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Removal of Noroviruses from Municipal Wastewater by Foam Separation using Dispersed Air-Bubbles and Surface-Active Substance

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Abstract: To prevent the spread of acute gastroenteritis, which prevails during winter and is called the winter vomiting disease, it is necessary to develop a technology for the detection and removal of noroviruses (NVs) from contaminated water. In this study, the removal from municipal wastewater and concentration in foam of NVs using dispersed bubbles and milk casein as a surface-active protein were examined. Real-time reverse transcription loop-mediated isothermal amplification (RT-LAMP) as a new nucleic acid amplification method was used for NV detection. Although NV-GII was detected in two wastewater samples, NV-GI was not detected. By foam separation using dispersed bubbles, NV-GII was removed from wastewater and concentrated into the generated foam. The concentration of NV-GII in the collapsed foam water was more than approximately 8-fold higher than that in the wastewater. Because the generated foam can be analyzed by real-time RT-LAMP, it is possible to sensitively monitor NV pollution in aqueous environments. Here, we show for the first time the

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removal from aqueous solution and concentration of NVs in foam using dispersed bubbles and a surface-active substance.

Keywords: Concentration effect, foam separation, municipal wastewater, noroviruses, removal, RT-LAMP

INTRODUCTION

Recently, it has been confirmed that acute gastroenteritis, which prevails during winter and is called the winter vomiting disease, is caused by noroviruses (NVs). NVs have emerged as one of the most virulent pathogens commonly associated with food related illnesses in Japan (1), the United States (2), Europe (3), China (4) etc. While it has been recognized that the cause of the winter vomiting disease is these small round viruses (5), the behavior of NVs in the aqueous environment is still unclear. After the sequence information of the NV genome was reported in 1990 (6), methods for the detection and quantitation of NVs by reverse transcription polymerase chain reaction (RT-PCR) as a gene amplification method were developed (7), and the number of studies on NVs rapidly increased. Although information on the determination and monitoring of NVs in sewage (8,9), tap water (10), river water (9,11,12), ground water (13), and coastal seawater (14) has been accumulating, the accurate concentration, the form of existence, and the fate of NVs in the aqueous environment are poorly understood.

Particularly large outbreaks of acute gastroenteritis associated with NVs have occurred after the consumption of contaminated raw shellfish such as oysters (15–17). However, the pollution sources of NVs are the feces and vomit of those infected; moreover, NVs spread from river to coastal areas via sewage treatment plants or community plants (18). As a result of coastal seawater contamination, NVs become concentrated in shellfish (15). To prevent the spread of the illness, it is necessary to develop a technology for the removal and monitoring of NVs, because NVs are associated with water and spread via the aqueous environment. Currently, there are only a few physicochemical purification methods for viral removal, and some possible method of nanofiltration for drinking water has been merely reported (19). In addition, a method using membrane filter for NV recovery requires extensive labor for application to wastewater before NV determination, because the filtration of wastewater containing suspended solids takes time (20).

When dispersed bubbles are added to a solution containing protein, protein accumulation in the generated foam is a well-known phenomenon (21). This is because proteins, which are composed of peptide chains of

both hydrophilic and strong hydrophobic amino acids, have high surface activity, and the hydrophobic sites strongly adsorb onto the gas-liquid interface of a bubble. For the application of dispersed bubbles, numerous studies have examined the recovery of dissolved proteins by foam separation (22–25). At present, foam separation is applied to the purification or collection of proteins from solutions in such fields as food processing (24) and industrial chemistry (26,27). Because enzymes are also proteins, foam separation can be applied to the isolation of enzymes from aqueous media (28,29). In addition, foam separation with coagulation, which is a solid-liquid separation method for the removal of suspended substances from wastewater, has been developed using both ferric chloride as a coagulant and protein as a chemical agent (collector and/or frother) (30). Therefore, foam separation using dispersed bubbles has the high potential for removing and concentrating proteins and protein-like substances from water into the generated foam. NVs are composed of a capsid shell containing RNA and can be regarded as ultrafine particles made of proteins (31). Viral particles seem to adsorb to the surface-active substances such as proteins quite easily. Therefore, when dispersed bubbles are supplied to wastewater containing NVs and surface-active substances such as protein, we can assume that NVs will become concentrated in the generated foam together with the protein and be removed from wastewater.

In this study, the removal from municipal wastewater and concentration in foam of NVs using dispersed bubbles and milk casein as a surface-active protein were examined. For NV determination, real-time reverse transcription loop-mediated isothermal amplification (RT-LAMP) (32), which has the significant advantages of simple operation and low cost compared with a conventional method such as real-time RT-PCR, was applied.

MATERIALS AND METHODS

Sample Collection and Preparation

Samples were collected from a municipal wastewater treatment plant (Miyazaki, Japan) in 2007 during the winter season. An influent wastewater sample was obtained from the outlet of the grit-removal tank and was sieved with a stainless mesh (opening, 150 μm, Toyo Kyogyo Co., Japan) immediately after collection. Experiments on foam separation were carried out using the screened sample. The basic water quality characteristics of the samples were as follows: pH 7.27–7.29; electrical conductivity, 1.59–1.70 mS/cm; turbidity 57.7–71.0 unit as kaolin standard. In winter season, the concentration of anionic surfactant in the

wastewater fluctuated within 6.8 to 12 mg/L (mean ± SD, 9.6 ± 2.1 mg/L; n = 5) determined by a spectrophotometry using methylene blue (33).

Foam Separation using Batch Equipment

In our previous research using batch equipment (30), the casein concentration necessary for generating sufficient foam was 10 mg/L in wastewater. Therefore, the casein dosage was fixed at 10 mg/L. An appropriate amount of casein (10 mg/L) was added to wastewater (500 mL). A stock solution of casein (milk casein, reagent grade, Wako Chemical, Japan) was prepared in 0.01 M NaOH to a final concentration of 10,000 mg/L. The wastewater sample was rapidly stirred (150 rpm) for 1 min using a jar tester. By transferring a sample to the cylindrical column (height, 84 cm; diameter, 3.0 cm) of the batch flotation equipment (Fig. 1), foam separation was carried out. Dispersed air was supplied from the bottom of the column using a glass-ball filter (Kinoshita Rika,

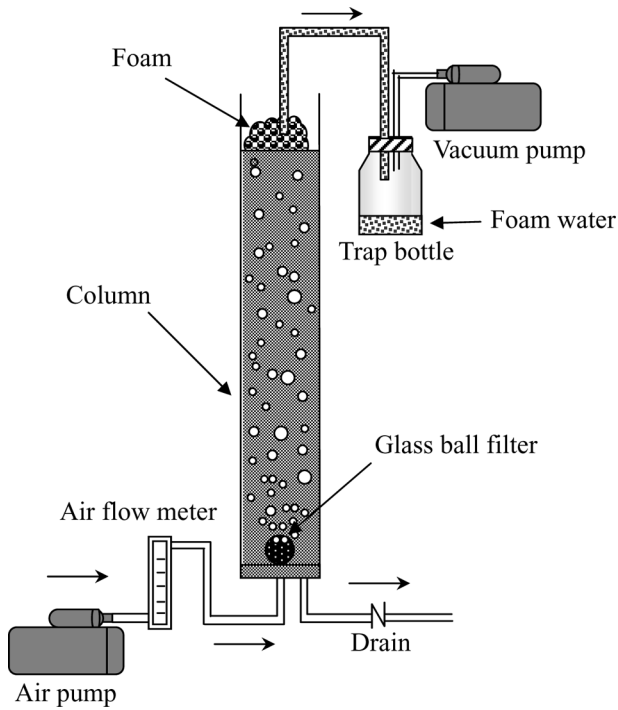


Figure 1. Schematic diagram (not to scale) of foam separation equipment for batch system.

Japan). Foam generated on the water surface was drawn into a trap bottle using a vacuum pump. The recovered foam was defoamed and designated as "foam water." The bubbling time for foam separation was set to 10 min, which is sufficient for foam generation. The treated water was sampled from the drain. To determine if the processing time can be shortened, the experiment with 3 min of bubbling time was also carried out under the same conditions. Because the wastewater contained detergents such as anionic surfactant at a concentration of approximately 10 mg/L, foam was generated without casein when the wastewater was bubbled using the batch equipment. Then, an identical experiment under the same conditions but without casein added was carried out.

Virus Concentration and RNA Extraction

NVs were recovered from wastewater, treated water, and foam water by the virus concentration method using negatively charged membrane with alkaline elution after acid rinsing (11). A sample volume was 50 mL. Although the foam water was less than 50 mL, the sample was diluted to 50 mL using sterilized Milli-Q water (Millipore, Japan). The cation concentration in the sample was made up to 25 mM $MgCl_2$ by adding $MgCl_2$ stock solution. The sample was filtered through a membrane filter (0.45 μm pore size, 47 mm diameter, HA type, Millipore). The filter was rinsed with 200 mL of 0.5 mM H_2SO_4 (pH 3.0), and NVs were eluted with 10 mL of 1.0 mM NaOH (pH 10.7–11.0) into a tube containing 50 μL of 100 mM H_2SO_4 and 100 μL of 100-fold tris-EDTA buffer (Wako Pure Chemical, Japan) for neutralization. The eluate was reconcentrated to 1.75 mL using a Centriprep TM-50 ultrafiltration device (Millpore). Viral RNA was extracted from the concentrated eluate using an RNA extraction kit (High Pure Viral RNA kit, Roche), according to the manufacturer's instructions.

Real-Time RT-LAMP Assay

The RT-LAMP assay of NVs was performed using Loopamp RNA amplification kits (norovirus GI detection kit and norovirus GII detection kit, Eiken Chemical, Japan). The amplification of nucleic acids using these two kits was conducted by the LAMP method using primers derived from the sequences of conservative genes in the RNAs of norovirus genogroup I (GI) and norovirus genogroup II (GII). With the preset time and temperature, the gene amplification occurs, and the detection is measured by monitoring white turbidity caused by magnesium pyrophosphate, an amplification by-product. The sequences of the primers have not been

revealed to the public. The assay was carried out following the manufacturer's instructions. For the real-time RT-LAMP assay monitoring, the reaction mixture prepared using the kit was incubated at 63°C for 60 min in a real-time turbidity meter (LA-200, Teramecs, Japan). The sample in which the turbidity increased within the reaction time of 60 min to over 0.1 was considered positive for the virus (32). The time until the turbidity exceeds the endpoint of 0.1 was defined as the threshold time (Tt).

Quantitative Analysis for Real-Time RT-LAMP

The RT-LAMP kits for NVs were for qualitative analysis, and not for quantitative analysis. However, it has been reported that viral quantification is possible by real-time RT-LAMP for the hepatitis A virus given that the viral quantity strongly correlated with Tt (34). Therefore, the quantitative analysis of NVs by real-time RT-LAMP was also carried out. Because it is impossible to obtain NV strains at present, the positive control (PC) solution included in the kit was used for the standard RNA solution of NV-GI or NV-GII. The PC solution contains the template RNAs of NV-GI or NV-GII obtained as a plasmid at a fixed concentration (copy number) although the concentration has not been revealed to the public by Eiken Chemical. A dilution series up to 10^{-9} was prepared using the PC solution together with the elution buffer included in the RNA extraction kit. Then, the relationship between the dilution rate and Tt was examined.

RESULTS AND DISCUSSION

Quantitative Analysis of NVs by Real-Time RT-LAMP

The results of the Tt measurement for the dilution series of the PC solution (GI) by real-time RT-LAMP are shown in Table 1. The turbidity of the samples diluted by 10^{-6} to 10^{-9} remained zero during the 60 min of reaction time, and all these samples were considered as negative. It was proven that the quantity of NV-GI template RNA diluted by less than 10^{-6} in the PC solution could not be determined by real-time RT-LAMP. Because the instruction manual provided by Eiken Chemical shows that the sensitivity of the NV-GI detection kit is 60 copies/test as the PCR copy number, the concentration of template RNA in the 10^{-6} dilution was presumed to be less than 60 copies. A comparison of Tt with dilution rate is shown in Fig. 2. Tt increased with increasing dilution factor at the range of 10^{0} to 10^{-3}. However, there was a case in which the dilution rate and Tt showed no correlation between the 10^{-4} dilution and the 10^{-5}

Table 1. Threshold time (Tt) measurement for a dilution series of GI type PC solution by the real-time RT-LAMP

Dilution factor	Tt (min)					
	GI trial 1	GI trial 2	GI trial 3	GI trial 4	GI trial 5	GI trial 6
10^{-9}	nd	nd	/	/	/	/
10^{-8}	nd	nd	/	/	/	/
10^{-7}	nd	nd	/	/	/	/
10^{-6}	nd	nd	/	/	/	/
10^{-5}	48.9	nd	nd	nd	nd	41.9
2×10^{-5}	/	/	44.3	nd	43.4	nd
5×10^{-5}	/	/	41.1	nd	36.4	47.5
10^{-4}	50.8	50.4	46.4	nd	nd	42.2
2×10^{-4}	/	/	46.2	38.1	nd	38.4
5×10^{-4}	/	/	42.6	36.9	39.8	35.3
10^{-3}	36.3	36.1	35.1	38.4	nd	34.5
10^{-2}	31.3	32.4	32.8	32.0	29.9	30.2
10^{-1}	28.1	28.9	27.9	28.6	26.8	26.7
1	25.3	25.5	25.1	26.3	22.4	23.9

nd: not detected.
/: not determined.

dilution, although the turbidity was more than 0.1 until 60 min (Fig. 2a). In the case of very low RNA concentration, it was difficult to correlate exactly between virus quantity and Tt. Hence, the data of Tt within 40 min were selected, and the relationship between the dilution rate of the GI PC solution and Tt is shown in Fig. 2b. The logarithm of dilution rate and Tt were strongly negatively correlated. In addition, the reproducibility of the real-time RT-LAMP results using the GI kit was very high, judging from the results of the independent experiments. In this method, it was not possible to determine the absolute quantity of NV-GI RNA, because the accurate quantity of template RNA in the PC solution was unknown. However, it is possible to semiquantitatively determine which sample has a high RNA concentration among the positive samples within 40 min of Tt. As the mean of a series of experiments repeated 6 times ($\pm$SD), a relationship between dilution rate (R_1) and Tt for NV-GI can be represented using the following equation (Eq. 1): $Tt = 24.5(\pm 1.15) - 3.63(\pm 0.14) * \log R_1$, $r^2 = 0.960(\pm 0.051)$, $n = 6$.

Similarly to the results of the quantitative analysis of NV-GI, the results of Tt measurement for the dilution series of PC solution (GII) by real-time RT-LAMP are shown in Table 2. For NV-GII, the samples diluted by 10^{-5} to 10^{-9} were considered negative. In comparison with

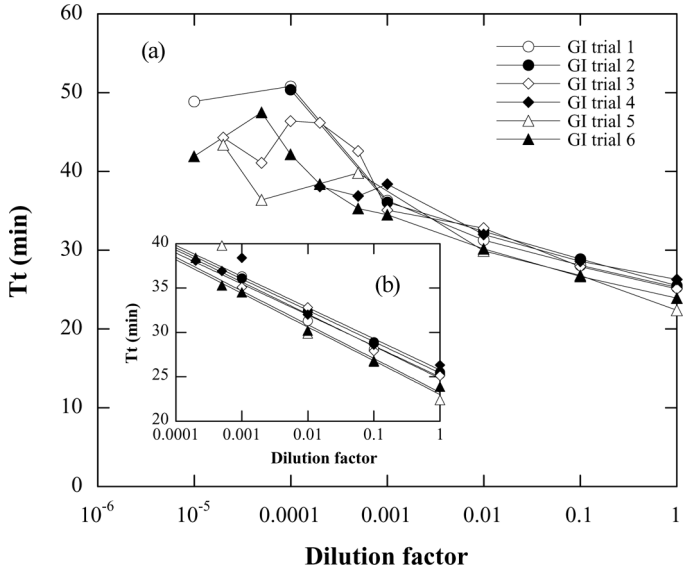


Figure 2. Estimation of quantitative analysis of NV-GI for real-time RT-LAMP. (a) Comparison of threshold time (Tt) with dilution rate of PC solution (GI) by the real-time RT-LAMP. (b) Relationship between dilution rate of PC solution (GI) and selected data of Tt within 40 min.

NV-GI, the detection sensitivity for NV-GII was lower by one order. In support of the result showing the low sensitivity for NV-GII compared with NV-GI, the sensitivity of the NV-GII detection kit in terms of PCR copy number (200 copies/test) as indicated in the manufacturer's instructions is lower than that of the NV-GI detection kit. The relationship between the dilution rate and Tt was uncorrelated within 40 to 60 min of the reaction time (Fig. 3a). The data of Tt within 40 min were selected in the same manner as that for the NV-GI analysis, and the relationship between the dilution rate of the GII PC solution and Tt is shown in Fig. 3b. The logarithm of the dilution rate and Tt for NV-GII were strongly negatively correlated, similar to the results of the GI quantitative analysis. The relationship between the dilution rate (R_2) and Tt for NV-GII can be represented using the following equation (Eq. 2): $Tt = 22.8(\pm 1.34) - 5.06(\pm 0.48) * \log R_2$, $r^2 = 0.982 \pm 0.011$, $n = 6$.

Concentration Effect of NVs in Foam

The generation volumes of foam water for casein-added and casein-free trials using sample A were 14.5 and 8.2 mL for 10 min, respectively.

Table 2. Threshold time (Tt) measurement for a dilution series of GII type PC solution by the real-time RT-LAMP

Dilution factor	Tt (min)					
	GII trial 1	GII trial 2	GII trial 3	GII trial 4	GII trial 5	GII trial 6
10^{-9}	nd	nd	/	/	/	/
10^{-8}	nd	nd	/	/	/	/
10^{-7}	nd	nd	/	/	/	/
10^{-6}	nd	nd	/	/	/	/
10^{-5}	nd	nd	nd	nd	nd	nd
2×10^{-5}	/	/	nd	nd	nd	nd
5×10^{-5}	/	/	nd	nd	nd	nd
10^{-4}	nd	nd	nd	56.3	nd	nd
2×10^{-4}	/	/	nd	44.8	44	nd
5×10^{-4}	/	/	55.9	41.1	nd	nd
10^{-3}	36.9	39.4	54.9	39.6	36.9	41.7
10^{-2}	32.5	36.9	32.0	32.6	33.2	33.3
10^{-1}	27.5	29.8	26.1	26.4	27.0	26.2
1	23.6	25.0	22.7	22.5	22.3	22.4

nd: not detected.
/: not determined.

Because casein functioned as a frother, the foam generation volume increased with casein added. NVs in wastewater, treated water using casein and foam water were determined by real-time RT-LAMP. GI-NVs were absent in all the samples (Fig. 4a), whereas GII-NVs were detected in all the samples (Fig. 4b). The Tt of treated water was 54.7 min, and the amplification time has markedly delayed in comparison with that of wastewater (Tt, 36.8 min). The NV-GII RNA concentration in the wastewater was markedly decreased by foam separation. In contrast, the reaction time for turbidity increase in the case of foam water was the most rapid (Tt, 32.5 min) among these samples. Thus, on the basis of Tt, foam water showed the highest RNA concentration among these samples. According to Eq. 2, the NV-GII RNA concentration in foam water was 7.1-fold higher than that of wastewater. In addition, those in treated water and foam water without casein were also determined (Fig. 5). The sample for the casein-free trial was identical to the wastewater (sample A) used in the case of casein added. The reaction time for turbidity increase in the case of the treated water was markedly delayed compared with that of wastewater. As in the case of casein added, the NV-GII RNA concentration in treated water also markedly decreased in the case of the casein-free condition. The reaction time for turbidity

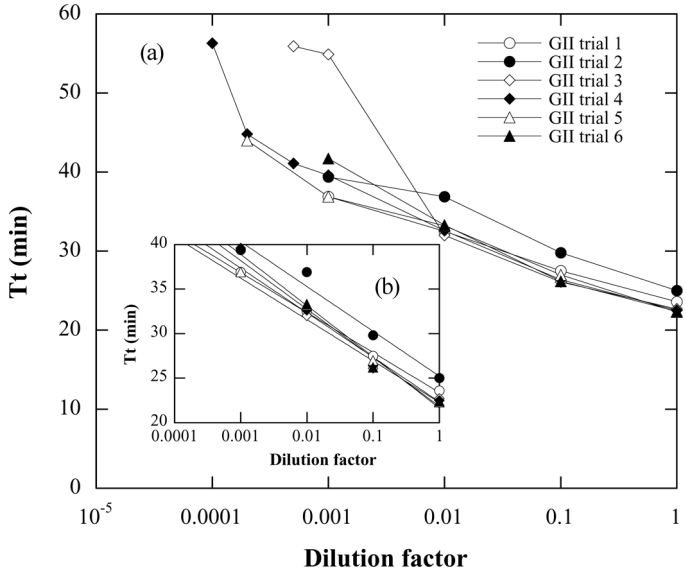
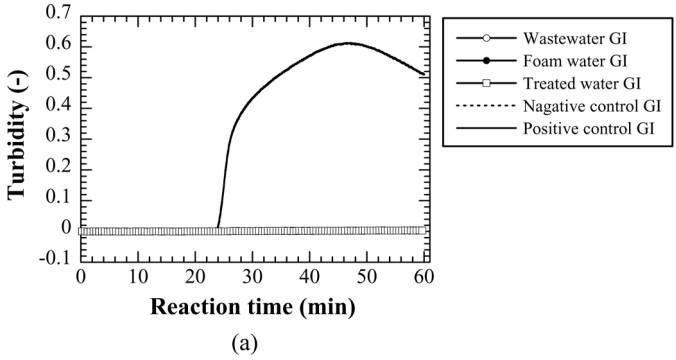


Figure 3. Estimation of quantitative analysis of NV-GII for real-time RT-LAMP. (a) Comparison of threshold time (Tt) with dilution rate of PC solution (GII) by the real-time RT-LAMP. (b) Relationship between dilution rate of PC solution (GII) and selected data of Tt within 40 min.

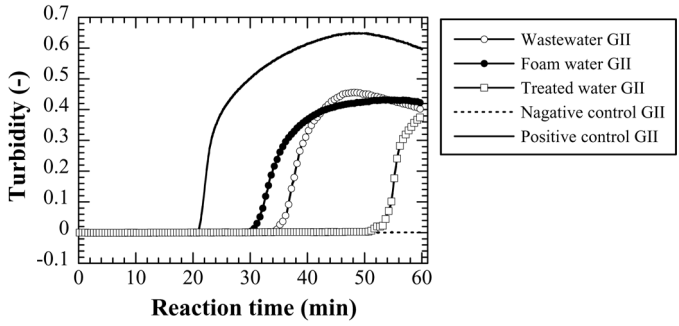
increase in the case of foam water (Tt, 32.1 min) was shorter than that of wastewater. According to Eq. (2), the NV-GII RNA concentration in foam water without casein was 8.5-fold higher than that in wastewater.

To confirm the concentration effect of NVs from wastewater into foam, the experiment on foam separation was repeated using a different sample B. Though, no NV-GI was detected in the sample B, the positive reaction of NV-GII with turbidity increase was clearly observed in wastewater and foam water. However, the treated water was negative for NV-GII. Then, the concentration effect of NVs in the foam was confirmed by a dilution method as positive or negative. The RNA extract for positive samples was serially diluted to 10^{-4} using the elution buffer, and the dilutions were analyzed again. The 10^{-1} to 10^{-4} dilutions of the wastewater extract resulted in negative results (Table 3). In contrast, the 10^{-1} dilution of the foam water extract showed a positive result. From the results of the dilution method for extracts, it was confirmed that the NV-GII RNA concentration in the foam water was much higher than that in the wastewater.

It was clear that NV-GII was removed from the wastewater and concentrated into the generated foam by foam separation using dispersed



(a)



(b)

Figure 4. Real-time turbidimetry of LAMP reactions for wastewater, treated water and foam water using casein. (a) Analysis for NV-GI, (b) analysis for NV-GII.

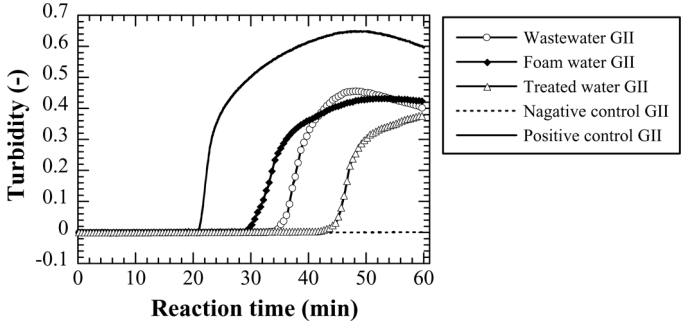


Figure 5. Real-time turbidimetry of LAMP reactions of NV-GII for wastewater, treated water and foam water without casein.

Table 3. Detection of NV-GII for a dilution series of RNA extract by the real-time RT-LAMP (Sample B)

	Dilution factor				
Sample	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Wastewater	+	−	−	−	−
Foam water	+	+	−	−	−
Treated water	−	/	/	/	/

+: positive.
−: negative.
/: not determined.

bubbles. Although casein as a surface-active protein was added to the wastewater before processing to increase the removal efficiency, there were no differences in the removal and concentration of NV-GII between the casein-added and casein-free conditions. Casein did not function as a collector of NV-GII. Since the hydrophobic sites existed on the NVs surface, NVs were able to adsorb onto the bubble interface without the collector. Foam separation without casein will be applied to the removal of NVs from the water in which stable foam is generated using dispersed bubbles, such as municipal wastewater.

Effect of Separation Time on NV Removal

The effect of separation time on NV removal was examined for separation times as short as 3 min as well as 10 min, which is sufficient for foam generation. The comparison of the samples obtained in the case of 3 min and 10 min is shown in Fig. 6. The reaction time for turbidity increase in the case of the treated water set for 3 min was much shorter than that for 10 min, and Tt was shortened from 54.7 min to 35.3 min (Fig. 6a). The residual concentration of NV-GII in the treated water under the short-time condition was higher than that under the long-time condition. The removal efficiency became poor with decreasing separation time. In addition, the Tt in the case of foam water increased with decreasing separation time, and the concentration effect of NV-GII into the foam water also decreased. In the case of casein-free condition, the removal efficiency of NV-GII from the wastewater and the concentration effect into foam water also decreased with decreasing separation time, as in the case of the casein-added condition (Fig. 6b). In the foam separation using dispersed bubbles, the separation time was an important

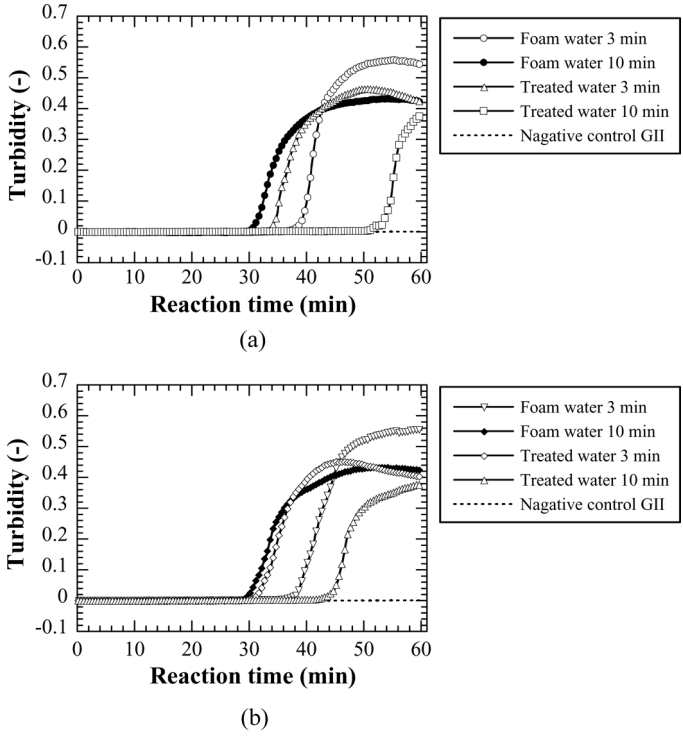


Figure 6. Comparison of real-time turbidimetry of LAMP reactions of sample obtained from separation time for 3 min and 10 min. (a) Casein added, (b) without casein.

factor, which determined the removal efficiency and the concentration effect for NVs.

CONCLUSIONS

The relationship between the dilution rate of the PC solution as a positive standard of NV RNA (GI and GII) and threshold time (Tt) as the endpoint of RNA amplification was examined by real-time RT-LAMP. The logarithm of the dilution rate and Tt were strongly negatively correlated for both NV-GI and NV-GII. The real-time RT-LAMP using the NV detection kits was not only for detection but also for the semiquantitative evaluation of NVs. Although NV-GII was detected in two wastewater samples, NV-GI was not detected. By foam separation using dispersed bubbles, NV-GII was removed from wastewater and concentrated into

the generated foam. According to the correlation of the dilution rate and Tt, the concentration of NV-GII RNA in the foam water was 8-fold higher than that in the wastewater.

For effervescent water, such as municipal wastewater and domestic drainage containing surfactants, foam separation using only dispersed bubbles can be used for the NV removal. The foam separation is easy to carry out for continuous processing (35) and can be applied to the removal and collection of NVs from a large volume of water. Therefore, it is highly possible to develop techniques of reducing NVs from various types of wastewater, city water, and industrial water. In addition, because the generated foam can be analyzed by real-time RT-LAMP, it is possible to sensitively monitor NVs in aqueous solutions.

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