Removal of Microbes from Raw Sewage by Stepwise Advanced Treatment

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Abstract

Both the stepwise advanced treatment method (experimental system) for reducing the biochemical oxygen demand, total nitrogen, and total phosphorus in secondary treated water and standard activated sludge method (control system) were compared for 14 months. Performance was evaluated by Student’s t-test. The results revealed that the water quality items in the experimental system met the target water quality standard for stepwise advanced treatment and were significantly reduced compared to those in the control system.

Next, the removal efficiency of hygienically relevant microbes (total coliform, Escherichia coli, fecal streptococci, and enterococcus form) was compared. The concentrations of these microbes and removal ratio in the experimental system were significantly reduced and improved compared to in the control system, respectively. Particularly, the concentration of total coliform bacteria met the effluent standards (≤3,000 colony-forming units/mL) before disinfection treatment. The results of Pearson product moment correlation analysis and hierarchical variable cluster analysis indicated that the removal ratio of hygienically relevant microbes in the secondary treatment process were highly correlated with mixed liquor suspended solids, aerobic-solids retention time, dissolved oxygen, sludge age, air-to-flow ratio, and activated sludge biota. These results clarify the operating factors that increase the hygienically relevant microbe removal ratio in the stepwise advanced treatment method.

Key words: stepwise advanced treatment method, logarithmic removal ratio, hygienically relevant microbes, activated sludge biota

1 INTRODUCTION

The standard activated sludge method (standard method) employed for sewage treatment is widely used in domestic sewage treatment plants to remove suspended solids (SS) and reduce the biochemical oxygen demand (BOD). However, removing nitrogen and phosphorus with the standard method can be inefficient, as the aerobic nature of the reaction tank becomes a rate-limiting factor. Here, advanced wastewater treatment facilities, which are said to have higher removal efficiency of BOD, total nitrogen (TN), and total phosphorus (TP) than the standard method, are 41%¹ of the nationwide as of 2013. Therefore, the Japanese government has adopted the “stepwise advanced treatment method” or “stepwise method” to obtain treated water quality similar to that of advanced wastewater treatment by partially modifying the standard method equipment and optimizing operational management. If the treated water quality of the standard method facility operated by the stepwise method is BOD ≤ 15, TP ≤ 3.0, or TN ≤ 20, this facility is regarded as an advanced treatment facility by the government¹. In addition, hygiene and safety, in terms of microbial content, of the

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Effluent water discharged from sewage treatment plants and flow into swimming areas, such as Odaiba marine Park in Tokyo Bay, has drawn negative attention.

In the US and EU, the maximum safety levels of hygienically relevant microbes (HRMs), such as total coliform (TC), fecal coliform (FC), *Escherichia coli* (EC), fecal streptococci (FS), and enterococcus form (EF) have already been designated; we are currently working to ensure hygienic safety by developing similar environmental and drainage standards. For operation of the stepwise method, the effects of partition walls in the reaction tank, sludge treatment wastewater, and rainfall have already been studied.

Although the TC concentration for discharged water is regulated, there are no reports on the removal of other HRMs due to a lack of a regulatory mandate. Therefore, we compared the operational results of the stepwise and standard methods for 14 months to evaluate water quality factors including BOD, TN, and TP. Additionally, the removal performance of HRMs (TC, EC, FS, and EF) was investigated. Finally, we clarified the associations between the HRM removal ratio, operation items, water quality items, and activated sludge biota in the secondary treatment process.

# 2 MATERIALS AND METHODS

## 2.1 Experimental site

From November 2013 to the end of December 2014, experiments were conducted at sewage treatment plants with separate-type regional systems in Saitama Prefecture. The maximum sewage treatment capacity of treatment facilities No.1-8 is 628,770 m³/day. Among treatment facilities using the standard method, No. 5 and No. 6 were experimental systems (capacity, 65,200 m³/day × 2 series), whereas No. 3 and No. 4 were control systems (65,200 m³/day × 2 series).

## 2.2 Stepwise method operating conditions

The operating conditions were as follows. (1) The daily inflow sewage volume of the experimental/control system was set to more than half of the design capacity, in accordance with the requirements for the stepwise method. (2) The sludge return ratio based on inflow sewage was set to approximately 45% for the experimental system and 30% for the control system. (3) In the experimental system, the mixed liquor suspended solids (MLSS) concentration was set to ≥1,500 mg/L, which is the standard value for anaerobic/aerobic operation of the reaction tank. On the other hand, in the control system, was set to ≥1,000 mg/L, which is a conventional control value in this treatment plant. (4) In the experimental system, anaerobic adjustment of the reaction tank was performed by reducing the opening of the end blower valve on the anaerobic tank (1/4 section of reaction tank) to approximately 10%. (5) The air-to-flow ratio of the aerobic tank (2/4–4/4 section of reaction tank) was set so that the nitrification reaction in the experimental system would maintain dissolved oxygen (DO) at ≥0.5–1.0, and other control systems were set to suppress the nitrification reaction.

## 2.3 Experimental method

### 2.3.1 Sewage and sludge sample collection and field measurements

The following sewage samples were collected for water quality testing and microbial level testing: sample of sewage flowing into the treatment plant (crude sewage), first sedimentation basin effluent (primary effluent), returned sludge, activated sludge at the 4/4 section of the reaction tank, secondary effluent (experimental/control treated water), and disinfected discharge water (discharge water). Samples were collected 2–4 times per month. Each sample or measurement was collected for a single sample at 10:00 h. Figure 1 shows the processing flow and sampling points. The DO was measured at the end of the 4/4 section, and the oxidation-reduction potential (ORP) was measured at the end of the 1/4 section. DO was measured by the diaphragm electrode method (galvanic cell type, YSI Pro20 long probe type, YSI Nanotech Co., Ltd., Kanagawa, Japan) and ORP was measured using the platinum electrode method (calomel comparative electrode, RM–30P long probe type, DKK-TOA Corporation, Tokyo, Japan). The indicated value was read when
each detection unit was immersed at 1.0 m below the water surface for approximately 10 min. In addition, the inflow sewage volume, air-to-flow ratio, and sludge return ratio were continuously measured with an automatic measuring device.

### 2.3.2 Sewage and sludge sample analysis

The collected samples were immediately returned to the test room and the sewage test was performed. The pH, SS, BOD, COD, TN, and TP were chemically analyzed, and ammonia nitrogen (NH$_4^+$-N) was analyzed by ion chromatography after serially filtering the sample through 1.0- and 0.2-μm pore membrane filters. The activated sludge samples were analyzed to determine the activated sludge concentration, return sludge concentration, and sludge settling velocity (SV). The sludge volume index (SVI), aerobic-solids retention time (A-SRT), and sludge age (SA) were calculated based on the water quality analysis results. Further, denitrification and dephosphorization ratios were calculated according to Eq. (1) and Eq. (2).

**Denitrication ratio**

\[
\frac{\text{Primary treated water TN} - \text{Secondary treated water TN}}{\text{Primary treated water TN}} \times 100
\]

**Dephosphorization ratio**

\[
\frac{\text{Primary treated water TP} - \text{Secondary treated water TP}}{\text{Primary treated water TP}} \times 100
\]

#### 2.3.3 Quantification of HRM

TC was cultured for 24 h at 37°C using desoxycholate-agar (Eiken Chemical Co., Ltd., Tokyo, Japan), EC was cultured for 24 h at 37°C using specific enzyme substrate agar-nutrient medium (ELMEX Co., Ltd., Tokyo, Japan), FS was cultivated at 37°C for 24 h using Escherin-agar (Oxoid Ltd., UK.), and EF was cultured for 48 h at 37°C using KF-agar (Oxoid Ltd., UK.). The lower limit of quantification was set to 1.0 CFU/mL.

#### 2.3.4 Identification and quantification of activated sludge organisms

Activated sludge in the 4/4 sections of the reaction tank was collected, and 0.05 mL of the sample was used for analysis. Biological species present were identified using an optical microscope (100 × E6F type; Nikon Corporation, Tokyo, Japan) and compared to photographs and illustrations in the sewage test method. Using the quantitative results from activated sludge organisms, the diversity index of the activated sludge of the experimental and control systems was calculated using Shannon’s formula.

#### 2.4 Analysis method

A box-and-whisker plot was constructed to compare the HRM concentration and log removal ratio of each process. After performing an F test (rejection rate 5%) on the experimental/control system to determine whether the analysis items had equal variance, Student’s t-test (rejection rate 5%) or Welch t-test (rejection rate 5%) was performed, and $p < 0.05$ was considered as
statistically significant. The relationship between the logarithmic removal ratio of HRM, operation items, water quality items, and activated sludge organisms was analyzed using the Pearson’ product moment correlation coefficient and $|r| \geq 0.4$ was determined to be correlated. Hierarchical cluster analysis was performed using the aggregation method to examine the relationships between correlated items. The word method was used as the distance calculation method after the merger, a tree diagram of each variable item was created from the distance after the merger, and the clusters for similar items were compared.

3 RESULTS AND DISCUSSION

3.1 Operation item status

The values obtained for the operational parameters during the experimental period are shown in Table 1. The crude sewage volumes per day were $\geq 59\%$ and $> 60\%$ in the experimental and control systems, respectively, relative to the target value for our system. Compared to the control system, items showing an increase in the experimental system were the air-to-flow ratio (0.6-fold), sludge return ratio (14%), MLSS (700 mg/L), returned sludge concentration (300 mg/L), DO (1.7 mg/L), A-SRT (3.6 d), SA (8 d), denitrification ratio (28%), and dephosphorization ratio (12%). In contrast, decreased items were SVI (100 points), ORP (20 mV), and BOD-SS loading (0.06 points).

The sludge return ratio, MLSS, and air-to-flow ratio of the experimental system met the operating conditions of the stepwise method described in section 2.2.

The actual air-to-flow ratio of the experimental system was 5.0 times, which was half of previously reported values\(^4,5\). This is because our reaction tanks had four compartments, allowing for different DO concentrations to be maintained between the anaerobic/aerobic zones and maintaining a higher DO in the aerobic zone. In addition, the effect of DO consumption via inflow of the return water from sludge treatment was minimized and the air-to-flow ratio was reduced.

The sludge return ratio in the experimental system was 44%, which is approximately 6% lower than that reported in previous studies\(^4,5\). This is because the return pump capacity of the experimental facility is rate-limiting. However, as the denitrification ratio was improved by 28% compared to in the control system, we concluded that the denitrification reaction in the sludge return line, which is the treatment principle of the stepwise method, was fully functioning. The dephosphorization ratio was $\geq 80\%$ in both

| Table 1 Operational parameters |
|-------------------------------|
| Items | Unit | Experimental system | Control system |
|-------|------|---------------------|----------------|
| Flow of influent | Q/d/ max volume (%) | 73 (59-93) | 74 (60-100) |
| Air-to-flow ratio | Flow ratio | 5.0 (4.3-5.8) | 4.4 (2.7-6.7) |
| Sludge return ratio | Rs (%) | 44 (20-47) | 30 (21-38) |
| Mixed liquor suspended solids | MLSS (mg/L) | 1,900 (1,700-2,300) | 1,200 (920-1,800) |
| Return sludge suspended solids | RSSS (mg/L) | 6,100 (4,700-8,400) | 5,800 (3,700-9,100) |
| Sludge volume index | SVI | 160 (100-270) | 260 (150-430) |
| ORP (1/4 section of the reaction tank) | (mV) | -140 (-46--220) | -120 (-6--190) |
| DO (4/4 section of the reaction tank) | (mg/L) | 3.0 (1.5-5.0) | 1.3 (0.6-2.7) |
| Aerobic-solids retention time | A-SRT (d) | 7.7 (5.2-9.8) | 4.10(2.6-5.6) |
| Sludge age | SA(d) | 22 (9.0-32) | 14 (8.5-26) |
| Hydraulic retention time | HRT (h) | 11 (7.1-13) | 11 (8.0-13) |
| BOD-SS loading | kg BOD/kg MLSS×d | 0.11 (0.03-0.20) | 0.17 (0.09-0.22) |
| Denitrification ratio | (%) | 52 (40-62) | 24 (4.5-33) |
| Dephosphorization ratio | (%) | 94 (87-98) | 82 (46-96) |

Note: n = 49; mean (minimum - maximum)
systems and showed only small differences. This is because the 1/4 section of the reaction tank consumed a large amount of DO because of the inflow of a large amount of organic substances. The anaerobic state may have been maintained even in the control system under aerobic conditions, and biological release of phosphorus and reabsorption occurred. It has been reported that phosphorus removal becomes unstable\(^6\)-\(^8\), but the distribution of ORP and DO in each part of the reaction tank likely contributed to improving the phosphorus removal ratio and was stable in this experiment. The maximum BOD-SS loading in the experimental/control system was in the range of 0.2–0.3, indicating moderate growth of activated sludge ciliates\(^9\).

3.2 Status of treated water quality

The treated water quality was compared using a box-and-whisker plot (Fig. 2). The maximum values (mg/L) in the experimental system were 4.7 for BOD, 14 for TN, and 0.5 for TP, satisfying the target water quality requirements. The pH of the experimental system was 6.8 (range, 6.5–7.5), BOD (mg/L) was 1.6 (range, 0.5–4.7), and fluctuation range was larger than that of the control system. This may be because of the consumption of alkalinity, and residual NH\(_4\)-N, as the nitrification reaction progresses. In addition, the fluctuation range of TN and TP were lowered by adjusting the air-to-flow ratio based on the processing principle of the stepwise method. According to t-tests, the experimental results were significantly different (\(p < 0.001\)) for pH, SS, COD, BOD, NH\(_4\)-N, TN, and TP compared with the control system.

3.3 HRM concentration reduction performance

3.3.1 HRM concentration of inflow sewage and each treatment process water

The HRM concentrations of influent sewage and each treated water sample are shown in Fig. 3.

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Fig. 2  Performance comparison of secondary treated water quality.

The secondary treated water quality of the experimental/control systems was compared with a box-and-whisker plot, and the concentrations of both were analyzed by Student’s t-tests (asterisk indicates significant difference between experimental/control system, * \(p < 0.05\); ** \(p < 0.01\), samples: 49 samples each).
Influent sewage and primary treated water

The order of HRM concentration (CFU/mL) in the influent sewage and primary treated water was the same: TC and FS were $10^5$ and EC and EF were $10^4$. Previous studies of influent sewage\textsuperscript{10,11} showed that TC was $10^5-10^6$, EC was $10^5$, FS was $10^4$ and EF was $10^4$. The order of TC and FS differed between the experimental results and previous studies. One reason for this is that FS is more abundant than FC in feces and its survival ratio in the aquatic environment is better than that of FC\textsuperscript{12}. Therefore, this difference may be because of the different survival ratio in sewage.

Secondary treated water

TC, EC, and FS counts (CFU/mL) were $10^2$ and EF was $10^1$ in the experimental system. In the control system, TC and FS were $10^4$ and EC and EF were $10^2$. Thus, the HRM of the experimental system was reduced by approximately one order of magnitude relative to the control system. Moreover, the HRM concentration in the experimental system was significantly reduced ($p < 0.001$) as compared to in the control system.

This may be because operation of the stepwise method improved the solid-liquid separation performance of the activated sludge and, as described below, increased the feeding efficiency of the activated sludge organisms for HRM.

In addition, the TC of the experimental system satisfied the effluent standards ($\leq 3,000$ CFU/mL)\textsuperscript{13} for discharged water. This is expected to lead to a reduction in the amount of disinfectant used and impact on aquatic organisms in the discharge area. The treated water of the standard method facility that promotes nitrification reaction may meet the drainage standard even before disinfection\textsuperscript{10}. The stepwise method is advantageous for not only BOD, TN, and TP removal, but also for HRM reduction.

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**Fig. 3** HRM removal performance in each water treatment process.

Removal performances of HRM in the experimental/control systems were compared with a box-and-whisker plot, and concentrations in the experimental/control systems were analyzed via Student’s t-tests (asterisk indicates significant difference between experimental/control system, * $p < 0.05$; ** $p < 0.01$, samples: 49 samples each). In addition, discharged water showed undetectable data (N.D.), and thus the mean and minimum (min.) of the elements could not be drawn. The items were added to the figure.
**Discharged water**

In discharged water, the concentration of TC, EC, FS, and EF were $10^1$ CFU/mL, corresponding to the levels reported previously\(^{11}\). The fluctuation range of HRM was largest compared to other processed waters. As the experimental facility uses a batch-type chemical injection system, rather than a continuous injection system, the injection volume of the chemical does not necessarily follow the fluctuations in the treated water volume and water quality. As a result, the disinfection effect on HRM likely changes because of differences in the contact time and chemical concentration required for disinfection. The FS showed higher resistance than TC to sodium hypochlorite\(^{14}\).

### 3.3.2 HRM log removal ratio for each process

The HRM removal ratio in each treatment process is shown in Fig. 4.

**Primary processing**

The primary treatment removal ratio based on inflow sewage ranged from $\leq -0.1$ Log for TC to $0.21$ Log for EF. The negative removal ratio may be related to excess sludge being returned to the primary settling tank\(^{10}\). However, in the experimental facility, sludge treatment system wastewater may return to the grid chamber. Considering the concentration levels of influent and primary effluent during the experiment, HRM may not have been removed by primary treatment.

**Secondary processing**

The logarithmic removal ratio (based on the primary effluent) was $2.5$–$2.7$ Log for the experimental system and $1.8$–$2.0$ Log for the control system. According to a survey of eight locations nationwide including advanced wastewater treatment facilities, the HRM removal ratio in the secondary treatment process was $2$–$3$ Log\(^{11}\). The removal ratio of

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Fig. 4  HRM removal ratio for each treatment process. For each treatment process, the logarithmic removal ratio of HRM was compared using a box-and-whisker plot. Thereafter, the logarithmic removal ratios of the experimental and control systems were analyzed via Student's *t*-test (asterisk indicates significant difference between experimental/control system, * $p < 0.05$; ** $p < 0.01$, samples: 49 samples each).
the experimental system was within the range of literature values and the same level as that after advanced wastewater treatment. The t-tests for the secondary treatment process showed that the experimental system had a significantly higher HRM removal ratio than the control system \((p < 0.001)\).

**Total inflow sewage-based removal ratio**

The overall removal ratio, including the disinfection process, was highest for TC (4.59), followed by FS (4.30) > EC (3.98) > EF (3.66). The maximum value removal ratio of all HRMs exceeded 5 Log.

3.4 Activated sludge biota and microbial species

HRM in the influent sewage was removed by more than two digits in the secondary treatment process. The removal efficiency is considered to depend largely on the activated sludge biota in the reaction tank\(^{15}\).

Therefore, the relationship between the activated sludge biota and HRM removal was examined by comparing the activated sludge biota in the experimental and control systems. The composition ratios (%) and primary species are shown in Table 2. The dominant species in activated sludge biota were ciliates (54%) in the experimental system and flagellates (51%) in the control system. The total number and range were considered as nearly identical in the experimental and control systems, whereas the proportions of the constituent species were significantly different and biodiversity index value was also significant at \(p < 0.001\). This may be due to the differences in operating conditions between the experimental and control system, as the amount of water flowing into the reaction tank and water quality was almost equal.

| Classification | Experimental system | Control system |
|----------------|---------------------|----------------|
| **Flagellates** |                     |                |
| (sessile)      | 12 (1-43)           | 51 (16-86)     |
|                | Peranema sp.         | Monas sp.      |
|                | Epistylis sp.        | Vorticella sp. |
|                | Opercularia sp.      | Vaginicola sp. |
|                |                     |                |
| **Ciliates**   | 28 (3-66)           | 10 (0-35)      |
| (floating)     | 13 (0-71)           | 9 (0-21)       |
|                | Aspidisca sp.        | Aspidisca sp.  |
| (swimming)     | 13 (1-51)           | 14 (2-47)      |
|                | Paramecium sp.       | Litonotus sp.  |
|                | Vorticella sp.       |                |
|                | Opercularia sp.      |                |
| **Rhizopods**  | 28 (8-54)           | 14 (0-51)      |
|                | Arcella sp.          | Arcella sp.    |
| **Metazoans**  | 6 (0-19)            | 2 (0-14)       |
|                | Rotaria sp.          | Rotaria sp.    |
|                | Lecane sp.           | Lecane sp.     |
|                | Chaetonotus sp.      | Chaetonotus sp.|
|                | Macrobiotus sp.      |                |
|                | Diplodaster sp.      |                |

**Activated sludge organism population (N/mL)**

- Experimental system: 4,600 (1,800-9,500)
- Control system: 4,300 (1,100-8,000)

**Shannon-Wiener index \((H')\)**

- Experimental system: 2.1 (1.4-2.5)**
- Control system: 1.8 (0.8-2.4)

**Note 1:** \(n = 49\); mean ( ): range of appearance ratio for the species during the experiment.

**Note 2:** \(n = 49\); mean ( ): range of population or Shannon-Wiener index \((H')\) value

**Note 3:** Shannon-Wiener index \((H') = - \sum P_i \times \log P_i\)

- \(S\) is the number of species contained in mL of activated sludge.
- \(P_i\) is the ratio of species i population to the total population of activated sludge (mL)

**Note 4:** Student’s t-tests. (asterisk indicates the significant difference between experimental/control system, * \(p <0.05\); ** \(p <0.01\))
The ciliate (sessile) Vaginicola sp., metazoan Macrobiotus sp., and Diplogaster sp. were characteristic organisms in the experimental system, whereas the flagellate Entosiphon sp. was characteristic in the control system. The flagellates likely became dominant in the control system because of their greater ability to resist low DO compared to ciliates. Previous studies of the selective dietary habits of activated sludge organisms against bacteria showed that the ciliate Vorticella sp. has a narrow dietary habit and consumes specific gram-negative and positive bacteria, whereas for metazoan Rotatoria sp., growth was good when gram-negative bacteria were fed. Additionally, when fed multiple bacterial species, the number of most ciliates was increased.

Considering the experimental results and the findings of previous studies, it is necessary to diversify the activated sludge biota by increasing various activated sludge organisms with different food habits to increase the removal ratio of HRM during the secondary treatment process. Thus, the stepwise method was found to be more suitable than the standard method.

### 3.5 Relationship between HRM removal ratio, reaction tank operation items, secondary treated water quality, and activated sludge organisms

#### 3.5.1 Correlation analysis

Pearson product-moment correlation analysis was performed to evaluate the HRM removal ratio, reaction tank operation items, secondary treated water quality, and activated sludge organisms in the experimental and control systems (Table 3).

### Table 3 Correlation between operation items, water quality items, biota, and logarithmic removal ratio of the hygienically relevant microbes

| Items               | TC      | EC      | FS      | EF      | average |
|---------------------|---------|---------|---------|---------|---------|
| MLSS                | 0.66**  | 0.53**  | 0.65**  | 0.73**  | 0.64    |
| A-SRT               | 0.67**  | 0.51**  | 0.62**  | 0.69**  | 0.62    |
| Denitrification ratio | 0.63**  | 0.49**  | 0.66**  | 0.71**  | 0.62    |
| DO (4/4section)     | 0.59**  | 0.55**  | 0.54**  | 0.56**  | 0.56    |
| Sludge age          | 0.57**  | 0.50**  | 0.49**  | 0.52**  | 0.52    |
| Air-to-flow ratio   | 0.50**  | 0.44**  | 0.47**  | 0.51**  | 0.48    |
| Dephosphorization ratio | 0.52**  | 0.36**  | 0.47**  | 0.51**  | 0.47    |
| RSSS                | 0.22*   | 0.22*   | 0.21*   | 0.27**  | 0.23    |
| HRT                 | 0.19    | 0.20    | 0.20*   | 0.18    | 0.19    |
| Influent flow       | −0.19   | −0.36** | −0.17   | −0.09   | −0.20   |
| ORP (1/4section)    | −0.19   | −0.17   | −0.24*  | −0.22*  | −0.21   |
| BOD-SS Loading      | −0.56** | −0.41** | −0.55** | −0.65** | −0.55   |
| SVI                 | −0.64** | −0.54** | −0.56** | −0.58** | −0.59   |
| BOD                 | −0.33** | −0.50** | −0.40** | −0.42** | −0.41   |
| TP                  | −0.50** | −0.33** | −0.43** | −0.47** | −0.43   |
| SS                  | −0.44** | −0.39** | −0.58** | −0.52** | −0.48   |
| TN                  | −0.62** | −0.45** | −0.65** | −0.70** | −0.61   |
| COD                 | −0.64** | −0.46** | −0.66** | −0.69** | −0.61   |
| pH                  | −0.70** | −0.57** | −0.65** | −0.72** | −0.66   |
| NH₃-N               | −0.71** | −0.56** | −0.72** | −0.76** | −0.69   |
| Metazoa             | 0.42**  | 0.28**  | 0.49**  | 0.48**  | 0.42    |
| Rhizopoda           | 0.44**  | 0.35**  | 0.40**  | 0.41**  | 0.40    |
| Ciliate (sessile)   | 0.38**  | 0.34**  | 0.39**  | 0.41**  | 0.38    |
| Ciliate (floating)  | 0.16    | −0.01   | 0.16    | 0.24*   | 0.14    |
| Ciliate (swimming)  | −0.01   | −0.05   | 0.01    | −0.02   | −0.02   |
| Flagellate          | −0.48** | −0.34** | −0.44** | −0.47** | −0.43   |

Note 1: Correlation matrix; The single correlation coefficient between the two variables of the HRM removal ratio and operation items, water quality items, and activated sludge organisms is summarized for each item.

Note 2: Uncorrelated test of population correlation coefficient, the null hypothesis that "the correlation coefficient is 0" was tested; * p < 0.05; ** p < 0.01, n = 98
Among the operation items (|r| ≥ 0.4) showing a correlation with the HRM removal ratio, the positively correlated variables were MLSS, A-SRT, denitrification ratio, DO, SA, air-to-flow ratio, and dephosphorylation ratio. An increase in each variable promotes the nitrification/denitrification reaction in the stepwise method. In contrast, negatively correlated variables included SVI and BOD-SS loading. A smaller SVI led to better solid-liquid separation performance of activated sludge, and the BOD-SS loading in nitrification/denitrification treatment is smaller than that of the standard method. Thus, the operations performed by the stepwise method contributed to improved the HRM removal ratio.

All secondary treatment water quality items were negatively correlated with the HRM removal ratio. NH₄-N, pH, and TN showed a higher correlation than other parameters. The decrease in NH₄-N and pH reflects the nitrification reaction, and that in TN reflects the denitrification reaction. For the correlation between the HRM removal ratio and increase/reduction in the number of organisms during diversification of activated sludge biota, metazoans, rhizopods, and ciliates (stickiness) showed positive correlations and flagellates showed a negative correlation. Rhizopods (Arcella sp.) feed on nitrifying bacteria, and an increase in their population indicates higher nitrification activity.

### 3.5.2 Cluster analysis

Regarding items correlated with the HRM removal ratio (|r| ≥ 0.4), the strength of the mutual relationship was divided into groups by cluster analysis and shown in a dendrogram (Fig. 5). In the cluster of the HRM removal ratio, the distance following merger calculation was 0.90. Values closer to this number showed a stronger relationship. That is, 0.91 was classified into the first cluster (operation item of the stepwise method including HRM removal ratio), 1.0 was classified into the second cluster (negative correlation items), and 1.25 was classified into the third cluster (activated sludge organisms and air-to-flow ratio). The fourth cluster is 1.45 because the BOD was predicted to contain a positive error due to the remaining NH₄-N. As described above, the distances between the first to third clusters were short. Therefore, to improve the HRM...
removal ratio by the stepwise method, it is necessary to improve the correlation between operation items, water quality items, and activated sludge organisms. It was found that MLSS had the closest relationship with the HRM removal ratio among the first cluster. The concentration of MLSS is about 1.5 times higher than that of the control system for the purpose of removing nitrogen and phosphorus by the stepwise method. Therefore, in order to improve the dephosphorization ratio by providing a difference in DO between the anaerobic tank and the aerobic tank, the DO concentration and air-to-flow ratio for maintaining aerobic conditions are also increased. Nitrification reaction is promoted by keeping A-SRT and SA higher than the control system. In addition, the denitrification ratio is improved by keeping the sludge return ratio higher than that of the control system. These operations in the reaction tank promote the aggregation of activated sludge flocs as they mature, which improves the solid-liquid separation performance in the final settling tank and improves the treated water quality. With respect to activated sludge organisms, it is considered that the predation action of gram-negative and positive bacteria on HRM is improved by the stabilization of activated sludge flocs and the diversification of biota. According to the activated sludge treatment experiments with and without protozoa, soluble BOD, viable cell count and E. coli were clearly reduced in the sewage treatment water in the presence of protozoa\(^{22,23}\). Moreover, not only protozoa, but also small metazoans, *Philodina* sp. prey on dispersive bacteria, and *Aeolosoma* sp. prey on cohesive bacteria\(^{24}\). In this way, we believe that the diversification of activated sludge biota will support a wide range of predation according to the existence form of gram-negative and positive bacteria, leading to improved HRM removal. In the present study, we found that operation items of the stepwise method also includes factor that promote diversification of activated sludge biota. Taken together, the stepwise method has a high HRM removal effect, and is expected to be widely used not only as a method for reducing eutrophication in the water environment, but also as a method for improving the sanitary status of water bodies.

### 4 CONCLUSIONS

To lower BOD, TN, and TP in secondary treated water, HRM removal performance and the relationship with operation items, water quality items, and activated sludge biota were investigated for 14 months in two treatment systems. The stepwise method was operated to increase the MLSS, air-to-flow ratio, and sludge return ratio compared to the standard method. As a result, the treated water quality (target mg/L) of the stepwise method was 6.6 (≤ 15) for BOD, 14 (≤ 20) for TN, and 0.5 (≤ 3.0) for TP. The HRM concentration (CFU/mL) of the treated water was 10² for TC, EC, and FS and 10¹ for EF. The logarithmic removal ratio of HRM in the secondary treatment process was 2.5–2.7 Log. The treated water quality, HRM concentration, and HRM removal ratio were significantly improved relative to those in the standard method. Particularly, TC met the effluent standards (≤ 3,000 CFU/mL) even before disinfection.

The improvement in the HRM removal ratio by the stepwise method likely resulted from the combined effect of MLSS, A-SRT, SA, DO, air-to-flow ratio, and activated sludge biota. It is important to manage the operation so that the biota of activated sludge feeding on bacteria, including the HRM, is enriched.

To understand the mechanism of HRM removal in the reaction tank, quantitative analysis of activated sludge, water quality, and HRM should be performed in each part of the reaction tank, and feeding experiments with activated sludge organisms and HRMs should be conducted.

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