chromatography (Nowack *et al.*, 1996; Kemmei *et al.*, 2009), ion chromatography/mass spectrometry (Bauer *et al.*, 1999; Knepper *et al.*, 2005), gas chromatography/mass spetrometry (Nishikawa and Okumura, 1995), and liquid chromatography/tandem spetrometry (Quintana and Reemtsma, 2007). To simplify Japan's recommended method of EDTA determination by using gas chromatography-mass spectrometry, we focused on the pretreatment of EDTA by using solid-phase extraction in the present study. In comparison with evaporation using rotary evaporator, solid-phase extraction is simple, rapid, and efficient.

MATERIALS AND METHODS

Chemicals

Ethylenediaminetetraacetic acid, disodium salt, dehydrate and trans-1, 2-cyclohexanediaminetetraacetic acid monohydrate (CyDTA) were obtained from Dojindo (Kumamoto, Japan) and Strem Chemicals, Inc. (Newburyport, MA, USA), respectively. Boron trifluoride methanol complex methanol solution, formic acid, potassium dihydrogenphosphate, sodium sulfate, sodium hydroxide, L(+)-ascorbic acid, methanol and dichloromethane were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All chemicals and solutions were of analytical grade. Milli-Q water was used in all experiments.

Gas chromatography/mass spectrometry analysis

Gas chromatography was carried out by using HP6890 series gas chromatography system (Hewlett Packard, Wilmington, DE, USA) with an HP6890 series auto sampler and split/splitless injector. The analytical column was a DB-5 fused-silica capillary column, 30 m × 0.25 mm i.d., 0.25 μm film thickness (J & W Scientific, Folsom, CA, USA). The temperature program for the column oven was 70°C as initial temperature for 2 min; ramped at 15°C/min to 300°C then held at 300°C for 3 min. The carrier gas (helium) flow rate was set at 1.2 mL/min.

Mass spectrometry was carried out using a 5973 Mass Selective Detector (Hewlett Packard, Wilmington, DE, USA) in electron-ionization mode with an ionization voltage of 70 eV and ion source temperature of 230°C. The instrument was operated in selected-ion monitoring (SIM) mode. The monitor ion of EDTA and CyDTA were *m/z* of 174 (for identification : *m/z* = 289, 348) and 402, respectively. CyDTA was used as an internal standard.

Sample collection

River water samples used in this study were collected from six rivers located in the Shikoku region (Kochi and Tokushima prefectures), Kansai region (Hyogo and Kyoto prefectures), and Kanto region (Kanagawa Prefecture and Tokyo Metropolis) (Fig. 1). River water sampling was conducted in January (for Shikoku region and Hyogo Prefecture) and April (Kyoto Prefecture and Kanto region) 2010. River water samples were collected in 300 mL glass bottles and stored in the dark at 4°C until analysis.

Standard and sample preparation

A standard stock solution of EDTA was prepared by dissolving 0.127 g of ethylenediaminetetraacetic acid, disodium salt, dehydrate in 1 L Milli-Q water. On the

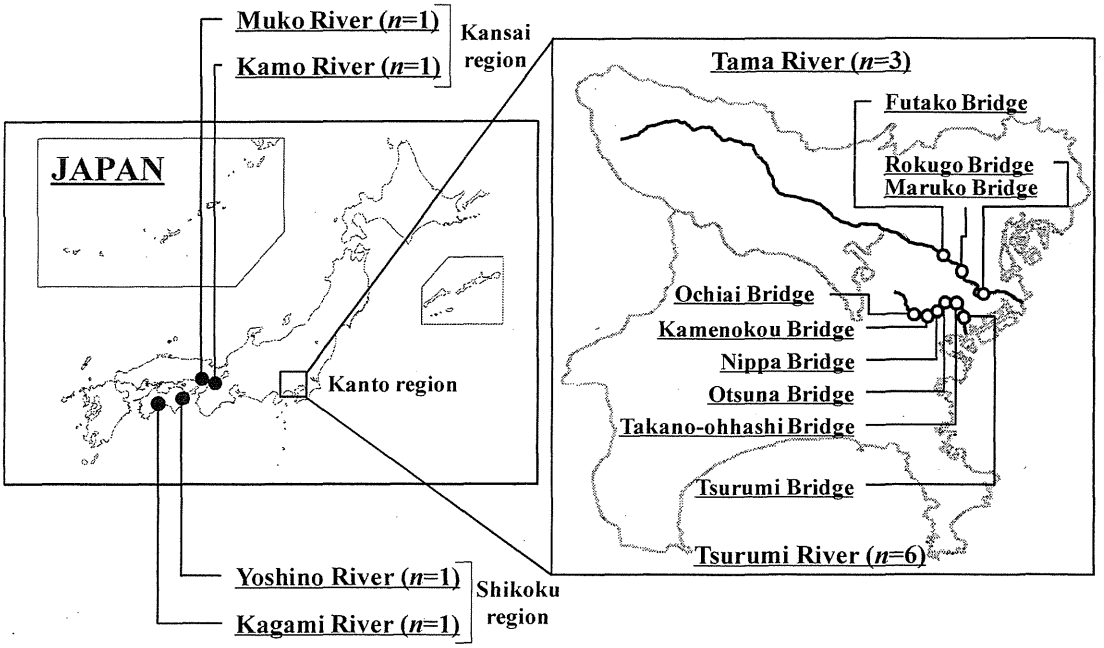

Fig. 1 - River water sampling locations

other hand, a standard stock solution of CyDTA was prepared by dissolving 0.01 g of trans-1, 2-cyclohexanediaminetetraacetic in 100 mL of 1 M sodium hydroxide.

Extraction of EDTA from water samples was performed according to the method of Knepper *et al.* (2005) with modifications. Water samples (100 mL each) were adjusted to pH 3 by 16 M formic acid and filtered with glass fiber filter (0.7 μm, Millipore, Billerica, MA, USA). Bond Elut Jr. –SAX (500 mg, Varian, Inc., Palo Alto, CA, USA) cartridges were conditioned with 3 mL methanol and 3 mL of Milli-Q water, respectively. Extraction of water samples was carried out at a flow rate of 10mL/min. After extraction, cartridges were rinsed with 3mL of Milli-Q water and eluted into stoppered glass test tubes with 3 mL of 16 M formic acid. The eluates combined with internal standard (CyDTA) were concentrated in a nitrogen stream at 80°C to complete dryness. After exsiccation, 1 mL of boron trifluoride-methanol-complex solution was added to the glass test tubes. Derivatization was carried out by 1 h heat treatment at 80°C using a water bath. After derivatization, 3 mL of 1 M phosphate buffer (pH 7) and 1mL of dichloromethane were added to the test tubes and rigorously shaken. Thereafter, test tubes were centrifuged for 5 min at 900 *g*. After centrifugation, the layer of dichloromethane was collected in a glass tube and dehydrated by sodium sulfate. The dehydrated dichloromethane solution was used for analysis. The limit of quantification (LOQ) was determined by analyzing the lowest level standard at least 5 times. The LOQ was calculated as 10-fold the standard deviation of these determinations. The LOQ value was 0.1 μg/L in sample water.

RESULTS AND DISCUSSION

Recovery experiments

To evaluate the efficiency, solid-phase extraction was performed as described below.

The EDTA standard solution was added to 100 mL Milli-Q water and tap water at concentrations of 0.1 mg/L (1/5 of EDTA standard value in drinking water) and 0.01 mg/L (1/50 of EDTA standard value in drinking water) and subsequent extraction and derivatization were carried out as described previously. Tap water samples were dechlorinated by L(+)-ascorbic acid before use. Control samples for recovery test by using Milli-Q water were prepared by adding the same amount of EDTA standard solutions to 3 mL Milli-Q water. In the case of using tap water for the recovery test, control samples were prepared by adding the same amount of EDTA standard solution to the eluate from tap water extraction. All controls, samples, and blanks were determined in triplicate. Recovery percentages of EDTA from Milli-Q and tap water samples are shown in Table 1. In the case of Milli-Q water samples, excellent recovery percentages at each concentration were obtained, and the values were 100.5% (concentration: 0.01 mg/L) and 100.2% (concentration: 0.1 mg/L). Moreover, the relative standard deviation (RSD) of the ratio of EDTA to CyDTA in each sample was within 5% (ranging from 0.3 to 4.2%) and the variability among samples was small. No influence of matrix from the SPE cartridge was observed in blank samples.

In order to apply the proposed method to actual tap water samples, a recovery test was performed using tap water (Table 1). Satisfactory results were obtained from recovery tests using tap water samples as well as those of Milli-Q water. Variability between samples was small and the pretreatment process of our method was simple and took a relatively short time compared with the existing Japanese Standard Methods for the Examination of Water. We therefore suggest that the SPE-derivatization-GC/MS method should be considered for the Japanese Standard Methods for the Examination of Water as an EDTA analytical method. The next stage to add this new SPE-derivatization-GC/MS method to the Japanese Standard Methods for the Examintion of Water would be to perform an inter-laboratory validation study of the proposed method.

Determination of EDTA in river water samples

For the application of this analytical method to environmental water samples, concentrations of EDTA in river water samples from urban and rural areas of Japan were investigated using SPE-derivatization-GC/MS. River water samples were taken from three regions of Japan (Fig 1). As shown in Fig 2, EDTA was detected in ten of thirteen river water samples. Although EDTA was not detected from river water samples of Kagami, Yoshino, and Kamo rivers (concentration: < 0.1 μg/L), EDTA was detected at comparatively high concentration in other river water samples. The median concentration of EDTA in river water samples was 115 μg/L and the concentrations detected ranged from 18.8 to 443 μg/L.

In the Kanto region, the highest concentration of EDTA (443 μg/L) was observed in a

Table 1 – Recovery of EDTA from Milli-Q water and tap water samples

Analyte	Vehicle	Concentration (mg/L)	Recovery of triplicate samples (%)				
			A	B	C	Mean	SD
EDTA	Milli-Q water	0.01	100.1	100.9	100.6	100.5	0.4
	Milli-Q water	0.1	100.0	99.8	100.8	100.2	0.5
	Tap water	0.01	102.0	98.7	99.8	100.1	1.7
	Tap water	0.1	98.3	98.9	97.2	98.1	0.9

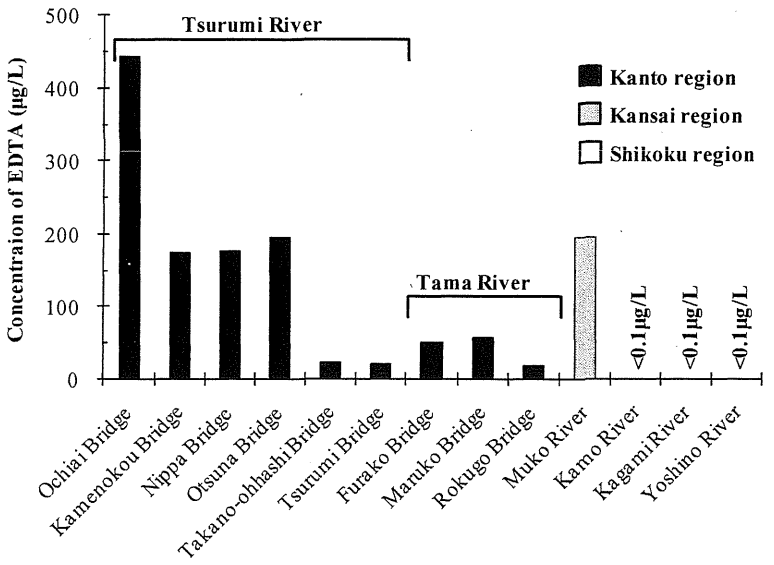

Fig.2 - Concentration of EDTA in river water samples from three regions of Japan

river water sample collected at Ochiai Bridge of Tsurumi River. It is generally considered that effluent from sewage treatment plants (STP) is one of the major sources of EDTA (Kari and Giger, 1996). This sampling site is located at about 150 m downstream from the effluent output of an STP and the contribution of effluent to the EDTA concentration is high. In Tsurumi River, there are three STPs near the sampling sites. Two STPs are located upstream of Ochiai Bridge and between Kamenokou Bridge and Nippa Bridge, respectively. Moreover an STP is located at Yagami River (tributary of Tsurumi River) between Otsuna Bridge and Takano-ohhashi Bridge. The river water sample taken from Nippa Bridge contained high levels of EDTA. The EDTA concentration of the river water sample taken from Takano-ohhashi bridge was lower than those of other river water samples collected downstream of STPs. Because the STP is located on a tributary of Tsurumi River, the EDTA concentration might be affected by dilution with the influent of Yagami River at this sampling site. The low EDTA concentration in the river water sample from Tsurumi Bridge (downstream of Tsurumi River) might also be caused by dilution. EDTA concentrations of Tama River samples ranged from 18.8 to 56.8 μg/L and the values were comparatively lower than those from Tsurumi River. These sampling sites were located at lower-middle and lower portions of Tama River while the STPs are located at the upper-middle portion of Tama River. Therefore, it was concluded that concentrations of EDTA decreased going downstream due to dilution. In Kansai region, EDTA was only detected in the river water sample of Muko River and the value (196 μg/L) was comparable to that of Tsurumi River. There is an STP in the upper part of Muko River and EDTA may originate from that source. However, because there is no STP upstream of the sampling site of Kamo River, EDTA was not detected in Kamo River sample. On the other hand, in Shikoku region, EDTA was not observed in the river water samples. The concentration of EDTA might be low due to these samples being collected in estuarine regions. In further studies, it will be necessary to survey the differences in EDTA contamination levels between urban and rural areas.

The results of the present study were compared to those of the previous studies. It was estimated that EDTA concentrations of 50-500 μg/L are present in wastewaters (WHO, 1998). According to Quintana and Reemtsma (2007), mean EDTA concentrations in various water samples from Germany, such as STP influent and effluent, tap water, and surface water, ranged from 1.1 (surface water) to 35 (STP effluent) μg/L. Knepper *et al.* (2005) reported EDTA concentrations ranging from 4 to 970 μg/L in wastewaters and concentrations ranging from 1 to 33 μg/L in surface water of European countries. Our results are comparable to those of wastewater in previous studies. These results suggested that Kanto region rivers, especially Tsurumi River, might be highly affected by STP effluents. It is necessary to further evaluate the contribution of STP effluents to EDTA levels in rivers.

CONCLUSIONS

To improve Japan's recommended method of EDTA determination to a simplified method by using gas chromatography/mass spectrometry, a new solid-phase extraction method of EDTA was developed. Satisfactory results were obtained in recovery tests using both tap water and Milli-Q water samples. Because the evaporation procedure was changed to solid-phase extraction, the analytical method used in this study is simple and rapid compared with Japan's existing standard method and is reasonable to recommend as an EDTA analytical method to the Japanese Standard Methods for the Examination of Water. Occurrence of EDTA in river water samples from three regions of Japan was examined. EDTA was detected in ten of thirteen river water samples. A high level of EDTA was observed in river water samples with significant STP effluents. Further evaluation of the contribution of STP effluents to EDTA levels in rivers is needed.

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資料

2009年の水道法に基づく水質基準改正について

西村哲治
Tetsuji NISHIMURA
国立医薬品食品衛生研究所環境衛生化学部部長

水道法に基づく水質基準の改正については，2003年の改正時から，これまでの数年おきにまとめて改正を行う大改正方式に換え，該当する項目について逐次検討を実施し，必要に応じて毎年改正を行う逐次改正方式になった．また，飲料水が担保すべき項目は，水道法に基づく水質基準項目(2009年改正後，50項目)のほか，水質管理設定項目(2009年改正後，28項目)，要検討項目(2009年改正後，44項目)の3階層に区分されて，設定されている(図1)．2009年の改正においては，基準項目の「有機物(全有機炭素(TOC)の量)」の基準値の変更，「1,1-ジクロロエチレン」の廃止，「シス-1,2-ジクロロエチレン及びトランス-1,2-ジクロロエチレン」の対象項目の変更が示され，その他，水質管理目標設定項目として「アルミニウム及びその化合物」及び「1,1-ジクロロエチレン」の追加，「ジクロロアセトトリル」，「抱水クロラール」，及び農薬類の「EPN」「クロロピリホス」の目標値の変更，要検討項目として「過塩素酸」，「パーフルオロオクタンスルホン酸(PFOS)」「パーフルオロオクタン酸(PFOA)」及び「*N*-ニトロソジメチルアミン(NDMA)」の項目が追加された(表1)．

改正内容の背景や根拠等についての概略

1　基準項目

1.「有機物(全有機炭素(TOC)の量)」の基準値の変更　厚生科学審議会答申(厚科審第5号：平成15年4月28日付け)において，これまで有機物指標としての項目に対する評価値の算出方法としていた過マンガン酸カリウム消費量が，全有機炭素(TOC)の量に変更された．これは，従来の有機物指標である過マンガン酸カリウム消費量が，水中の有機物を定量的に酸化しておらず，水中に存在する有機炭素化合物の被酸化性によって大きく変動することが変更の理由であった．これまでの検査結果との継続性を維持することから，有機物指標の評価値の算出方法として，TOC，溶解性有機炭素量，紫外線吸光度(E 260)，濁度，色度，トリハロメタン生成能及び全有機ハロゲン化合物量などの項目について，全国の代表的な水道原水や浄水に関してそれぞれの項目の間と過マンガン酸カリウム消費量との間における相関性を調査した結果，水中の有機物の指標としては，TOCが最も有効であり，精度，感度のいずれにおいても優れていた．過マンガン酸カリウム消費量とTOCとの相関性は，水域や水系が異なった場合，相関性が良好でなかった．しかし，過マンガン酸カリウム消費量とTOCとの相関性は，同一水源では良好な相関関係が認められた．以上の検討内容から，過マンガン酸カリウム消費量に換え，TOCを有機物指標としての項目に対する評価値の算出方法として採用された．

水質基準の改定にあわせて実施した調査及び既往の文献における過マンガン酸カリウム消費量とTOCとの回帰式の調査から，TOCは過マンガン酸カリウム消費量の約1/3程度の3～4 mg/Lであった．環境水におけるCODMnからTOCの概略を換算すると約2 mg/Lであった．また，薬局方における過マンガン酸カリウム消費量10 mg/LはTOCとして1.58 mg/L，快適水質項目3 mg/Lは0.474 mg/Lであった．以上の試算の値から，これまでの基準値であった過マンガン酸カリウム消費量10 mg/Lに相当する値は，全有機炭素量の評価値として1～4 mg/Lと算出された．長期間運用されていた過マンガン酸カリウム消費量を新しい評価方法で

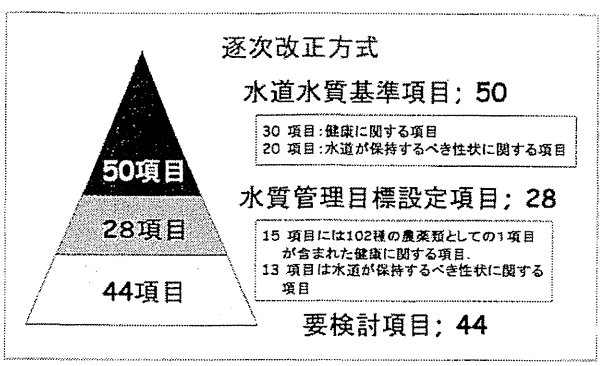

図1　飲料水の水質基準の枠組み

表1　改正内容のまとめ

区分	項目	内容	変更後	変更前
基準項目	有機物(全有機炭素(TOC)の量)	基準値の変更	3 mg/L 以下であること	5 mg/L 以下であること
	1,1-ジクロロエチレン	廃止	水質管理目標設定項目に設定	0.02 mg/L 以下であること
	シス-1,2-ジクロロエチレン及び トランス-1,2-ジクロロエチレン	対象物質の変更	シス-1,2-ジクロロエチレン及び トランス-1,2-ジクロロエチレンの合計	シス-1,2-ジクロロエチレンのみが対象
水質管理目標設定項目			アルミニウムの量に関して	
	アルミニウム及びその化合物	追加	0.1 mg/L 以下	
	1,1-ジクロロエチレン	追加	0.1 mg/L 以下	
	ジクロロアセトニトリル	目標値の変更	0.01 mg/L 以下	0.04 mg/L(暫定)
	抱水クロラール	目標値の変更	0.02 mg/L 以下	0.03 mg/L(暫定)
	EPN	目標値の変更	0.004 mg/L	0.006 mg/L
	クロロピリホス	目標値の変更	0.003 mg/L	0.03 mg/L
要検討項目	過塩素酸	追加		
	パーフルオロオクタンスルホン酸 (PFOS)	追加		
	パーフルオロオクタン酸(PFOA)	追加		
	N-ニトロソジメチルアミン(NDMA)	追加		

あるTOCによる評価値に変更をするために，設定時の全有機炭素に関するデータの集積状況を考慮して，危険率を見込んでおく必要から，上限値である4 mg/Lに危険率25%を見込んで，当面の評価値は5 mg/Lが適当であるとして設定された．快適水質項目としての過マンガン酸カリウム消費量3 mg/Lは，全有機炭素量2 mg/Lに相当すると算出された．また，従前の過マンガン酸カリウム消費量については，移行措置として，水質管理目標設定項目に当面の間10 mg/L，目標値3 mg/Lとして継続すべきであるとして残された．

設定時に，以上の評価値は当面の間のもので，データの集積状況に応じ適宜改定されるものであるとされていた．TOCが有機物指標としての項目に対する評価値に変更された後，導入された装置と適用されている測定条件におけるTOCの定量下限値を調査した結果，0.05 mg/L前後であった．さらに，超純水製造装置の保守管理に注意し，容器の汚染などがないように十分留意することにより，ブランク水を一定の低濃度のTOCに保つことができることが明らかとなった．また，主要な水道事業者における浄水のTOC濃度の中央値は0.5～0.6 mg/Lであった．浄水のTOC濃度と過マンガン酸カリウム消費量の関係については，TOC濃度が低いこともあり相関性は低い結果であったが，原水と浄水を含めた場合は高い相関性が認められることが確認された．TOCが設定された以降で集積されたデータによると，過マンガン酸カリウム消費量10 mg/Lに相当するTOCは3～4 mg/Lであった．原水のTOC濃度と総トリハロメタン生成能の関係は，高い相関がみられ，TOC濃度と総トリハロメタン濃度との関係式から，総トリハロメタンの基準値0.1 mg/Lに相当するTOC濃度は1.5～2.72 mg/Lであった．すなわち，トリハロメタン対策の観点からTOCを少なくとも3 mg/L程度以下にすることが必要であることが明らかとなった．さらに，TOC濃度とハロ酢酸類の濃度との間にも相関性が認められ，TOC濃度が消毒副生成物の生成濃度を予測できる指標となる可能性が示唆された．

以上の情報から，TOCについては現行基準を強化して，「5 mg/L以下」から「3 mg/L以下」にすることが適切であるとされた．平成20年12月22日公布の「水質基準に関する省令の一部を改正する省令」(平成20年度厚生労働省令第174号)で「水質基準に関する省令」が一部改正され，「有機物(全有機炭素(TOC)の量)」に係る水質基準を「5 mg/L

以下」から「3 mg/L 以下」に強化された．

また，水質基準の見直しに伴い，「水道施設の技術的基準を定める省令」に基づく薬品基準及び資機材材質基準並びに「給水施設の構造及び材質の基準に関する省令」に基づく給水装置浸出性能基準について，平成21年3月6日公布の「水道施設の技術的基準を定める省令の一部を改正する省令」(平成21年度厚生労働省令第26号)と「給水施設の構造及び材質の基準に関する省令の一部を改正する省令」(平成21年度厚生労働省令第27号)で改正された．このうち，TOCに係る資機材材質基準及び水栓その他給水装置の末端に設置されている給水用具の給水装置浸出性能基準に関しては，塗料等からの溶出量を低減させることが技術的に困難なことなどを考慮して，現行基準の0.5 mg/Lを維持することとしている．

2.「1,1-ジクロロエチレン」の廃止　内閣府食品安全委員会から食品健康影響評価(清涼飲料水中の化学物質)により，Quastら[1]によるラットの2年間飲水投与試験における肝小葉中心性の脂肪変性に基づき，$BMDL_{10}$(10%の影響に対するベンチマーク用量の95%信頼下限値)から求められた4.6 mg/kg体重/日に，種差及び個体差を各々10とした不確実係数100を適用して算出された耐容1日摂取量(TDI)46 μg/kg体重/日が示された．「1,1-ジクロロエチレン」に係る水質基準については，寄与率10%とすると基準値が0.1 mg/Lとすることが適切であると算出できる．

しかし，これまでの浄水における検出実態では，近年基準値の10%である0.01 mg/L(現行基準の50%値に相当)を超過する報告がないことから，水質基準を廃止し，新たに水質管理設定項目に設定された．

なお，この判断は，水質基準項目の改廃を行う際には，食品安全基本法に基づき，内閣府食品安全委員会の意見を聴くこととされていることに基づいている．

3.「シス-1,2-ジクロロエチレン」　内閣府食品安全委員会から食品健康影響評価(清涼飲料水中の化学物質)により，Barnesら[2]によるマウスを用いたトランス異性体の90日間の飲水投与試験による血清中ALPの上昇に基づき，NOAELを17 mg/kg体重/日とし，種差，個体差を各々10と短期試験であることによる10から不確実係数1,000を適用して算出されたTDI 17 μg/kg体重/日が示された．水道水の評価においては，この内閣府食品安全委員会による食品健康影響評価(清涼飲料水中の化学物質)でシス体及びトランス体について行った評価を適用して，シス体及びトランス体の和で毒性評価が設定されたことを受けて，「シス-1,2-ジクロロエチレン及びトランス-1,2-ジクロロエチレン」に変更された．基準値はシス-1,2-ジクロロエチレンとトランス-1,2-ジクロロエチレンの和とし，値の変更はなかった．また，この変更に伴い，水質管理目標設定項目から「トランス-1,2-ジクロロエチレン」が削除された．

2　水質管理目標設定項目

1.「アルミニウム及びその化合物」の追加　水道水質管理上留意すべき項目として，水質管理目標設定項目が通知により示されている．2009年4月から「アルミニウム及びその化合物」及び「1,1-ジクロロエチレン」が追加された．

「アルミニウム及びその化合物」については，2004～2006年度に実施された厚生労働科学研究「最新の科学的知見に基づく水質基準の見直し等に関する研究」(主任研究者 眞柄泰基北海道大学大学院教授)の研究結果で，硫酸添加により低pH側へ制御することで，アルミニウム濃度を0.1 mg/L以下とすることが可能であることが幾つかの浄水場で実証された．しかし，同研究において，アンケートによる実態調査を行った結果，浄水中のアルミニウム濃度が0.1 mg/Lを超過もしくは超過のおそれがある浄水場では，基準値が0.1 mg/Lへ引き下げられた場合，低水温，低濁度，高濁度，藻類，高pH等に起因し，対応が困難あるいは難しいとした事業体が80%以上であった．これらの状況から，アルミニウムについては，「0.1 mg/L以下」を水質管理目標値とし，例えば腐食性の指標であるランゲリア指数に留意しつつ，水道事業者において目標値を超過しないような浄水処理の工程管理に努めることが適切であると考えられる．また，アルミニウム低減策

についての技術的な検討や，水道システム全体としての効果とコスト等について，更に知見の収集を図ることが望まれることから，目標値として「0.1 mg/L 以下」として水質管理目標設定項目に追加された．

2.「1, 1-ジクロロエチレン」の追加　「1, 1-ジクロロエチレン」については，前記の判断から水質基準項目が廃止されたことにともない，目標値として「0.1 mg/L 以下」として水質管理目標設定項目に追加された．

3.「ジクロロアセトニトリル」の目標値の変更　内閣府食品安全委員会から食品健康影響評価(清涼飲料水中の化学物質)により，Hayes ら[3]によるラットを用いた 90 日間の経口投与試験における有意な相対肝重量の増加に基づき，LOAEL を 8 mg/kg 体重/日を評価の根拠とし，種差，個体差を各々 10，短期試験であることによる 10 及び NOAEL に近い LOAEL の結果であるとの判断による 3 を適用した不確実係数 3,000 から算出された TDI 2.7 μg/kg 体重/日が示された．目標値は，この TDI に寄与率を 20% として，0.01 mg/L が算出されている．

4.「抱水クロラール」の目標値の変更　内閣府食品安全委員会から食品健康影響評価(清涼飲料水中の化学物質)により，George ら[4]による雄マウスを用いた 2 年間の飲水投与試験における肝腺腫の発生頻度と発生数の増加に基づき，LOAEL を 13.5 mg/kg 体重/日を評価の根拠とし，種差，個体差を各々 10，NOAEL の代わりに LOAEL を用いたことによる 10 及び毒性の重篤性による 3 を適用した不確実係数 3,000 から算出された TDI 4.5 μg/kg 体重/日が示された．目標値は，この TDI に寄与率を 20% として，0.02 mg/L が算出されている．

5.「EPN」及び「クロロピリホス」目標値の変更　「農薬類」の対象リスト中の「EPN」及び「クロロピリホス」については，内閣府食品安全委員会の食品健康影響評価で ADI が変更されたことにより，目標値を改めた．

「EPN」については，ADI が 2.3 μg/kg 体重/日から 1.4 μg/kg 体重/日に変更されたことにより，目標値を現行の「0.006 mg/L」から「0.004 mg/L」に改められた．

「クロロピリホス」については，ラット，マウス及びイヌの各種試験における赤血球コリンエステラーゼ活性の低下に基づいて求められた NOAEL 0.1 mg/kg 体重/日に，種差，個体差を各々 10 とした不確実係数 100 を適用して算出された ADI 1 μg/kg 体重/日が示された．これまでの ADI 10 μg/kg 体重/日から 1 μg/kg 体重/日に毒性評価が変更されたことにより，目標値を現行の「0.03 mg/L」から「0.003 mg/L」に改められた．

「農薬類」の対象リストには含まれていないが，殺菌剤のフルアジナム(第 2 群)についても，ADI が 10 μg/kg 体重/日が設定されたことにより，目標値を「0.03 mg/L」と設定されている．

なお，農薬類については，10% の寄与率を適用している．

3　要検討項目の追加

未規制物質として，「過塩素酸」「パーフルオロオクタンスルホン酸(PFOS)」「パーフルオロオクタン酸(PFOA)」及び「*N*-ニトロソジメチルアミン(NDMA)」を要検討項目に位置づけ，科学的知見の集積を図ることとされた．

本資料は，環境・衛生部会内に設置された関連法規情報委員会(委員長：姫野誠一郎 徳島文理大学薬学部教授)が衛生薬学関連法規の改正等に関する情報を提供するものである．

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水道法水質基準の改正について—塩素酸—

1. はじめに

消毒剤や水の異臭味制御用として使用される二酸化塩素の消毒副生成物として，亜塩素酸イオンとともに生成されることが知られている[1]．二酸化塩素は水中で亜塩素酸，塩素酸，塩化物イオンに分解し，この分解はアルカリ性でより促進する．塩素酸ナトリウムとしては，雑草の除草剤，分析用試薬，酸化剤，パルプ漂白用二酸化塩素の原料などに使用されている[2]．

ヒトに対する影響は，除草剤の使用による塩素酸の中毒事故例として，メトヘモグロビン血症，無尿，腹痛および腎不全の症状が観察されている[3]．

2. 水道法水質基準の改正の経緯

平成16年水質管理目標設定項目等基準化検討調査において，塩素酸の水道水からの検出状況として，最高検出値が水質管理目標値（0.6 mg/L以下）の100%超過地点が6地点/248地点あったが，調査地点の大部分は水質管理目標値の40%以下（220地点/248地点）であった．しかし，調査地点の50%以上は水質管理目標値の10%を超過していた（132地点/248地点）．このように，浄水において，多くの地点でこれまでの評価値の1/10を超えて検出されていることから，平成18年8月4日に厚生科学審議会生活環境水道部会において，食品安全基本法（平成15年法律第48号）第24条第1項第7号の規定に基づき，水道法第4条第2項の規定に基づく水質基準として「塩素酸」を追加することについて，食品安全委員会に意見を求めることとなった．このことを受けて，平成18年8月31日に内閣府食品安全委員会に対して，水道により供給される水の水質基準の設定に係る食品影響評価について意見が求められたところ，平成19年3月15日に，内閣府食品安全委員会から「塩素酸の耐容一日摂取量を30 mg/kg/日と設定する」等の通知があった[4]．この耐容一日摂取量は，ラットを用いた90日間の飲水投与試験[5]における甲状腺のコロイド枯渇の無毒性量を30 mg/kg/日とした判断から，不確実係数1,000を適用して求められている．水質基準値は，耐容一日摂取量（30 mg/kg/日）に占める飲料水の寄与率を80%とし，体重50 kgのヒトが1日2L飲むという仮定から，「0.6 mg/L以下」と算出されている．

水質基準の設定に伴い，水質検査はイオンクロマトグラフ法により，おおむね3か月に1回以上検査を行い，消毒副生成物であることから検査回数を減らすことはできず，かつ省略は不可とされている．二酸化塩素を使用する場合には，これまでどおり，毎日検査を行わなければならない．

3. 諸外国等の水質基準値またはガイドライン値

WHO飲料水水質ガイドライン（2005年第3版）では，ガイドライン値（暫定）として「0.7 mg/L以下」が示されている[1]．このガイドライン値（暫定）は，耐容一日摂取量（30 mg/kg/日）に占める飲料水の寄与率を80%とし，体重60 kgのヒトが1日2L飲むという仮定から算出されている．ヒトへの影響については，酸化力による赤血球への障害を重視している．米国環境保護庁(USEPA)安全飲料水法水質規準では，最大許容濃度(Maximum Contaminant Level)として「0.8 mg/L以下」，目標最大許容濃度(Maximum Contaminant Level Goal)として「1.0 mg/L以下」とされている．

4. 水道水からの検出原因と対策

平成16年水質管理目標設定項目等基準化検討調査において，塩素酸の水道原水からの検出状況として，最高検出値として水質管理目標値の10%超過から20%以下の範囲で1か所あったが，調査地点の大部分（約99%）は水質管理目標値の10%以下（70地点/71地点）であった．これらの結果から，水道水から検出される塩素酸は，水道原水から混入してくる可能性もあるが，浄水工程の間に増加することが示唆される．

次亜塩素酸ナトリウム中の有効塩素濃度の減少と塩素酸濃度の増加との間には負の相関関係があり，塩素酸濃度上昇の主な原因は消毒用次亜塩素酸ナトリウムに含まれる不純物である塩素酸に由来するものが多いと考えられる．消毒用次亜塩素酸ナトリウムを長期間保存すると，酸化により塩素酸が生成し，塩素酸濃度の上昇が起こることがあり，特に高温下での貯蔵は塩素酸の上昇が顕著である．分解が進み，塩素酸濃度が上昇したため有効塩素濃度が低下した次亜塩素酸ナトリウムを消毒用に使用すると，一定の有効塩素濃度を保つために相対的に次亜塩素酸ナトリウムの注入量が増加し，塩素酸濃度を上昇させる結果となる．

次亜塩素酸ナトリウム中の塩素酸濃度の上昇を防止するためには，含有成分が保管温度20℃以下において比較的安定であることから，貯蔵温度を20℃以下に保つことが有効であるといえる．同時に，一度の購入量を少量とし購入頻度を増やすこと，夏季などにおける高温下での貯蔵期間が長期間となることがないように配慮することも有効である．また，貯蔵槽内の汚れも酸化を促進することがあることから，内部を清浄な状態に保つことも有効であるといえる．

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執筆者の PROFILE

西村哲治 (Tetsuji NISHIMURA)
国立医薬品食品衛生研究所　環境衛生化学部　部長
(National Institute of Health Sciences Division of Environmental Chemistry)
東京大学大学院博士課程　昭和 55 年 3 月修了
(専門分野) 環境衛生学

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Estimation of norovirus removal performance in a coagulation–rapid sand filtration process by using recombinant norovirus VLPs

N. Shirasaki, T. Matsushita, Y. Matsui, A. Oshiba, K. Ohno*

Division of Built Environment, Graduate School of Engineering, Hokkaido University, N13W8, Sapporo 060-8628, Japan

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ABSTRACT

Norovirus (NV) is an important human pathogen that causes epidemic acute nonbacterial gastroenteritis worldwide. Because of the lack of a cell culture system or an animal model for this virus, studies of drinking water treatment such as separation and disinfection processes are still hampered. We successfully estimated NV removal performance during a coagulation–rapid sand filtration process by using recombinant NV virus-like particles (rNV-VLPs) morphologically and antigenically similar to native NV. The behaviors of two widely accepted surrogates for pathogenic waterborne viruses, bacteriophages Qβ and MS2, were also investigated for comparison with that of rNV-VLPs. Approximately 3-$\log_{10}$ removals were observed for rNV-VLPs with a dose of 40 μM-Al or -Fe, as polyaluminum chloride at pH 6.8 or ferric chloride at pH 5.8, respectively. Smaller removal ratios were obtained with alum and ferric chloride at pH 6.8. The removal performance for MS2 was somewhat larger than that for rNV-VLPs, meaning that MS2 is not recommended as an appropriate surrogate for native NV. By comparison, the removal performance for Qβ was similar to, or smaller than, that for rNV-VLPs. However, the removal performances for rNV-VLPs and Qβ differed between the coagulation process and the following rapid sand filtration process. Therefore, Qβ also is not recommended as an appropriate surrogate for native NV.

1. Introduction

Norovirus (NV), which has been previously termed Norwalk-like virus or small round structured virus, is an important human pathogen that causes epidemic acute nonbacterial gastroenteritis worldwide. This virus belongs to the genus *Norovirus* in family Caliciviridae (Zheng et al., 2006). On the basis of the molecular characterization of complete gene sequences, the *Norovirus* genus has been classified into seven distinct genogroups (GI to GVII) (Phan et al., 2007). Among the seven genogroups, the GI, GII, GIV, GVI, and GVII strains are found in humans (Phan et al., 2007).

Acute gastroenteritis is one of the leading causes of morbidity and mortality in children in the developing countries, and NV is known to be present in a large fraction of stool samples from diarrhea hospitalizations (Ramani and Kang, 2009). NV outbreaks are caused mainly by consumption of contaminated food (Daniels et al., 2000; Fankhauser et al., 2002), or they can be spread person-to-person (Fankhauser et al., 2002). NV outbreaks due to contaminated drinking water have also been reported (Kukkula et al., 1999; Nygård et al., 2003; Maunula et al., 2005). The presence of NV in drinking water sources is a public health concern owing to the potential for widespread NV outbreaks. However, because of the lack of a cell culture system or an

* *Corresponding author.* Tel.: +81 11 706 7281; fax: +81 11 706 7279.
E-mail address: nobutaka@eng.hokudai.ac.jp (N. Shirasaki).
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animal model for NV (Parker et al., 2005; Zheng et al., 2006), studies of drinking water treatments such as separation and disinfection processes are still hampered. Accordingly, the removal performance of NV in the coagulation–rapid sand filtration process, which is commonly used in drinking water treatment facilities, has not been investigated fully.

Feline calicivirus (FCV) has been widely used as an NV surrogate in studies of drinking water treatment processes to predict the treatability of NV (Thurston-Enriquez et al., 2003, 2005; Duizer et al., 2004; Abbaszadegan et al., 2007; Mayer et al., 2008), because FCV has a similar genome organization and capsid architecture to NV and it can be easily grown in cell cultures (Thurston-Enriquez et al., 2003). However, FCV belongs to the genus *Vesivirus*, family Caliciviridae, and it causes respiratory illness (Hashimoto et al., 1999); thus, FCV is unlike an enteric virus that is spread by the fecal–oral route. More recently, murine norovirus (MNV), genus *Norovirus*, family Caliciviridae, has been successfully propagated in cell culture (Karst et al., 2003). MNV is an enteric virus, and some researchers have reported MNV to be more suitable as a surrogate for NV than FCV in the aquatic environment (Cannon et al., 2006; Bae and Schwab, 2008). However, the suitability of MNV as a surrogate for NV in physical removal processes such as coagulation and filtration processes has not been investigated.

On the other hand, expression of the NV genome in a baculovirus expression system has made possible the production of recombinant NV virus-like particles (rNV-VLPs) (Jiang et al., 1992) that are morphologically and antigenically similar to native NV (Jiang et al., 1992; Green et al., 1993). Because the native NV source is the stools of human volunteers infected with NV, it is difficult to obtain a large amount of NV for spiking experiments of drinking water treatment processes, but the baculovirus expression system can produce enough rNV-VLPs to conduct spiking experiments. In addition, because rNV-VLPs lack RNA, which is necessary for infection and replication in host cells, they are harmless to humans during experiments, so they are easy to handle without any special facilities. In fact, rNV-VLPs have been used with the sandy aquifer treatment process to estimate the behavior of native NV (Redman et al., 1997). On the other hand, the fate of infectivity of NV in the treatment process, especially in response to disinfection processes such as chlorination, ozonation, and UV radiation, cannot be studied by using rNV-VLPs because of their lack of RNA. Thus, we can only discuss the fate of NV as particles.

Here, we used rNV-VLPs to investigate the removal performance of NV as particles during laboratory-scale coagulation–rapid sand filtration. We also experimentally investigated the behaviors of the F-specific RNA bacteriophages Qβ and MS2 for comparison with those of rNV-VLPs and to assess the suitability of these bacteriophages as surrogates for NV. This study represents the first attempt to apply rNV-VLPs to estimate the removal of native NV in a drinking water treatment process.

2. Materials and methods

2.1. Source water, coagulants, and filter media

River water was sampled from the Toyohira River (Sapporo, Japan, water quality shown in Table 1) on 12 June 2008. The coagulants used for the coagulation process were two commercial aluminum coagulants, polyaluminum chloride (PACl) (PACl 250A; 10.5% Al_2O_3, relative density 1.2 at 20 °C; Taki Chemical Co., Ltd., Hyogo, Japan) and alum (8.1% Al_2O_3, relative density 1.3 at 20 °C; Taki Chemical Co., Ltd.), and one laboratory-made ferric chloride solution, which was prepared by dilution of regent-grade iron (III) chloride ($FeCl_3$, Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in Milli-Q water (Milli-Q Advantage, Millipore Corp., Billerica, MA, USA). Silica sand (effective size 0.6 mm, uniformity coefficient <1.3; Nihon Genryo Co., Ltd., Kanagawa, Japan) was used as the filter medium for the rapid sand filtration process.

Table 1 – Water quality of the Toyohira River.

pH	7.2
DOC (mg/L)	0.76
OD260 (cm^{-1})	0.019
Turbidity (NTU)	0.63
Alkalinity (mg-$CaCO_3$/L)	17.2

2.2. rNV-VLPs

rNV-VLPs were produced by a baculovirus in silkworm, *Bombyx mori* (Katakura Industries Co. Ltd., Saitama, Japan). Subgenomic cDNA fragments of Chiba virus (AB042808, GI/4, Chiba407/1987/JP) genome were artificially synthesized and used for the expression of Chiba virus rNV-VLPs. Chiba virus, a Japanese strain in genogroup I of genus *Norovirus*, family Caliciviridae, was first identified as the cause of an oyster-associated outbreak of gastroenteritis that occurred in Chiba Prefecture, Japan, in 1987 (Kasuga et al., 1990). Someya et al. (2000) have determined the complete nucleotide sequence of the Chiba virus genome. Chiba virus has a positive-sense, single-stranded RNA of 7697 bases composed of a 5′ untranslated region (UTR), three open reading frames (ORFs), and a 3′UTR; the large 5′-terminal ORF (ORF1) encodes a polyprotein with 1785 amino acids that is likely processed into functional proteins. ORF2 encodes the capsid protein with 544 amino acids, and the small 3′-terminal ORF (ORF3) encodes a basic protein with 208 amino acids (Someya et al., 2000). Because Bertolotti-Ciarlet et al. (2003) reported that expression levels of NV capsid protein are enhanced by the presence of ORF3 and 3′UTR in recombinant baculovirus, compared with expression in the absence of ORF3, 3′UTR, or both, we synthesized 2352 (positions 5346–7697) bases of Chiba virus genome containing ORF2, ORF3, and 3′UTR with *att*B1, *Eco*RI, *att*B2, and *Pst*I restriction sites (total 2422 bases). The synthesized cDNA fragment was inserted into the vector (pDONR221, Invitrogen Japan K. K., Tokyo, Japan) by the Gateway BP reaction. After *Eco*RI and *Pst*I digestion of the plasmid, the digested cDNA fragment was ligated into the baculovirus transfer vector (pM0NHT04, Katakura Industries Co. Ltd.). The transfer vector was co-transfected with the linearized genomic DNA of baculovirus (*B. mori* nucleopolyhedrovirus; CPd strain, Suzuki et al., 1997) into the *B. mori*-derived cell line (BmN, Maeda, 1989) to generate the recombinant baculovirus, and then the recombinant baculovirus was injected into silkworm pupae to express the rNV-VLPs. Six days after

inoculation, the expressed rNV-VLPs were separated from the pupal homogenate by centrifugation and dialysis to prepare the rNV-VLP stock solution.

2.3. Bacteriophages

F-specific RNA bacteriophages Qβ (NBRC 20012) and MS2 (NBRC 102619) were obtained from the NITE Biological Research Center (NBRC, Chiba, Japan). The bacteriophages Qβ (Dowd et al., 1998; Matsui et al., 2003; Matsushita et al., 2004; Shirasaki et al., 2009) and MS2 (Nasser et al., 1995; Redman et al., 1997; Dowd et al., 1998; Abbaszadegan et al., 2007; Mayer et al., 2008; Shirasaki et al., 2009) are widely used as surrogates for pathogenic waterborne viruses in the coagulation, coagulation–rapid sand filtration, and sandy aquifer treatment processes because of their morphological similarities to hepatitis A viruses and polioviruses, which are important to remove during the treatment of drinking water. Qβ is the prototype member of the genus *Allolevivirus* in the virus family Leviviridae, and MS2 is the prototype member of the genus *Levivirus* in the Leviviridae. The genomes of these two bacteriophages contain a single molecule of linear, positive-sense, single-stranded RNA, which is encapsulated in an icosahedral protein capsid with a diameter of 24–26 nm (The Universal Virus Database of the International Committee on the Taxonomy of Viruses). Each bacteriophage was propagated for 22–24 h at 37 °C in *Escherichia coli* (NBRC 13965) obtained from NBRC. The bacteriophage culture solution was centrifuged (2000 × *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; Amicon Ultra-15, Millipore Corp.) to prepare the bacteriophage stock solution.

2.4. Coagulation experiments

Batch coagulation experiments were conducted with 400 mL of rNV-VLPs and bacteriophage-spiked river water in glass beakers at 20 °C. The rNV-VLPs (see Section 2.2) and the stock solutions (see Section 2.3) of both bacteriophages were simultaneously added to the beaker at approximately 10^{11} VLPs/mL and 10^8 plaque-forming units (PFU)/mL, respectively, and mixed with an impeller stirrer. PACl, alum, or $FeCl_3$ was injected into the water as a coagulant at 20 μM (0.54 mg-Al/L or 1.12 mg-Fe/L), 40 μM (1.08 mg-Al/L or 2.24 mg-Fe/L), or 60 μM (1.62 mg-Al/L or 3.36 mg-Fe/L). In the PACl and alum experiments, the pH of the water was immediately adjusted to, and maintained at, 6.8 using hydrochloric acid or sodium hydroxide. In contrast, in the $FeCl_3$ experiments, the pH was adjusted to one of three levels, 5.8 (minimum pH level established by drinking water quality standards in Japan), 6.3, or 6.8. The water was stirred rapidly for 2 min ($G = 200\ s^{-1}$, 77 rpm) and then slowly for 28 min ($G = 20\ s^{-1}$, 17 rpm). The water was then left at rest for 20 min to settle the floc particles generated. Samples were taken from the beaker before coagulant dosing (C_{c0}) and after settling (C_{cs}) for quantification of the rNV-VLP, Qβ, and MS2 concentrations. Statistical analysis by t-test (two-tailed) based on a 0.05 level of significance was performed to determine whether the removal performances of rNV-VLPs, Qβ, and MS2 differed when the conditions of the coagulation process were different. Because of the absence of experimental replication for any coagulation condition with $FeCl_3$, statistical analysis could not be performed for $FeCl_3$.

2.5. Rapid sand filtration experiments

After the coagulation experiments, filtration experiments were carried out with a glass column (diameter 0.8 cm, length 20 cm) packed with silica sand. Silica sand was 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 10 cm filter depth. Next, to saturate the filter media, Milli-Q water was pumped through the column by a peristaltic pump for 15 min, and then the excess Milli-Q water was drained off from the column just before the filtration experiment. Approximately 350 mL of the supernatant of the settling sample (see Section 2.4) was withdrawn from the beaker by the peristaltic pump, and transferred to another glass beaker as raw water for the sand filtration experiments. The raw water was continuously mixed with a magnetic stirrer at 200 rpm during the filtration experiment, and it was 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 column filtrate (C_{rf}) after 15 and 30 min of filtration time for quantification of the rNV-VLP, Qβ, and MS2 concentrations. Statistical analysis as described above was also performed on the results of the coagulation–rapid sand filtration process.

2.6. rNV-VLP assay

rNV-VLPs were detected by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (NV-AD (II), Denka Seiken Co., Ltd., Tokyo, Japan). The assay was performed according to the manufacturer's instructions. Optical densities at wavelengths of 450 nm and 630 nm in a 96-well microplate were measured with a microplate reader (MTP-300, Corona Electric Co., Ltd., Ibaraki, Japan).

2.7. Bacteriophage assay

Viral RNA of bacteriophages was quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR) method. This method detects viruses regardless of their infectivity. For quantification of bacteriophages in the samples, viral RNA was extracted from 200 μL of sample with a QIAamp MinElute Virus Spin Kit (Qiagen K. K., Tokyo, Japan) to obtain a final volume of 20 μL. The extracted RNA solution was added to a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems Japan Ltd., Tokyo, Japan) for the reverse transcription (RT) reaction, which was conducted at 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 s, followed by cooling to 4 °C in the thermal cycler (Thermal Cycler Dice Model TP600, Takara Bio Inc., Shiga, Japan). The cDNA solution was then amplified by a TaqMan Universal PCR Master Mix with UNG (Applied Biosystems Japan Ltd.), 400 nM of each primer (HQ-SEQ grade, Takara Bio Inc.), and 250 nM of TaqMan probe (Applied Biosystems Japan Ltd.). The oligonucleotide sequences of the primers and the probes are shown in Table 2. Amplification was conducted at 50 °C for 2 min, 95 °C for 10 min, and

Table 2 – Oligonucleotide sequences of the primers and probes used in real-time RT-PCR quantification of Qβ and MS2.

Viruses		Oligonucleotide sequences	Positions	References
Qβ	Forward primer	5′-TCA AGC CGT GAT AGT CGT TCC TC-3′	49–71	Katayama et al., 2002
	Reverse primer	5′-AAT CGT TGG CAA TGG AAA GTG C-3′	187–208	
	TaqMan probe	5′-CGA GCC GCG AAC ACA AGA ATT GA-3′	147–169	
MS2	Forward primer	5′-GTC GCG GTA ATT GGC GC-3′	632–648	O'Connell et al., 2006
	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	

then 40 cycles of 95 °C for 15 s and 60 °C for 1 min in an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems Japan Ltd.).

The standard curve for the real-time RT-PCR method was based on the relationship between the infectious bacteriophage concentration of a freshly prepared stock solution measured by the plaque-forming unit (PFU) method (Adams, 1959) and the number of cycles of PCR amplification.

2.8. *Electron microscopy*

Negative-stain electron microscopy was used to analyze the presence, integrity, and morphology of the rNV-VLPs. Ten microliters of rNV-VLP stock solution (see Section 2.2) was put on a 400-mesh copper grid with collodion membrane (Nissin EM Corp., Tokyo, Japan) and adsorbed to the grid for 1 min. Excess solution on the grid was drained from the side of the grid with filter paper, and rNV-VLPs were negatively stained with 10 μL of 2% phosphotungstic acid (pH 5.5) for 45 s. After the excess stain was drained off, the grid was examined with a transmission electron microscope (TEM, H-7650, Hitachi High-Technologies Corp., Tokyo, Japan). The particle diameter of rNV-VLPs was expressed as the mean and standard deviation of 10 randomly chosen particles on the electron micrograph.

2.9. *Cesium chloride density gradient*

The densities of rNV-VLPs and bacteriophages were analyzed in a cesium chloride (CsCl) density gradient. rNV-VLPs and both bacteriophages were simultaneously suspended at approximately 10^{12} VLPs/mL and 10^{8} PFU/mL, respectively, in a 1.2-g/cm^3 CsCl solution using the stock solutions (see Sections 2.2 and 2.3), and layered on top of a 1.3–1.6-g/cm^3 CsCl gradient. The gradient including rNV-VLPs and bacteriophages was ultracentrifuged (100,000 × *g*, 18 h) in a preparative centrifuge (CP80MX, Hitachi Koki Co., Ltd., Tokyo, Japan) with a P28S swing rotor, and then divided into 1-mL fractions. The density of each fraction was measured with an electronic balance (Mettler-Toledo AG245 Balance, Mettler-Toledo K. K., Tokyo, Japan), and rNV-VLP and bacteriophage (Qβ and MS2) concentrations were quantified by ELISA and real-time RT-PCR method, respectively.

2.10. *Electrophoretic mobility*

The electrophoretic mobility of rNV-VLPs and bacteriophages was measured in filtered river water. River water in a stirred ultrafiltration cell (Model 8400, Millipore Corp.) was filtered through an ultrafiltration membrane (molecular weight cutoff 100,000, regenerated cellulose; Ultrafiltration Disks, YM-100, Millipore Corp.) to exclude the large particles, and the pH was adjusted to 6.8 with HCl. The river water samples were kept for 1 day at 20 °C to stabilize the pH. Just before the measurement of electrophoretic mobility, the rNV-VLPs and each bacteriophage were suspended at approximately 10^{10} VLPs/mL or 10^{10} PFU/mL in the filtered river water using the stock solution (see Sections 2.2 and 2.3). The electrophoretic mobility of the rNV-VLPs and both bacteriophages was measured with an electrophoretic light-scattering spectrophotometer (Zetasizer Nano ZS; 532 nm green laser, Malvern Instruments Ltd., Malvern, Worcestershire, UK) at 25 °C and at a 17° measurement angle.

3. Results and discussion

3.1. *Characteristics of the produced rNV-VLPs*

Fig. 1 shows an electron micrograph of rNV-VLPs produced by the baculovirus–silkworm expression system. The presence of particles was confirmed on the electron micrograph, and rNV capsid proteins spontaneously self-assembled into VLPs during expression. The rNV-VLP particle diameter was 35.7 ± 3.2 nm, measured on 10 randomly chosen particles on the electron micrograph. This value roughly corresponds to the particle diameter (approximately 38 nm) previously reported for native NV (Someya et al., 2000).

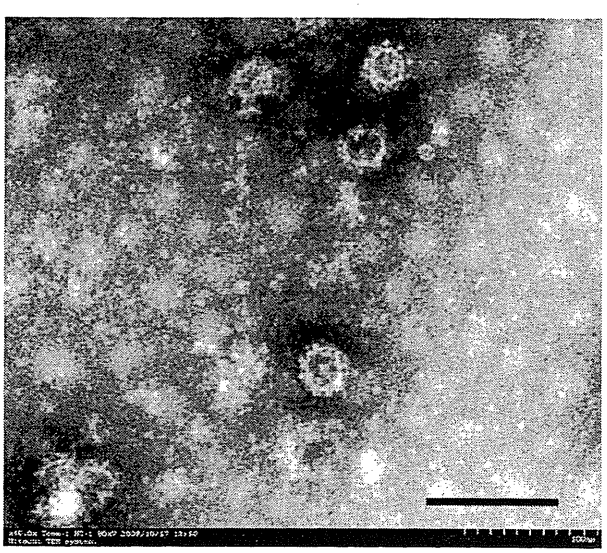

Fig. 1 – Negatively stained electron micrograph of rNV-VLPs. The scale bar corresponds to 100 nm.

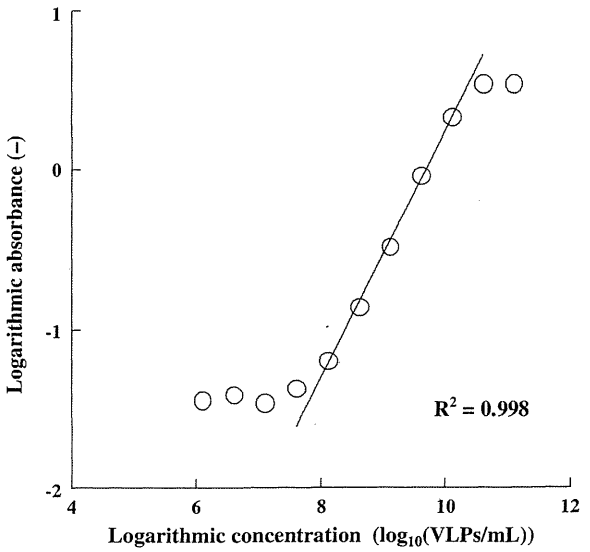

Fig. 2 – Sensitivity of the ELISA kit for the detection of rNV-VLPs.

A commercially available ELISA kit was used for quantification of rNV-VLPs. The sensitivity of the ELISA kit was determined by using 0.5-log_{10}-fold serial dilutions of the rNV-VLP stock solution (see Section 2.2) with river water (Fig. 2). An excellent linear correlation between the rNV-VLP concentration and absorbance was observed in the range from 10^8 to 10^{10} VLPs/mL. The rNV-VLP quantification limit with the ELISA kit was approximately 10^8 VLPs/mL (Fig. 2). This result suggests that the rNV-VLPs produced here could be quantified by the commercially available ELISA kit.

The CsCl density of the fraction with the highest rNV-VLP concentration, quantified by ELISA, was 1.28 g/cm^3 (Fig. 3), meaning that the rNV-VLPs produced had a density of 1.28 g/cm^3. Although this value was somewhat smaller than that of native NV (1.38 g/cm^3, Jiang et al., 1992; 1.36–1.37 g/cm^3, Utagawa et al., 1994), probably owing to the lack of RNA, it is similar to the previously reported values for rNV-VLPs (1.31 g/cm^3, Jiang et al., 1992; 1.27 g/cm^3, Katayama et al., 2006). In addition, the density of rNV-VLPs was smaller than that of either bacteriophage: the CsCl densities of the fractions with the peak Qβ and MS2 concentrations, quantified by real-time RT-PCR method, were 1.42 and 1.40 g/cm^3, respectively, roughly corresponding to the densities previously reported for Qβ (1.46 g/cm^3, Engelberg-Kulka et al., 1979) and MS2 (1.38 g/cm^3, Kuzmanovic et al., 2003).

These results suggest that rNV-VLPs were successfully produced by the baculovirus–silkworm expression system, and that the produced rNV-VLPs were morphologically similar to native NV. Accordingly, it is possible to estimate removal performance for NV as particles of the coagulation–rapid sand filtration process by using the rNV-VLPs produced here.

3.2. *Simultaneous removal of rNV-VLPs and bacteriophages in the coagulation process*

Fig. 4 shows the removal ratios ($log_{10}[C_{c0}/C_{cs}]$) of the model viruses (rNV-VLPs, Qβ, and MS2) after settling in the coagulation process with PACl at pH 6.8 (the measurement errors were less than 0.1-log_{10} for rNV-VLPs, Qβ, and MS2). Because of the small sizes of the model viruses and the stability resulting from electrical repulsion in the river water, no removal (<0.1-log_{10}) of rNV-VLPs or either bacteriophage was observed in the absence of a coagulant. Even with 20 μM-Al of PACl, no removal was observed. In contrast, the coagulation process removed model viruses at PACl doses of 40 and 60 μM-Al. This result indicated that the stably monodispersed model viruses in the river water were destabilized by the addition of PACl and became adsorbed to/entrapped in the aluminum floc particles generated during the coagulation process, and then the aluminum floc particles with the adsorbed/entrapped model viruses settled from suspension by gravity during the settling process. The removal ratios of rNV-VLPs were only <0.3-log_{10} at a PACl dose of 20 μM-Al, whereas with a dose of 40 μM-Al or more the removal ratios significantly ($P < 0.05$) increased to approximately 1-log_{10} for rNV-VLPs. Increasing PACl dose to more than 40 μM-Al also increased the removal ratios of Qβ and MS2 to approximately 2-log_{10}, although no

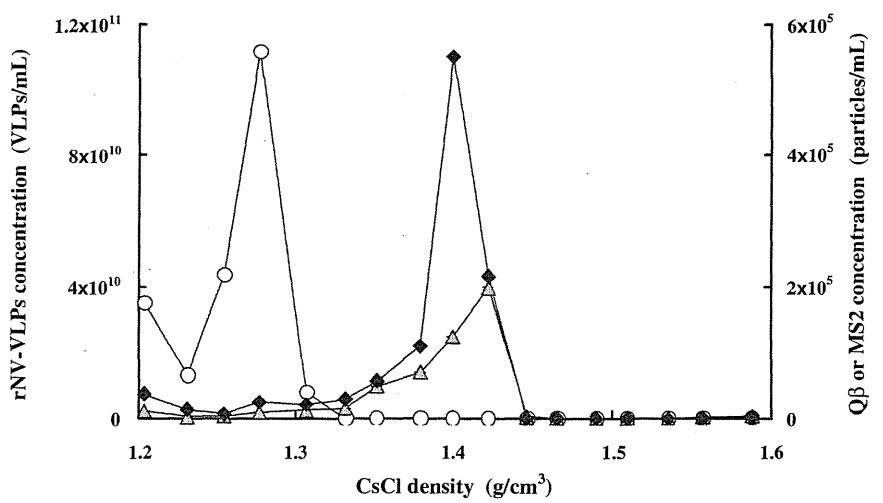

Fig. 3 – Densities of rNV-VLPs, Qβ, and MS2 analyzed by CsCl density gradient. Circles, triangles, and diamonds represent rNV-VLPs, Qβ, and MS2, respectively.

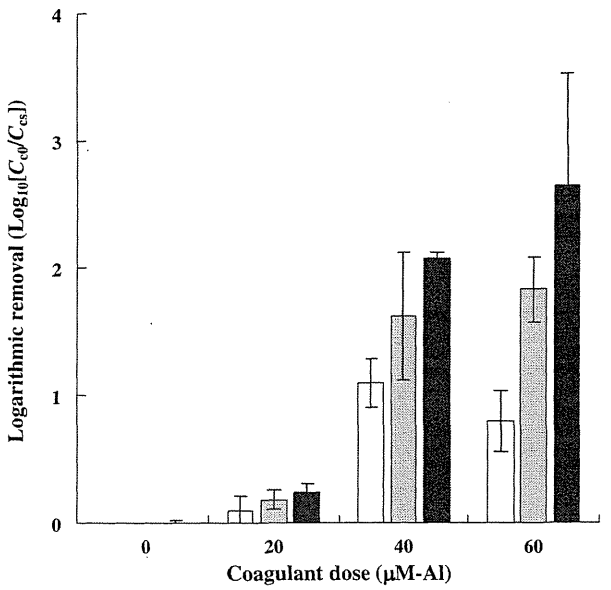

Fig. 4 – Effect of coagulant dose on rNV-VLP, Qβ, and MS2 removals after settling in the coagulation process with PACl at pH 6.8. White, gray, and black columns represent rNV-VLPs, Qβ, and MS2, respectively. Values are means and standard deviation of two or three replications.

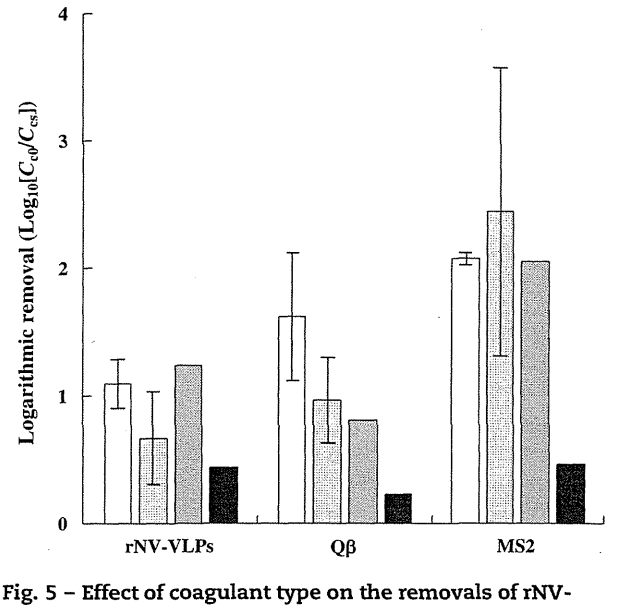

Fig. 5 – Effect of coagulant type on the removals of rNV-VLPs, Qβ, and MS2 after settling in the coagulation process. White, light gray, dark gray, and black columns represent PACl (pH 6.8), alum (pH 6.8), $FeCl_3$ (pH 5.8), and $FeCl_3$ (pH 6.8), respectively. Values are means and standard deviation of two or three replications for PACl and three or four replications for alum. Values for $FeCl_3$ were determined from one experiment. Coagulant dose was 40 μM-Al or -Fe.

significance ($P = 0.06$) was observed for Qβ (40 μM-Al) and MS2 (60 μM-Al). Accordingly, a PACl dose of 40 μM-Al is recommended to remove the model viruses in the present coagulation process. Similar trends were observed when alum and $FeCl_3$ were used as the coagulant (data not shown).

To investigate the effect of coagulant type on the removals of model viruses, the removal performances of the coagulation process with a coagulant dose of 40 μM-Al or -Fe were compared among rNV-VLPs, Qβ, and MS2 after settling (Fig. 5). Because the optimal coagulation pH for $FeCl_3$ is generally lower than that for aluminum coagulants (American Water Works Association, 1990), the coagulation pH for $FeCl_3$ was adjusted to 5.8 and 6.3 (data not shown) as well as to 6.8. The coagulation processes with PACl and $FeCl_3$ (pH 5.8) obtained approximately 1-log_{10} removals of rNV-VLPs. These removals are somewhat larger than those obtained with alum and $FeCl_3$ (pH 6.8) although no significant difference between PACl and alum was observed ($P > 0.05$). The efficacy of PACl for the removal of negatively charged colloids, including viruses, compared with that of alum is attributed to soluble polycationic species, which are abundant in PACl. Because the widely accepted mechanism of negatively charged colloid removal is by charge neutralization by soluble polycationic species such as $Al_2(OH)_2^{4+}$, $Al_3(OH)_4^{5+}$, and $Al_{13}O_4(OH)_{24}^{7+}$ (Stewart et al., 2009), the difference in the abundances of polycationic species probably can explain the differences in the characteristics of the aluminum floc particles generated during the coagulation process between PACl and alum. In fact, Gregory and Dupont (2001) reported that aluminum floc particles formed with PACl are larger, stronger, and more readily separated by the settling process than those formed with alum. Accordingly, PACl could more effectively remove the rNV-VLPs than alum.

$FeCl_3$ (pH 5.8) also removed rNV-VLPs more effectively than alum in the present coagulation process. Rao et al. (1988) investigated the removal performance for hepatitis A virus and poliovirus during the coagulation process, and demonstrated that greater removals of both viruses were obtained with $FeCl_3$ than with alum. Chang et al. (1958) also reported effective removal of coxsackie virus with $FeCl_3$, and floc particles formed with $FeCl_3$ were more compact and settled more rapidly than those formed with alum. Therefore, the difference in the characteristics of the floc particles between $FeCl_3$ (pH 5.8) and alum led to the difference in rNV-VLP removal performances in the present coagulation process. However, $FeCl_3$ could not effectively remove rNV-VLPs at pH 6.8: the removal ratio of rNV-VLPs decreased as pH increased, with removal performance in the order pH 5.8 > 6.3 > 6.8. Abbaszadegan et al. (2007) and Mayer et al. (2008) investigated the effect of pH on the removal of five viruses, including FCV, during enhanced coagulation processes with $FeCl_3$; they reported that the removal ratios of these viruses were improved as pH decreased (optimum pH range 5–6). Our findings are consistent with these previous results.

The removal performance for Qβ obtained with PACl was higher than that obtained with alum or $FeCl_3$. By comparison, approximately 2-log_{10} removals of MS2 were achieved with all coagulant types except $FeCl_3$ (pH 6.8). Consequently, PACl more effectively removed the model viruses than alum or $FeCl_3$ in the present coagulation process. Additionally, it was difficult to remove the model viruses with $FeCl_3$ at pH 6.8, although the removal performances for rNV-VLPs and MS2 with $FeCl_3$ at pH 5.8 were equal to those with PACl.

Differences in the removal performances among rNV-VLPs, Qβ, and MS2 were observed in the coagulation process: although the removal ratio of Qβ was similar to that of rNV-VLPs at a coagulant dose of 40 μM-Al or -Fe, the removal ratio of MS2 was approximately 1-$\log_{10}$ larger than that of rNV-VLPs (Fig. 5). In general, the surface charge on virus particles is often invoked to explain virus removal by physicochemical water treatment processes, including coagulation processes (Matsushita et al., 2004): more negatively charged viruses may resist aggregation, with the result that they are more difficult to destabilize and aggregate by charge neutralization during the coagulation process than less negatively charged viruses. However, rNV-VLPs were less negative than Qβ or MS2 in the filtered river water at pH 6.8 (Fig. 6), a result that is not in accordance with a previous result in which rNV-VLPs were more negative than MS2 in an NaCl solution at around pH 7 (Redman et al., 1997), possibly owing to the difference in the electrolyte solution. The difference in electrophoretic mobility among the model viruses did not strongly affect removal performances in our study. We imagine that the differences in other characteristics of the model viruses, such as hydrophobicity, affected the removal performances of the coagulation process. Because the hydrophobic force contributes to the adsorption of protein on aluminum phosphate adjuvant (Al-Shakhshir et al., 1995), the difference in hydrophobicity among surface proteins of the model viruses might cause differences in the interaction between surface proteins and the floc particles generated during the coagulation process. Further investigation is needed.

Much discussion of possible surrogates for pathogenic waterborne viruses in the aquatic environment has not yet resulted in any overall agreement. MS2 has been widely used as a surrogate for pathogenic waterborne viruses in coagulation treatments (Nasser et al., 1995; Abbaszadegan et al., 2007; Mayer et al., 2008; Shirasaki et al., 2009), mainly because of its morphological similarities to those viruses. However, as described above, the removal performance for MS2 was approximately 1-$\log_{10}$ larger than that for rNV-VLPs in the present coagulation process, meaning that the removal performances for native NV would be overestimated if MS2 were selected as a surrogate for native NV. Accordingly, MS2 is not recommended as an appropriate surrogate for native NV. By comparison, although the removal performance for Qβ was similar to that for rNV-VLPs at a coagulant dose of 40 μM-Al or -Fe (Fig. 5), the removal performance for Qβ was somewhat larger than that for rNV-VLPs at PACl dose of 60 μM-Al (Fig. 4). Therefore, Qβ also is not recommended as an appropriate surrogate for native NV.

3.3. *Simultaneous removal of rNV-VLPs and bacteriophages in the coagulation–rapid sand filtration process*

Fig. 7 shows the removal ratios ($\log_{10}[C_{c0}/C_{cs}] + \log_{10}[C_{r0}/C_{rf}]$) for model viruses in the coagulation–rapid sand filtration process with PACl at pH 6.8. Because the removal ratios of model viruses in the coagulation–rapid sand filtration process were almost constant during the filtration, the removal ratios in Fig. 7 are represented by the averages of the values after 15 and 30 min filtration. Even though the rapid sand filtration process was introduced after the coagulation process, no removal (<0.1-$\log_{10}$) of rNV-VLPs or either bacteriophage was observed in the absence of a coagulant. Even with the PACl dose of 20 μM-Al, no removal was observed. In contrast, improvement of removal ratios of the model viruses were observed in the rapid sand filtration process at PACl doses of 40 and 60 μM-Al compared with the coagulation process alone (Fig. 4). Therefore, the entrapped model viruses in the suspended aluminum floc particles were effectively removed

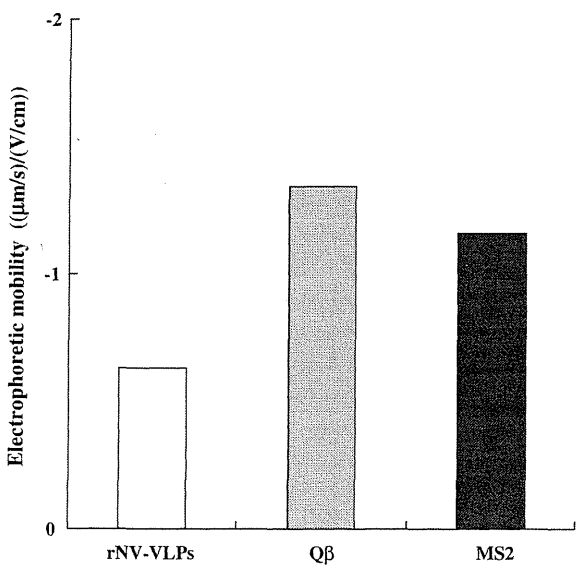

Fig. 6 – Electrophoretic mobility of rNV-VLPs, Qβ, and MS2 in filtered river water.

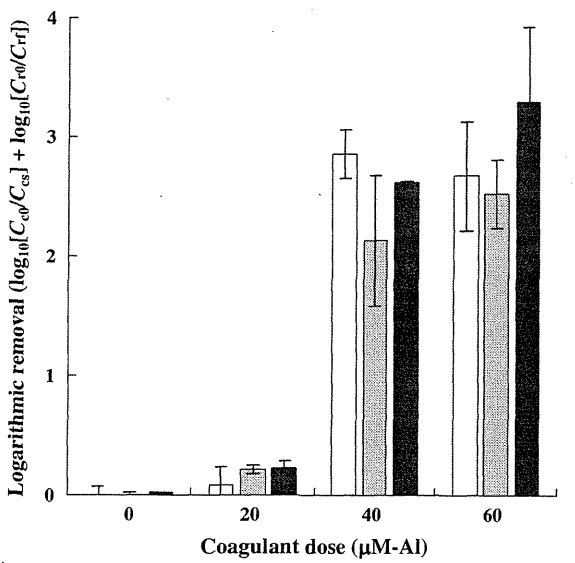

Fig. 7 – Effect of coagulant dose on rNV-VLP, Qβ, and MS2 removals in the coagulation–rapid sand filtration process with PACl at pH 6.8. White, gray, and black columns represent rNV-VLPs, Qβ, and MS2, respectively. Values are means and standard deviation of two or three replications.

by the subsequent rapid sand filtration process. Even monodispersed model viruses might have been adsorbed to the negatively charged sand surface by the electrostatic attractive force/van der Waals attractive force, because the surface charges of the model viruses probably changed from negative to neutral or positive by charge neutralization during the coagulation process. The removal ratio of rNV-VLPs was increased more by the introduction of the rapid sand filtration process than were those of Qβ and MS2 at PACl doses of 40 and 60 μM-Al: an approximately 2-$\log_{10}$ improvement was obtained for rNV-VLPs, whereas approximately 0.5-$\log_{10}$ improvements were obtained for the two bacteriophages. In the sandy aquifer treatment process, many factors affect virus adsorption. Dowd et al. (1998) demonstrated that the larger bacteriophages PRD1 and PM2 (60–63 nm) show greater retardation than the smaller bacteriophages Qβ, MS2, and φX174 (24–27 nm). Our findings agree with this previous result: the removal ratio of rNV-VLPs (35.7 ± 3.2 nm) was larger than those of Qβ and MS2 (24–26 nm), as described above. Accordingly, a difference in the particle diameter between rNV-VLPs and the two bacteriophages possibly affected the removal performance in the present rapid sand filtration process. In addition, Redman et al. (1997) reported that the removal ratio of less negatively charged rNV-VLPs was higher than that of more negatively charged ones owing to the reduction of electrostatic repulsive interactions between rNV-VLPs and the negatively charged sand surface. Although the electrophoretic mobility of rNV-VLPs and both bacteriophages after the coagulation process was not measured, a difference in the surface charge between rNV-VLPs and the bacteriophages after the coagulation process also might have affected removal performance in the present rapid sand filtration process.

Ultimately, the removal ratios of model viruses were only <0.3-$\log_{10}$ at a PACl dose of 20 μM-Al, whereas, with a dose of 40 μM-Al or more, the removals significantly ($P < 0.05$) increased to approximately 2–3-$\log_{10}$ for rNV-VLPs, Qβ, and MS2 in the present coagulation–rapid sand filtration process. Accordingly, a PACl dose of 40 μM-Al is recommended to remove the model viruses in this coagulation–rapid sand filtration process. Similar trends were observed when alum and $FeCl_3$ were used as the coagulant (data not shown).

To investigate the effect of coagulant type on the removals of model viruses, the removal performances for rNV-VLPs, Qβ, and MS2 were compared in the coagulation–rapid sand process between a 40 μM-Al and a 40 μM-Fe of coagulant dose (Fig. 8). The coagulation–rapid sand filtration process with PACl and $FeCl_3$ (pH 5.8) achieved approximately 3-$\log_{10}$ removals of rNV-VLPs, whereas the removal ratios of rNV-VLPs obtained with alum and $FeCl_3$ (pH 6.8) were smaller; in particular, only a 0.5-$\log_{10}$ removal was observed with $FeCl_3$ (pH 6.8). In addition, a significant difference between PACl and alum was observed ($P < 0.05$). By comparison, the removal ratio of Qβ with $FeCl_3$ (pH 5.8) was the highest obtained, followed by those obtained with PACl, alum, and $FeCl_3$ (pH 6.8). A similar trend was observed with MS2. Consequently, $FeCl_3$ (pH 5.8) more effectively removed model viruses than PACl or alum in the present coagulation–rapid sand filtration process, although a decreased coagulation pH is required when $FeCl_3$ is used as the coagulant compared with the use of PACl or alum.

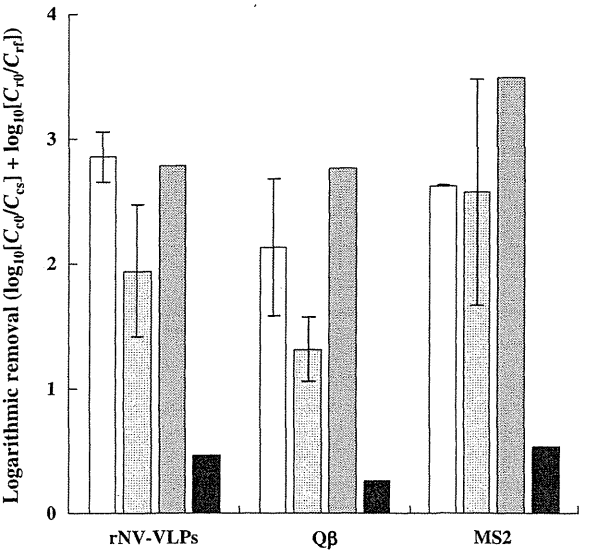

Fig. 8 – Effect of coagulant type on removal of rNV-VLPs, Qβ, and MS2 in the coagulation–rapid sand filtration process. White, light gray, dark gray, and black columns represent PACl (pH 6.8), alum (pH 6.8), $FeCl_3$ (pH 5.8), and $FeCl_3$ (pH 6.8), respectively. Values are means and standard deviation of two or three replications for PACl and three or four replications for alum. Values for $FeCl_3$ were determined from one experiment. Coagulant dose was 40 μM-Al or -Fe.

The removal performance for MS2 was somewhat larger than that for rNV-VLPs in the present coagulation–rapid sand filtration process. Accordingly, MS2 is not recommended as an appropriate surrogate for native NV. By comparison, the removal performance for Qβ was similar to, or smaller than, that for rNV-VLPs at a coagulant dose of 40 μM-Al or -Fe (Fig. 8). However, the removal performances for rNV-VLPs and Qβ differed between each unit process, i.e., the coagulation process removed Qβ rather than rNV-VLPs, while vice versa in the following rapid sand filtration process. Accordingly, Qβ also is not recommended as an appropriate surrogate for native NV, even though its removal ratio in total unit process was similar to, or smaller than, that of rNV-VLPs at any condition. To propose appropriate surrogates, further investigation is needed.

According to the U.S. Environmental Protection Agency (USEPA) National Primary Drinking Water Standards (USEPA, 2001), enteric viruses must be removed or inactivated by 4-$\log_{10}$ from source water by filtration, disinfection, or a combination of these technologies. This 4-$\log_{10}$ removal ratio was not obtained for rNV-VLPs by the present coagulation–rapid sand filtration process alone. To achieve 4-$\log_{10}$ removal or inactivation of native NV, further separation or disinfection processes will be required after coagulation–rapid sand filtration. On the other hand, virucidal activity of PACl during the coagulation process has been reported by our research group (Matsui et al., 2003; Matsushita et al., 2004; Shirasaki et al., 2009): an approximately 4-$\log_{10}$ difference between the total (infectious + inactivated) concentration measured by real-time RT-PCR method and the infectious concentration