Notification of the Ministry of Public Health

On Determination of the Number of Helminth Eggs and Escherichia Coli and Sampling Methods and Examination of Helminth Eggs and Escherichia Coli in Effluent and Sludge Already Undergone Sewage Disposal System

B.E. 2561 (A.D. 2018)

Whereas it is deemed expedient to determine the number of helminth eggs and Escherichia coli and sampling methods and examination of helminth eggs and Escherichia coli in effluent and sludge that have already been undergone sewage disposal system.

By virtue of the provisions of Clause 15 of the Ministerial Regulation on Sewage Management Hygiene, B.E. 2561 (A.D. 2018), the Minister of Public Health by advice of the Public Health Committee hereby issues this Notification as follows:

Clause 1. This Notification shall be referred to as "Notification of the Ministry of Public Health on Determination of the Number of Helminth Eggs and Escherichia Coli and Sampling Methods and Examination of Helminth Eggs and Escherichia Coli in Effluent and Sludge Already Undergone Sewage Disposal System, B.E. 2561 (A.D. 2018)".

Clause 2. This Notification shall come into force after the expiration of three hundred sixty days from the date of its promulgation in the Government Gazette.

Clause 3. In this Notification,

"Helminth eggs" means living helminth eggs.

Clause 4. In discharging water and sludge that have already been undergone the sewage disposal system, the quantity of helminth eggs and Escherichia coli contained in such effluent and sludge must be as follows:

Test	Type	Criteria
Helminth eggs	Effluent	Less than 1 egg per L
	Sludge	Less than 1 egg per g (dry weight)
Escherichia coli	Effluent	Less than 1,000 MPN (Most Probable Number) per 100 ml
	Sludge	Less than 1,000 MPN (Most Probable Number) per g (dry weight)

(Translation)

Clause 5. Sampling methods are as follows:

(1) A sampling method to examine helminth eggs shall be grab sampling, by scooping up water from the last tank of a disposal system, or from the final point before discharging water to the environment. A sampling of effluent shall be collected at the depth center point for tanks of which the depth does not exceed 2 m and collected at 1 m depth. For tanks of which the depth exceeds 2 m, the sampling shall be collected at 3 L of effluent and contained in a plastic container of which the capacity is 4 to 5 L.

(2) For the sampling method to examine Escherichia coli, it prescribes that a sampling of effluent shall be collected from the last tank at the depth of 30 cm, or from its receptacle at the assessment point by collecting approximately 100 mm of effluent and containing it in a glass bottle of which the capacity is 125 ml. In this regard, the glass bottle must be sterilised at a temperature range of 160-180^{o} C for 2 hours and stained internally with 0.1 ml of 10% concentrated Sodium Thiosulphate solution. Its cork shall be wrapped with an aluminum sheet and the bottle itself shall be contained in a can made of stainless steel.

In the case where the examination cannot be carried out immediately, it prescribes that the sampling of effluent shall be kept in a container and the temperature shall be maintained between 4 to 10^{o} C. In this regard, the examination shall be carried out within 24 hours.

Clause 6. As for the sampling method employed for sludge, it prescribes that samplings shall be collected from a pile of sludge by radomising at least 100 g from each of the 10 points and using it as specimens. Mix all the sludge samplings thoroughly into one pile and divide it into 4 piles of equivalent quantity. Then, randomise two opposite piles and mix them. Grab 400 g of sludge and put it in a clean plastic bag for helminth eggs examination, and grab another 100 g of sludge and put it into a plastic bag or a clean and sterilised container for Escherichia coli examination.

In the case where the examination cannot be carried out immediately, it prescribes that the samplings of effluent shall be kept in containers and the temperature shall be maintained between 4 to 10 ^{o} C. In this regard, the examination shall be carried out within 24 hours.

Clause 7. In examining the quantity of helminth eggs and Escherichia coli contained in effluent and sludge that have already been undergone a sewage disposal system, it prescribes that the person responsible for sewage management shall examine the quantity of helminth eggs contained in effluent and sludge based on the manual annexed hereto, and shall examine the quantity of Escherichia coli contained in effluent and sludge based on the Most Probable Number (MPN) method, or Multiple Tube Fermentation Technique (Standard Method Part 9221) at least once a year, and shall report the examination result to its local government. In

the case where a local government is responsible for the examination itself, it shall report the result to the Provincial Public Health Committee, or Bangkok Metropolitan Public Health Committee, as the case may be.

Announced on the 19th Day of November 2018

Piyasakol Sakolsatayadorn

Public Health Minister

(Translation)

Manual Attachment

(a) Examination of the quantity of helminth eggs contained in effluent from which sewage has already been removed shall be as follows:

1. Tools, equipment, and chemical substances used are as follows:

1.1 1000-ml conical cylinder

1.2 200-ml beaker

1.3 50-ml plastic centrifuge tube

1.4 15-ml plastic centrifuge tube

1.5 Vortex

1.6 Suction pump, or other equipment that can pump liquid

1.7 Centrifuge

1.8 Automatic pipette

1.9 Microscope slides

1.10 Parafilm

1.11 22x22 mm microscope cover glass

1.12 0.1% TritonX-100 solution

1.13 Formal saline (100 ml of 40% formalin, 9 g of sodium chloride per l)

1.14 Ethyl acetate

1.15 Specific gravity of sodium chloride solution 1.20 (SG 1.20), specific gravity of saturated sugar solution 1.27 (SG 1.27), specific gravity of saturated zinc sulfate 3.0 (SG 3.0)

1.16 0.85% sodium chloride

2. Procedures

The examination shall be divided into 3 steps. The first step shall be a simple test, or simple-centrifugal sedimentation by concentrating the samplings of effluent through centrifugal sedimentation and reviewing the obtained sediment in a microscope. If helminth eggs are found, such result must be reported, and the next testing step shall not be required. However, if none of helminth eggs are found, the second step shall be performed by using formalin-ethyl acetate sedimentation, removing grease and dirt from the remaining sediment, and reviewing the obtained sediment in a microscope to examine helminth eggs. If helminth eggs are found, such result must be reported, and the next testing step shall not be required. However, if none of helminth eggs are found, the third step shall be performed through a method of floatation by using a solution that has a proper specific gravity, examining floating helminth eggs through a microscope and reporting the result.

(Translation)

2.1 Examination by the use of simple-centrifugal sedimentation

2.1.1 Prepare a 1000-ml conical cylinder and label a sampling code number.

2.1.2 Shake the bottle that contains the sampling of effluent and pour 1 L of the water sampling into the prepared conical cylinder. However, if there are large pieces of sediment contained in the sampling, such sampling shall then be first filtered through a 2-layered piece of gauze.

2.1.3 Leave it at the room temperature for at least 12 hours and carry out 2.1.4, or spin it at 1,000xg for 15 minutes in order to settle it, and skip to 2.1.8.

2.1.4 When the time is served, supernatant shall be pumped out and 200 ml of liquid at the bottom of the container shall be remained.

2.1.5 Spin the conical cylinder in order to mix the liquid and the sediment, and to wash away the pellets sticking on the side of the container, and then pour it into a 500-ml beaker.

2.1.6 Spray 0.1% TritonX-100 solution in order to wash away the pellets sticking on the side of the container and pour it into the beaker which contains the water sampling.

2.1.7 Pour the water sampling from the beaker into a 50-ml plastic centrifuge tube and spin it by using the centrifuge at the speed of 1,000xg for 15 minutes.

2.1.8 Pump out supernatants and spin the water sampling again until the supernatant is entirely removed.

2.1.9 After the last spin, the supernatant shall be pumped out until its quantity is approximately 3 times of that of the sediment, and then use an automatic pipette by cutting the pipette tip to obtain a wide hole. Pump all the mixture into a 15-ml plastic centrifuge tube. Measure the quantity of the mixture of sediment in ml.

2.1.10 Examine helminth eggs from the sampling by using a microscope based on the following details:

2.1.10.1 Prepare 2 microscope slides and label numbers on them.

2.1.10.2 Drop 50 µl of 0.85 sodium chloride solution on the microscope slides.

2.1.10.3 Use the automatic pipette to pump 50 µl of the sampling by cutting the pipette tip to obtain a wide hole. Then drop it on the microscope slides. Stir the sampling with sodium chloride solution and close it with 22x22 mm microscope cover slides.

2.1.10.4 Examine helminth eggs from both microscope slides by using the microscope.

- In the case where none of helminth eggs are found, it prescribes that the remaining sampling in the beaker shall be concentrated through the use of Formalin-Ethyl Acetate Sedimentation according to 2.2.

- If helminth eggs are found, the number of helminth eggs per 1 L of water shall be calculated based on the number of helminth eggs counted and the quantity of the mixture of sediment under 2.1.9 in ml (V_1) by using the following formula:

Helminth eggs per 1 L of water = total number of helminth eggs counted from 2 slides x V_1 x 10

If the calculation result turns out to be less than 1 egg per L, the number of helminth eggs shall be further calculated according to 2.2. However, if the calculation result turns out to be more than 1 egg per L, the number of helminth eggs found per 1 L of water shall be reported.

2.2 Examination by the use of formalin-ethyl acetate sedimentation

2.2.1 Spin the sediment remained from 2.1.10 by using the centrifuge at the speed of 1,000xg for 15 minutes, and then remove supernatants.

2.2.2 Add 4 ml of formal saline. Shake it by using Vortex.

2.2.3 Add 2 ml of ethyl acetate by using a volumetric plastic test tube, cap it with a parafilm or an enclosure. Then, firmly shake it for 30 times in order to thoroughly mix it and leave it untouched for 10 minutes.

2.2.4 Spin it by using the centrifuge at the speed of 1,000xg for 15 minutes. Then, use a stick to remove the pellets sticking on the side of the container, wherein there is a connecting line between the layers of ethyl acetate and formalin, and pour out the supernatants.

2.2.5 Add 0.85% sodium chloride, the quantity of which is 3 times of that of the sediment. Use a stirrer to mix sodium chloride and sediment and note down the capacity of the sediment mixture in ml.

2.2.6 Examine helminth eggs in the sampling under 2.2.5 by following 2.1.10.1 to 2.1.10.4 and calculate the result based on the formula provided.

- If none of helminth eggs are found, it prescribes that the sediments remained from 2.2.6 shall be further examined by employing the method of floatation according to 2.3.

- If helminth eggs are found, it prescribes that the number of helminth eggs per 1 L of water shall be further calculated based on the number of helminth eggs counted and on the capacity of sediment mixture under 2.2.5 (V_2) in ml by using the following formula:

Helminth eggs per 1 L of water = total number of helminth eggs counted from 2 slides x V_2 x 10

If the calculation result turns out to be less than 1 egg per L, the number of helminth eggs shall be further calculated according to 2.3. However, if the calculation result turns out to be more than 1 egg per L, the number of helminth eggs found per 1 L of water shall be reported.

2.3 Examination by the use of floatation

2.3.1 Execute the following steps to the sampling of sediment under 2.2.6.

2.3.2 Pour 0.85% Sodium Chloride into a centrifuge tube until it reaches 14 ml and seal the tube with a parafilm or an enclosure. Invert the tube 5 times in order to mix the sediment with sodium chloride.

2.3.3 Spin it by using the centrifuge at 1,000xg for 15 minutes, and then pour out the supernatants.

2.3.4 Repeat Steps 2.3.2 to 2.3.3 one more time in order to wash out the sediment and completely eradicate formalin and ethyl acetate.

2.3.5 Add specific gravity of saturated sodium chloride 1.20 (SG 1.20) or specific gravity of saturated sugar solution 1.27 (S.G 1.27) or specific gravity of saturated zinc sulphate 3.0 (SG 3.0) into a centrifuge tube until it reaches 6 ml. Use a stirrer to stir it and add the solution until the liquid reaches the upper edge of the tube.

2.3.6 Place both pieces of 22x22 mm microscope cover slides on the mouth of the tube. Make sure that there is no space or a bubble under the microscope cover slides. In the case where saturated sodium chloride or saturated sugar solution is used, wait 15 minutes. Or, in case of saturated zinc sulphate, wait 10 minutes, and then examine helminth eggs that float to the microscope cover slides by using the microscope.

2.3.7 Examine helminth eggs in the sampling by using the microscope and report the result obtained.

- If none helminth eggs are found, report “Not Found”.

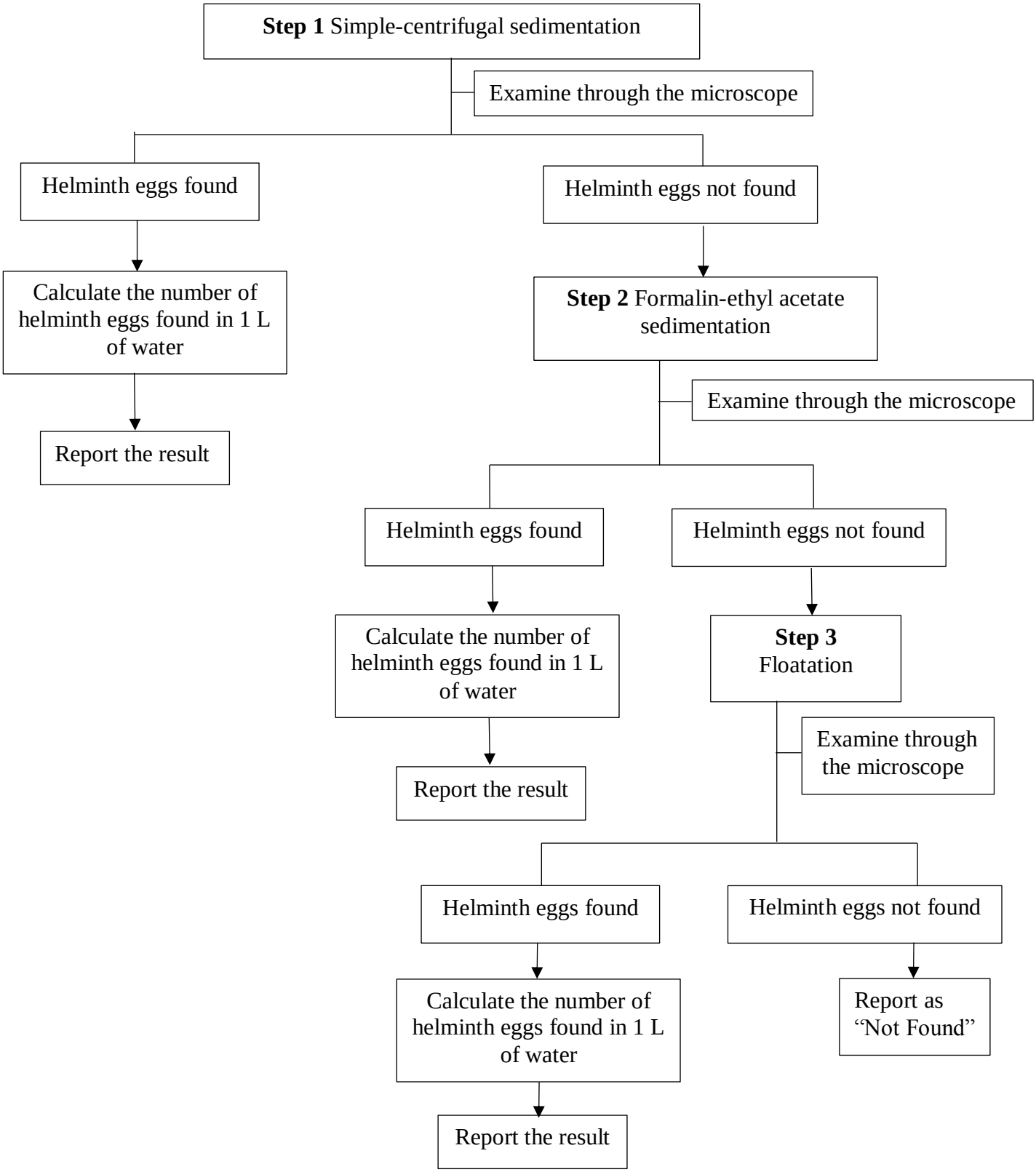
- If helminth eggs are found, it prescribes that the number of helminth eggs per 1 L of water shall be further calculated based on the number of helminth eggs counted by using the following formula.

Helminth eggs per 1 L of water = Total number of helminth eggs counted from 2 slides

If the calculation result indicates less than 1 egg per L, report “Not Found”. However, if the calculation result indicates more than 1 egg per L, the number of helminth eggs found per 1 L of water shall be reported.

(Translation)

Procedures to examine helminth eggs in effluent that has already been disposed of sewage



(b) Examination of the quantity of helminth eggs in sludge shall be carried out as follows:

1. Equipment and chemical substances to be used are:

1.1 1,000-ml conical cylinder

1.2 500-ml beaker

1.3 500-ml cylinder

1.4 15-ml plastic centrifuge tube

1.5 Balance analytical tool

1.6 Suction pump, or any equipment that can pump liquid

1.7 Centrifuge

1.8 Inoculation needle

1.9 Gauze

1.10 Parafilm

1.11 Plastic bag

1.12 Scissors

1.13 Glass rod

1.14 Plasteur pipette

1.15 Bulb plastic pipette

1.16 Automatic pipette

1.17 Microscope glass slide

1.18 22x22 ml microscope cover slide

1.19 Distilled water

1.20 5% Sodium Hypochlorite

1.21 Formal saline (100 ml of 40% formalin, Sodium chloride 9 g per L)

1.22 Ethyl acetate

1.23 Solution for floatation, such as specific gravity of sodium chloride solution 1.20 (SG 1.20), specific gravity of saturated sugar solution 1.27 (SG 1.27), specific gravity of saturated zinc sulfate 3.0 (SG 3.0)

1.24 0.85% sodium chloride

2. Procedures

The operation shall be divided into 3 steps. The first step shall be a simple test, or simple-centrifugal sedimentation by dissolving a sampling of sediment and filtering it in order to remove large-sized garbage, and settle it for 12 hours, or spinning it, and examining the obtained sediment by using a microscope. If helminth eggs are found, such result must be

reported without undertaking the next step. However, if none of helminth eggs are found, the second testing step shall be carried out through a method of formalin-ethyl acetate sedimentation by removing grease and dirt from the remaining sediment and reviewing the obtained sediment in a microscope to examine helminth eggs. If helminth eggs are found, such result must be reported without undertaking the next step.

However, if none of helminth eggs are found, the third step shall be carried out through a method of floatation by using a solution that has a proper specific gravity and examining floating helminth eggs through a microscope and report the result.

2.1 Simple-centrifugal sedimentation

2.1.1 Prepare a 1000-ml conical cylinder and label the sampling number.

2.1.2 Weigh 50 g of sediment sampling and place it into the prepared conical cylinder.

2.1.3 Measure 175 ml of distilled water by using the cylinder and pour the water into the conical cylinder that contains the sampling of sediment.

2.1.4 Measure 75 ml of 5% sodium hypochlorite by using the cylinder and pour it into the conical cylinder that contains the sampling of sediment.

2.1.5 Stir the sediment with the solution by using a glass rod for 30 minutes and filter the mixture through a two-layered piece of gauze which is placed on the 1,000-ml conical cylinder.

2.1.6 Pour 100 ml of distilled water into the same container in order to wash out the remaining sediment, and gradually pour it through the two-layered piece of gauze in order to wash out the sediment.

2.1.7 Leave it at the room temperature for at least 12 hours or spin it at 800xg for 3 minutes to settle.

2.1.8 When the time is served, the supernatants shall be pumped out and the quantity of those remained shall be one time of that of the sediment. Then, note down the capacity of sediment mixture in ml.

2.1.9 Spin the conical cylinder in order to mix the water and the sediment, and to wash away the pellets sticking on the side of the container, and then pour it into a 500-ml beaker.

2.1.10 Examine helminth eggs from the sampling by using the microscope according to the following details:

2.1.10.1 Prepare 2 microscope slides and label a number on them.

(Translation)

2.1.10.2 Drop 50 μL of 0.85 sodium chloride solution on the microscope slides.

2.1.10.3 Sway the beaker to mix the sediments. Use the automatic pipette to pump 50 μL of the sampling by cutting the pipette tip to obtain a wide hole. Then drop it on the microscope slides. Stir the sampling with the sodium chloride solution and close them with 22x22 mm microscope cover slides.

2.1.10.4 Review both microscope slides in the microscope.

- In the case where none of helminth eggs are found, it prescribes that the sampling remained in the beaker shall be concentrated through the method of formalin-ethyl acetate sedimentation according to 2.2.

- If helminth eggs are found, the number of helminth eggs per 1 g of sludge shall be calculated based on the number of helminth eggs counted and on the quantity of the mixture of sediment under 2.1.8 in ml (V_1) by using the following formula:

Helminth eggs per 1 g of sludge = Total number of helminth eggs counted from 2 slides x V_1 x 0.2

If the calculation result indicates less than 1 egg per g, the number of helminth eggs shall be further calculated according to 2.2. However, if the calculation result indicates more than 1 egg per g, the number of helminth eggs found per 1 g of sludge shall be reported.

2.2 Examination by the use of formalin-ethyl acetate sedimentation

2.2.1 Shake the sediment remained from 2.1.10 and pour it into two 15-ml plastic centrifuge tubes until it almost fills up the tube, or reaches approximately 14 ml.

2.2.2 Spin the 2 tubes of sediment sampling by using the centrifuge at 800xg for 3 minutes and then remove the supernatants.

2.2.3 Add formal saline into the tube until the liquid contained therein reaches 9 ml and use the inoculation needle to break down the sediment at the bottom of the tube.

2.2.4 Add ethyl acetate into the tubes until the liquid reaches 13 ml by using the bulb plastic pipette. Seal the tube with a parafilm or an enclosure and then shake it firmly for approximately 30 times in order to mix up the substances. Leave it untouched for 10 minutes.

2.2.5 Spin it by using the centrifuge at the speed of 800xg for 3 minutes. Then, use a stick to remove the pallets sticking on the side of the tube, wherein there is a connecting line between the layers of ethyl acetate and formalin, and pour out the supernatants.

2.2.6 Add 0.85% sodium chloride, the quantity of which is 3 times of that of the sediment. Use a stirrer to mix sodium chloride and sediment and note down the capacity of sediment mixture in both tubes in ml.

2.2.7 Examine helminth eggs in the sampling under 2.2.6 by following 2.1.10.1 to 2.1.10.4. Examine 2 slides per tube.

- If none of helminth eggs are found, it prescribes that the sediments remained from 2.2.6 shall be further examined by employing the method of floatation according to 2.3.

- If helminth eggs are found, it prescribes that the number of helminth eggs per 1 g of sludge shall be further calculated based on the number of helminth eggs counted and on the quantity of sediment mixture under 2.1.8 (V_1) and under 2.2.6 (V_2) in ml unit by using the following formula:

$$\text{Helminth eggs per 1 g of sludge} = \frac{\text{Total number of helminth eggs counted from 4 slides} \times V_1 \times V_2}{280}$$

If the calculation result indicates less than 1 egg per g, the number of helminth eggs shall be further calculated according to 2.3. However, if the calculation result indicates more than 1 egg per g, the number of helminth eggs found per 1 g of water shall be reported.

2.3 Examination by the use of floatation

2.3.1 Applies the following steps to both tubes of the sediment sampling under 2.2.6.

2.3.2 Pour 0.85% sodium chloride into a centrifuge tube until it reaches 14 ml and seal the tube with a parafilm or an enclosure. Invert the tube for 5 times in order to mix the sediment with sodium chloride.

2.3.3 Spin it by using the centrifuge at 800xg for 3 minutes, and then pour out the supernatants.

2.3.4 Repeat Steps 2.3.2 to 2.3.3 two more times in order to wash out the sediment and completely eradicate formalin and ethyl acetate.

2.3.5 Add specific gravity of saturated sodium chloride 1.20 (SG 1.20) or specific gravity of saturated sugar solution 1.27 (S.G 1.27) or specific gravity of saturated zinc sulphate 3.0 (SG 3.0) into a centrifuge tube until it reaches 6 ml. Use a stirrer to stir it and add the solution until the liquid reaches the upper edge of the tube.

2.3.6 Place both pieces of 22x22 mm microscope cover slides on the mouth of the tube. Make sure that there is no space or bubbles under the cover glasses. In the case where saturated sodium chloride or saturated sugar solution is used, wait 1 minute. Or, in case of

saturated zinc sulphate, wait 10 minutes, and then examine helminth eggs that float to the microscope cover slides by using the microscope.

2.3.7 Apply 2.3.6 to the other tube of sampling.

2.3.8 Examine helminth eggs in the sampling by using the microscope and report the result.

- If none helminth eggs are found, report “Not Found”.

- If helminth eggs are found, it prescribes that the number of helminth eggs per 1 g of sludge shall be further calculated based on the number of helminth eggs counted and on the quantity of sediment mixture under 2.1.8 in the ml unit (V_1) by using the following formula.

$$\text{Helminth eggs per 1 g of sludge} = \frac{\text{Total number of helminth eggs counted from 4 slides} \times V_1}{1{,}400}$$

If the calculation result indicates less than 1 egg per g, report “Not Found”. However, if the calculation result indicates more than 1 egg per g, the number of helminth eggs found per 1 g of sludge shall be reported.

(Translation)

Procedures to examine helminth eggs in sludge that has already been disposed of sewage