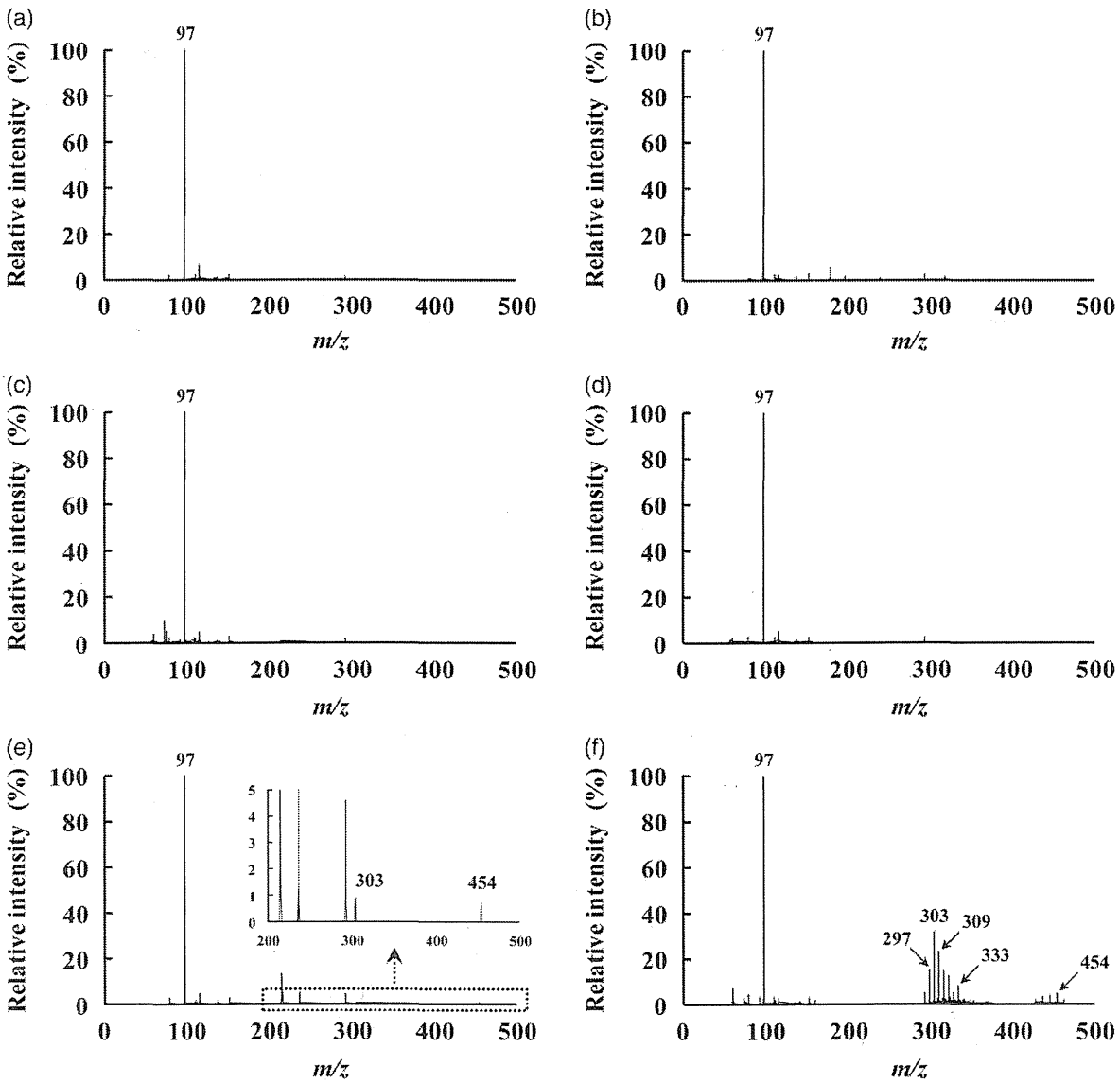

Figure 3 | ESI-TOF-MS spectra of coagulants: $AlCl_3$ (a), alum (b), PACl-1.5 (c), PACl-1.8 (d), PACl-2.2 (e), and PACl-2.5 (f).

PACl-2.2 (the relative intensities of these two peaks were 0.7–0.9%) and PACl-2.5. In contrast, no peak for Al_{13} species was confirmed in the spectra of $AlCl_3$, alum, PACl-1.5, and PACl-1.8. Moreover, other strong peaks of Al_{13} species at *m/z* 297 $[Al_{13}O_4(OH)_{28}]^{3+}$, 309, 315, 321, 327, and 333 $[Al_{13}O_4(OH)_{28}(H_2O)_{2-6}]^{3+}$ (Stewart *et al.* 2009) were observed in the spectrum of PACl-2.5. These results suggest that high-basicity PACls such as PACl-2.2 and PACl-2.5 contain Al_{13} species, but these species are not present in $AlCl_3$, alum, PACl-1.5, and PACl-1.8.

Some researchers have demonstrated that the content of Al_{13} species in a coagulant is approximately equal to that of Al_b species categorized by the ferron method (Chen *et al.* 2006). In the present study, whereas strong peaks related to Al_{13} species were observed for PACl-2.5, the content of Al_b in PACl-2.5 was very similar to those in other

aluminum-based coagulants used in the present study (Table 2). In contrast, the content of Al_c varied as a function of the coagulant type and was highest in PACl-2.5 (Table 2). Because the Al_{30} species $[(Al_{30}O_4(OH)_{56}(H_2O)_{24})]^{18+}$ does not react with ferron reagents within 120 min, it is categorized as Al_c by the ferron method (Chen *et al.* 2007). In addition, the Al_{30} species is known to consist of two Al_{13} species connected by four monomeric aluminum species (Chen *et al.* 2007), the implication being that the peaks of monomeric aluminum species and fragment ions of Al_{13} species will also be observed when the Al_{30} species in a coagulant is decomposed by fragmentation in the ESI-TOF-MS analysis. Actually, these peaks were clearly confirmed in the ESI-TOF-MS spectrum of PACl-2.5, as shown in Figure 3. Therefore, PACl-2.5 possibly contained not only Al_{13} species but also Al_{30} species.

The positive colloid charges of the coagulants were also determined by using a colloid titration technique (Figure 4). The colloid charge densities of the $AlCl_3$ and alum were very small and almost constant, regardless of the aluminum concentration. In contrast, the colloid charge densities of the PACls increased with increasing concentrations of aluminum. In addition, the colloid charge density of PACl-2.5 was the highest among the aluminum-based coagulants used in the present study. Because Al_{13} species and Al_{30} species are known to be effective coagulants because of their strong charge neutralization capability and high structural stability (Chen *et al.* 2006; Zhang *et al.* 2008), the presence of Al_{13} species, Al_{30} species, or both in PACl-2.5 is possibly associated with the large amount of positive colloid charge. In addition, the absence of sulfate in PACl-2.5 probably also contributed to its high colloid charge density compared with that of other sulfated PACls such as PACl-1.5, PACl-1.8, and PACl-2.2, because the presence of sulfate during the coagulation process reduces the charge neutralization capability of coagulants (Wang *et al.* 2002). These characteristics of PACl-2.5 account for its high capability to neutralize the negative charges on viruses during coagulation pretreatment. Actually, the in-line coagulation–ceramic MF process with PACl-2.5 could effectively remove viruses not only under neutral pH conditions but also under weakly alkaline conditions, as described above.

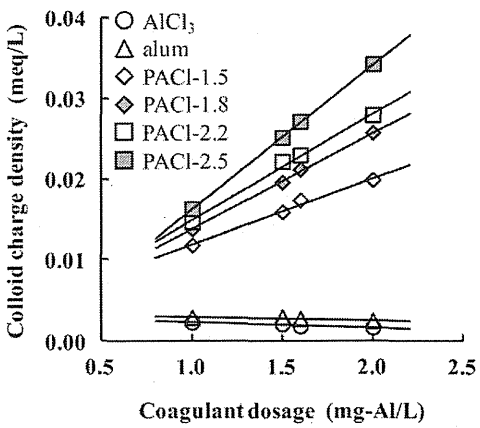

Figure 4 | Colloid charges densities of the coagulants as evaluated by the colloid titration technique.

We have shown through this study that coagulation pretreatment with PACl-2.5 followed by ceramic MF has a variety of advantages, including effective removal of viruses, DOC, and UV260-absorbing NOM, and the process results in very low residual aluminum concentrations compared with other aluminum-based coagulants, even under weakly alkaline conditions. To elucidate whether the in-line coagulation–ceramic MF process with high-basicity PACl is actually useful for drinking water treatment, further investigations using a wide variety of water sources are needed, because the concentration of viruses in the treated water may be affected by the characteristics of the source water.

CONCLUSIONS

1. The basicity of PACls affects the virus removal performance of the in-line coagulation–ceramic MF process. The high-basicity PACls, PACl-2.2 and PACl-2.5, could effectively remove viruses not only under neutral pH conditions but also under weakly alkaline conditions.
2. Among the various aluminum-based coagulants used in the present study, PACl-2.5, which has a high Al_c content (including Al_{13} species and possibly Al_{30} species) and a high colloid charge density, removed viruses more efficiently (>8-log for infectious viruses; >6-log for total viruses) than the other aluminum-based coagulants in the pH range 6.8 to 7.8.
3. The in-line coagulation–ceramic MF process with PACl-2.5 removed not only viruses but also DOC and

UV260-absorbing NOM more efficiently and resulted in a lower residual aluminum concentration than did commercially available PACls, especially under weakly alkaline conditions.

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Original Article

Repeated dose and reproductive/developmental toxicity of perfluoroundecanoic acid in rats

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ABSTRACT — Perfluoroalkyl acids (PFAAs) are environmental contaminants that have received attention because of their possible effects on wildlife and human health. In order to obtain initial risk information on the toxicity of perfluoroundecanoic acid (PFUA), we conducted a combined repeated dose toxicity study with the reproduction/developmental toxicity screening test (OECD test guideline 422). PFUA was administered by gavage to rats at 0 (vehicle: corn oil), 0.1, 0.3 or 1.0 mg/kg/day. At 1.0 mg/kg/day, body weight gain was inhibited in both sexes, and there was a decrease in fibrinogen in both sexes and shortening of the activated partial thromboplastin time in males. An increase in blood urea nitrogen and a decrease in total protein in both sexes and increases in alkaline phosphatase and alanine transaminase and a decrease in albumin in males were observed at 1.0 mg/kg/day. Liver weight was increased in males at 0.3 mg/kg/day and above and in females at 1.0 mg/kg/day, and this change was observed after a recovery period. In both sexes, centrilobular hypertrophy of hepatocytes was observed at 0.3 mg/kg/day and above and focal necrosis was observed at 1.0 mg/kg/day. In reproductive/developmental toxicity, body weight of pups at birth was lowered and body weight gain at 4 days after birth was inhibited at 1.0 mg/kg/day, while no dose-related changes were found in the other parameters. Based on these findings, the no observed adverse effect levels (NOAELs) for the repeated dose and reproductive/developmental toxicity were considered to be 0.1 mg/kg/day and 0.3 mg/kg/day, respectively.

Key words: Perfluoroundecanoic acid, Repeated dose toxicity, Reproductive and developmental toxicity, Screening test, Rat

INTRODUCTION

Perfluoroalkyl acids (PFAAs) are environmental contaminants that have received attention because of their possible effects on wildlife and human health in recent years; PFAAs are very stable in the environment, have bioaccumulation potential, and have been detected in environmental media and biota in many parts of the world, including oceans and the Arctic; and many researchers have revealed their toxic effects, including hepatotoxicity and reproductive/developmental toxicity in laboratory animals, as reviewed by ATSDR (2009) and Hirata-Koizumi *et al.* (2012). In particular, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are the most effective surfactants among PFAAs (Lau *et al.*, 2007), and many toxicological effects of PFOS and PFOA have been revealed (reviewed in ATSDR, 2009, and fully introduced in Hirata-Koizumi *et al.*, 2012). PFOS and PFOA have now been regulated worldwide, and the manufacture, import and use of PFOS were essentially prohibited in the EU in 2008 (DIRECTIVE 2006/122/EC) and in Japan in 2010 (Japanese law, 2009). As with PFOS, there is growing momentum to strengthen the regulation of PFOA.

Perfluoroundecanoic acid (PFUA, C11) is one of the higher homologue chemicals of PFOA, and PFUA is used as an alternative to PFOA, which is used as a processing aid in the manufacture of fluoropolymers (EPA, 2013a). Although the annual production and import volume of PFUA was not available, that of perfluoroalkyl carboxylic acids (PFCAs, C2-C10) in Japan was reported to be 1,000

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to 10,000 tons in 2007 and less than 1,000 tons in 2010 (CHRIP, 2013). The production and import volume of PFUA is considered to have fallen in recent years globally (EPA, 2013b). However, it is necessary to be concerned about the toxicological potential of PFUA even though its production and import volume has been reduced, due to its very persistent and highly bioaccumulative characteristics (ECHA, 2012). Moreover, long-chain (C9-C20) PFCAs can be detected in the environment as degradates from commercial fluorotelomers (Environment Canada, 2010). In humans, total exposure to PFUA is not available, but the mean concentration of PFUA in human serum collected in the U.S. was < 1 ng/ml (Calafat *et al.*, 2006, 2007a and 2007b; Kuklenyik *et al.*, 2004), and the maximum concentration in breast milk was 0.056 ng/ml (So *et al.*, 2006), as summarized by ATSDR (2009). In Sweden, estimated dietary exposure to PFUA increased (88, 158 and 212 pg/kg/day in 1999, 2005 and 2010, respectively) along with an increase in the quantified concentration of PFUA in fish products (Vestergren *et al.*, 2012). Domingo *et al.* (2012) summarized that the major dietary source of the estimated intake of PFUA was fish and shellfish.

In order to obtain initial risk information on the toxicity of PFCAs, which have a longer chain than PFOA (C8), we have carried out a series of screening tests on the toxicity of PFCAs (C11-C18), and the result for perfluorooctadecanoic acid (PFOdA, C18) has been already published (Hirata-Koizumi *et al.*, 2012). Here, we show initial risk information on the repeated dose and reproductive/developmental toxicity of PFUA (C11).

MATERIALS AND METHODS

This study was performed in compliance with OECD guideline 422 "Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test," and in accordance with the principles for Good Laboratory Practice (MOE *et al.*, 2003, 2008) at the BOZO Research Center (Shizuoka, Japan). The experiment was performed in accordance with the Japanese regulations on animal welfare (Japanese law, 2005).

Animals and housing conditions

Crl:CD(SD) rats (8 weeks old) were purchased from Atsugi Breeding Center (Charles River Laboratories Japan, Inc., Kanagawa, Japan). This strain was chosen because it is most commonly used in toxicity studies, including reproductive and developmental toxicity studies, and historical control data are available. The animals were acclimatized to the laboratory for 15 days and subjected to treatment at 10 weeks of age. They were carefully observed during the acclimation period, and male and female rats found to be in good health were selected for use. In addition, vaginal smears of each female were recorded, and only females showing a normal estrous cycle were used in the experiment. One day before the initial treatment, the rats were distributed into four main groups of 12 males and 12 females, and two additional satellite groups (control and highest dose groups) of five females, each by stratified random sampling based on body weight. For males, 5/12 animals each in the main groups of control and highest dose were used as the satellite groups.

Throughout the study, animals were maintained in an air-conditioned room set at 20-27°C, with relative humidity set at 31-69%, a 12-hr light/dark cycle, and ventilation with > 10 air changes/hr. A basal diet (NMF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were provided *ad libitum*. The rats were housed individually, except for mating and nursing periods. From day 17 of pregnancy to the day of sacrifice, individual dams and/or litters were reared using wood chips as bedding (White Flake; Charles River Laboratories Japan, Inc.).

Chemicals and dosing

PFUA (CAS RN: 2058-94-8) was obtained from Wako Chemical, Ltd. (Miyazaki, Japan), stored in a light-blocking bottle and kept at room temperature. The PFUA (Lot no. TSM0481) used in this study was 98.5% pure, and stability during the study was verified by gas chromatography. The test article was suspended in corn oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and administered to the animals by gastric intubation. Control rats received the vehicle alone. Dosing solutions were prepared at least once per eight days, stored under refrigeration until dosing, and dosed at room temperature, as stability under these conditions has been confirmed. The concentrations of PFUA in the formulations were within the acceptable range (97.0-101.8%).

The dose levels were chosen based on the results of a 14-day dose range-finding study conducted at levels of 2, 6, 20, 60, 200, and 600 mg/kg/day. In this range-finding study, deaths were observed in 5/5 males and 4/5 females at 20 mg/kg/day, and in all animals at 60 mg/kg/day or more, and an increase in liver weight in both sexes and increases in ALP and BUN in males were observed at 2 and 6 mg/kg/day. PFAAs including PFUA are persistent and bioaccumulative (ATSDR, 2009). Taking into account that the length of the dosing period in the present study was about three times than that in the dose range-finding study, the highest dose in the present study was set at 1.0 mg/kg/day. Finally, the dose levels of PFUA in

the present study were set as 0.1, 0.3 or 1.0 mg/kg/day.

Twelve males per group were dosed for 42 days, beginning 14 days before mating. After the administration period, 5 of 12 males per group were reared for the recovery period of 14 days without administration of PFUA, as satellite groups. The main group females were dosed for 41-46 days, beginning 14 days before mating to day 4 of lactation throughout the mating and gestation period. Females in the satellite group were given PFUA for 42 days, followed by the recovery period of 14 days. The first day of dosing was designated as day 0 of administration and the day after the final dose was designated as day 0 of the recovery period. The volume of each dose was adjusted to 5 ml/kg body weight based on the latest body weight.

Observations

All rats were observed daily for clinical signs of toxicity. Body weight was recorded twice a week in all males and in the satellite group females, and twice a week during the premating period, on days 0, 4, 7, 11, 14, 17, and 20 of pregnancy and on days 0 and 4 of lactation in main group females. Food consumption was recorded twice a week in all males and in satellite group females, and twice a week during the premating period, on days 1, 4, 7, 11, 14, 17, and 20 of pregnancy and on days 2 and 4 of lactation in main group females. Functional observation battery (FOB) in all animals was recorded once a week during the administration period, as follows: (i) home cage observation; posture, convulsion, and abnormal behavior, (ii) in-the-hand observation; ease of removal from cage and handling, fur and skin condition, eye ball, secretion from nose and/or eye, visible mucous membrane, lacrimation, salivation, piloerection, pupil diameter, and respiration, and (iii) open field observation; arousal, ambulation, posture, shivering, convulsion, rearing frequency, excreta, stereotypical behavior, and abnormal behavior.

Five animals in each group were subjected to the following observations and examinations unless noted otherwise. Sensory reactions for pupillary reflex, approximation reflex, tactile reflex, auditory reflex, pain reflex, righting reflex and width of the landing legs, grip strength of fore and hind limbs, and spontaneous motor activity were tested in main group males on day 37 of administration, in main group females on day 4 of lactation, and in satellite group males and females on day 37 of administration and on day 8 of the recovery period. Fresh urine was sampled from animals using a urine-collecting cage during the last weeks of the dosing and recovery periods. The 4-hr urine samples were collected soon after dosing under fasting (water was allowed *ad libitum*), and the 20-hr urine samples were collected, food and water being allowed *ad libitum*.

After 16-20 hr (overnight) of fasting, the main group of rats was euthanized by exsanguination under anesthesia on the day after the final administration in males and on day 4 of lactation in females, and satellite group rats were euthanized on the day of the completion of the recovery period. The external surfaces of the rats were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. Blood samples were drawn from the abdominal aorta. Major organs were removed from all animals, and the brain, thyroid, thymus, heart, liver, spleen, kidney, adrenal glands, testis, epididymis were weighed. The numbers of corpora lutea and implantation sites were counted in all main group females. The testes and epididymides were fixed with Bouin's solution and in 10% phosphate-buffered formalin. Other organs were stored in 10% phosphate-buffered formalin. The cerebrum and cerebellum, pituitary gland, spinal cord, sciatic nerve, thyroid, parathyroid, adrenal glands, thymus, spleen, mandibular lymph nodes, mesenteric lymph node, heart, lung, trachea, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, kidney, bladder, testis, epididymis, uterus, seminal vesicle, sternum, and femur were histopathologically evaluated for five males and females in the control and the highest groups, and organs with macroscopically abnormal findings were also examined histopathologically. The organs for histopathological evaluations were processed routinely for embedding in paraffin, and sections were prepared for staining with hematoxylin–eosin. Test substance-related histopathological changes were found in the liver in males and females, and in the stomach in males; therefore, the liver in all animals and the stomach in all males were also examined histopathologically.

The 4-hr urine samples were tested for color, pH, protein, glucose, ketone body, bilirubin, occult blood, urobilinogen, and urinary sediment. Urinary sediment was stained and examined microscopically. The 20-hr urine samples were tested for osmotic pressure. Urine volume for 4-hr and 20-hr was measured. In the collected blood samples the red blood cell (RBC) count, hemoglobin, platelet count, and white blood cell count were measured. In addition, mean corpuscular volume (MCV), hematocrit, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte rate, and differential leukocyte rates were calculated. Prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen were determined. Blood chemistry was tested for alkaline phosphatase (ALP), total protein, albumin, albumin/globulin (A/G) ratio, total bilirubin, blood

urea nitrogen (BUN), creatinine, glucose, total cholesterol, triglycerides, phospholipid, Na, K, Cl, Ca, inorganic phosphate, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactase dehydrogenase (LDH), and gamma-glutamyltransferase (γ-GTP).

In the main group, daily vaginal lavage samples of each female were evaluated for estrous cyclicity throughout the premating period. Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred or the 2-week mating period had elapsed. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of sperm in the vaginal smear and/or a vaginal plug was considered as evidence of successful mating. Once insemination was confirmed, the females were checked twice a day for signs of parturition from day 21 to day 24 of pregnancy. One female in the 0.1 mg/kg/day treatment group did not deliver and did not have implantation. Because of infertility, data for that female for the period corresponding to gestation were excluded from statistical analysis. Other females were allowed to deliver spontaneously and nurse their pups until postnatal day (PND) 4. The day on which parturition was completed by 17:00 was designated as PND 0. Litter size and numbers of live and dead pups were recorded, and live pups were sexed and individually weighed on PNDs 0 and 4. Pups were inspected for external malformations on PND 0. On PND 4, the pups were euthanized by exsanguination under anesthesia, and gross internal examinations were performed.

Data analysis

Statistical analysis of pups was carried out using the litter as the experimental unit. Mean and standard deviation in each dose group were calculated for body weight, food consumption, water consumption, number of feces, rearing frequency, width of the landing legs, grip strength, spontaneous motor activity, urine volume, hematological test results, blood biochemical test results, absolute and relative organ weights, estrous cycle length, length of gestation, numbers of corpora lutea and implantations, implantation index, total number of pups born, number of male and female pups, number of live and dead pups, live birth index, live pups and viability index on day 4 of lactation, and body weight of pups. These were analyzed with Bartlett's test or F-test for homogeneity of variance. If they were homogeneous, the data were analyzed using Dunnett's test or Student's t-test to compare the mean of the control group with that of each dosage group, and if they were not homogeneous, a Dunnett-type rank test or Aspin-Welch t-test was applied. The copulation index, fertility index, gestation index, sex ratio of pups, and data for sensory reactions of reflexes were analyzed with Yates' chi-square test. The 5% levels of probability were used as the criterion for significance. Unless otherwise noted, there are statistically significant differences in the changes described in the following Results section.

RESULTS

Parental toxicity

No deaths were observed in any of the groups. A decrease in grip strength of the forefoot was observed in males and females at 1.0 mg/kg/day in the recovery period. No other treatment-related effects on clinical signs of toxicity, FOB, sensory reactivity, or spontaneous motor activity were observed in males and females in the main and satellite groups (data not shown).

Body weight changes in each group are shown in Figs. 1 and 2. In males at 1.0 mg/kg/day, body weight gains decreased during the dosing period and during the recovery period. In females at 1.0 mg/kg/day, body weight gains decreased during the lactation period in the main group and during the dosing period and the recovery period in the satellite group, and lowered body weight was observed on days 38 and 41 of the dosing period and on days 0-13 of the recovery period in the satellite group. No effects on body weight in male and female groups were observed at any other dosing. Food consumption (data not shown) was decreased on day 4 of the delivery period at 1.0 mg/kg/day in females. Urinalysis revealed no significant differences in any parameters between the control and treatment groups in males and females in the main and satellite groups (data not shown).

Table 1 shows hematological findings in male and female rats. At 1.0 mg/kg/day, low values of fibrinogen and APTT were observed in males of the main and satellite groups, and a low value of fibrinogen was observed in females of the main group. The other significant changes in hematological findings were incidental because they were slight without related changes or did not occur in a dose-dependent manner.

Blood biochemical findings are shown in Table 2. At 1.0 mg/kg/day in the main group, increases in BUN and ALP and decreases in total protein and albumin were observed in males, and an increase in BUN and a decrease in total protein were observed in females. At 1.0 mg/kg/day in the satellite group, increases in BUN and ALP in males and females, and a decrease in total protein in females were observed. The other changes with statistical significances in blood biochemical findings were incidental because they were slight without related changes or did not occur in a dose-dependent manner.