and estimated parameters are summarized in Table 1.

The data used to determine the C/E ratio were the *E. coli* and *Campylobacter* concentrations in the Meuse River that were measured 22 times in 1994 (Medema *et al.*, 2006). In general, there is a large variation in pathogen to *E. coli* ratios. Therefore, PDFs were applied to the distribution of these ratios. As a result, the Lognormal distribution was best fitted as shown in Table 1. The variation of C/E ratio given by the Lognormal distribution includes all factors such as faecal contamination by livestock and waterfowl, possible contamination caused by combined sewer overflows, agricultural activity, rainfall, snow melting, water temperature, and so on (Hijnen *et al.*, 2005). However, this is not the C/E ratio in the Bethune Polder water. Since it is assumed that the C/E ratio has large uncertainty, the impact of the C/E ratio on the yearly risk of infection was analyzed.

Overall removal efficacy and yearly risk of infection

The mean and median of the overall log reduction were estimated to be 7.46 $\log_{10}$ and 6.22 $\log_{10}$, respectively, by the Monte Carlo simulation as shown in Table 2. It is noteworthy that the overall log reduction was estimated to be 7.46 $\log_{10}$ (median) instead of setting the overall removal to 100%, although most of the *E. coli* concentrations in SSF-treated water are 0. Since this calculated overall reduction is applied to the *E. coli* concentrations in the source water, the *E. coli* concentrations in the treated water were not estimated to be 0 as shown in Table 2. As a result, the mean was calculated to be 1.64×10^{-4} *E. coli*/100 mL, and the median was 4.35×10^{-6} *E. coli*/100 mL. The *E. coli* dose is calculated by multiplying the *E. coli* concentration in the drinking water by water consumption. The mean and median were estimated to be 2.99×10^{-4} and 1.24×10^{-8} *E. coli*/day, respectively.

After translating the *E. coli* dose into the *Campylobacter* dose using the C/E ratio, the *Campylobacter* dose-response relationship was applied. As a result, the daily risk of *Campylobacter* infection was estimated to be a mean of 6.51×10^{-6} infection/person/d and a median of 9.24×10^{-11} infection/person/d. As a result of calculating the yearly risk of infection from the daily risk of infection, a mean of 1.68×10^{-3} infection/person/yr and a median of 3.37×10^{-8} infection/person/yr were obtained. This estimate is called the base case (shown in Table 3). The Dutch drinking water regulations require water companies to comply with a 10^{-4} yearly risk of infection

Table 1 - Probability density functions (PDF) fitted to the target variables.

		PDF type	Estimated parameters
E.coli in the source water (*E.coli*/100 mL)		Gamma	$\mu = -2.50$; $\lambda = 383$; $\rho = 0.674$
Treatment efficacy (log reduction) of *E.coli*	Coagulation-storage	Logistic	$\mu = 1.48$; $\lambda = 0.15$
	RSF	Weibull	$\mu = 1.74$; $\lambda = 0.59$; $\rho = 2.38$
	Ozonation	Normal	$\mu = 1.91$; $\sigma = 0.88$
	SSF	Triangular	Min. = 2.00; MEC (Mean Elimination Capacity) = 2.40; Max. = 4.20
C/E ratio		Lognormal	$\mu = 0.0415$; $\sigma = 0.104$

Table 2 - Statistics estimated in the QMRA.

	Lower 95% CI boundary	Median	Mean	Upper 95% CI boundary
Overall log reduction	5.41	7.46	6.22	9.58
E. coli in the treated water (*E. coli*/100 mL)	1.07×10^{-8}	4.35×10^{-6}	1.64×10^{-4}	9.25×10^{-4}
E. coli dose (*E. coli*/day)	0	1.24×10^{-8}	2.99×10^{-4}	1.36×10^{-3}
Campylobacter dose (*Campylobacter*/day)	0	1.35×10^{-10}	9.52×10^{-6}	3.36×10^{-5}
Daily risk of infection (infection/person/d)	0	9.24×10^{-11}	6.51×10^{-6}	2.30×10^{-5}
Yearly risk of infection (infection/person/yr)	0	3.37×10^{-8}	1.68×10^{-3}	9.06×10^{-3}
DALYs (yr)	0	6.37×10^{-5}	2.71	1.49×10

Table 3 - Uncertainty analysis of the yearly risk of infection.

		Yearly risk of infection (infection/person/yr)			Note
		Lower 95% CI boundary	Mean	Upper 95% CI boundary	
Base case		0	1.68×10^{-3}	9.06×10^{-3}	
Amsterdam-Rhine canal water (ARK-water)		0	1.72×10^{-3}	8.60×10^{-3}	
Removal efficacy by SSF with high temperatures		0	1.01×10^{-3}	3.72×10^{-3}	
C/E ratio	0.001 (0.1%)	0	6.59×10^{-5}	3.54×10^{-4}	Min. (WHO, 2004)
	1 (100%)	0	2.53×10^{-2}	2.84×10^{-1}	Max. (Smeets, 2008)
Dose-response model	Maximum risk curve	0	2.30×10^{-3}	1.27×10^{-2}	
	Beta-Poisson	0	4.24×10^{-3}	2.77×10^{-2}	$\alpha = 0.024$, $\beta = 0.011$ (Tenuis *et al.*, 2005)
Date method		0	3.18×10^{-2}	4.72×10^{-1}	

target by a site-specific QMRA. Therefore, a mean of 1.68×10^{-3} infection/person/yr would not meet this requirement.

Sensitivity analysis

Sensitivity analyses of the estimated yearly risk of infection were performed (Itoh, 2010). For a sensitivity analysis, Spearman's rank correlation coefficients are computed between the assumed variables and predicted variables. The contribution to variance that is calculated by squaring the rank correlation coefficients indicates the relative importance by showing the percentage of the variance of the predicted variable contributed by each variable in the model.

It was found that the statistical methods used to analyze the water consumption data have large impacts on the results of the sensitivity analysis, although they do not have large effects on the probability of infection. It should be noted that statistical methods

used to analyze water consumption data may complicate the results of sensitivity analysis if the water consumption data are not analyzed by an appropriate statistical method. To avoid this problem, it is preferable to apply a continuous model like the Exponential model rather than a discrete model like the Poisson model.

Among the four treatment steps of coagulation-storage, RSF, ozonation and SSF, ozonation has the greatest impact, which means that the rank correlation coefficient between the inactivation efficacy by ozonation and the yearly risk of infection was the largest. This is because the *E. coli* inactivation efficacy by ozonation varies greatly from 0 to 4 $\log_{10}$. Therefore, the stable performance of ozonation inactivation is the most important to stably produce safe drinking water at the water treatment plant. The mean value of the yearly risk of infection of this treatment plant was estimated to be 1.68×10^{-3} infection/person/yr, which was larger than the required probability of infection ($< 10^{-4}$ infection/person/yr). In order to improve the protection against *Campylobacter* infection among consumers, it can be suggested that it is most effective to stably inactivate organisms by ozonation.

Uncertainty analysis

Impact of ARK-water intake

The volume of ARK-water is 5% of the annual total production at the plant, and the ARK-water is more polluted than the Bethune Polder water. However, detailed data regarding the daily quantity of ARK-water intake, fluctuations in microbe concentrations, etc. are insufficient. Therefore, only *E. coli* concentrations in the Bethune Polder water were used to analyze the base case, and the effects of ARK-water intake were examined in the uncertainty analysis. ARK-water is used mainly in the summer for three months from June to August. Therefore, it is assumed that 20% of ARK-water is added to water from the Bethune Polder during this time frame. The *E. coli* concentration in ARK-water was set to 1.75 times higher than the concentrations in Bethune Polder water (Hijnen *et al.*, 2005). The results of calculating the yearly risk of infection are shown in Table 3. Although the mean value slightly increased to 1.72×10^{-3} infection/person/yr, ARK-water intake did not greatly affect the risk of infection.

Impact of the removal efficacy by SSF with high water temperatures

The removal efficacy of SSF under conditions with water temperatures above 13°C was determined three times using a pilot-scale plant, while the removal efficacy under conditions with water temperatures below 13°C was determined six times (Dullemont *et al.*, 2006). When incorporating this data, it is possible to expect that the removal efficacy by SSF is not constant but can change in a year, depending on the water temperature. However, the influence of water temperature on the removal efficacy has not been investigated for the three other treatment steps. Therefore, the removal efficacy by SSF with high water temperatures was examined in the uncertainty analysis.

A maximum value of 5.6 $\log_{10}$, MEC of 3.6 $\log_{10}$ and minimum value of 3.1 $\log_{10}$ were obtained when the removal efficacy was determined three times. A triangular distribution with these parameters was constructed. This means that the removal efficacy increases when water temperatures are high compared to the removal efficacy when water temperatures are below 13°C (MEC 2.4 $\log_{10}$). The water temperature is

above 13°C for approximately five months from May to September. Therefore, the removal efficacy by SSF during this time period was set to the above values. The results of calculating the yearly risk of infection are shown in Table 3. The mean value slightly decreased to 1.01×10^{-3} infection/person/yr. The effect of increasing the removal efficacy of SSF more than one $\log_{10}$ over five months within one year is apparent.

Impact of the C/E ratio

For the base case, a Lognormal distribution with a mean value of 0.0415 and a standard deviation of 0.104 was assigned to the distribution of the C/E ratio obtained from measurements from the Meuse River. On the other hand, the concentration ranges of enteric pathogens and faecal indicators in different types of source water are described by reviewing scientific literatures in the WHO Guidelines for Drinking Water Quality (2004). Based on thisinformation, the minimum C/E ratio was set at 0.001 (0.1%). In contrast, recontamination by waterfowl, etc. is observed in the reservoir where the water is stored for an average of 89 days. At another treatment plant, Leiduin of Waternet, the water abstracted from the dunes is stored in an open pond. It has been reported that recontamination occurs in this pond. The yearly variation in the *Campylobacter* and *E. coli* concentrations in this pond shows that the mean *Campylobacter* concentration was approximately 50% of the mean *E. coli* concentration (Smeets, 2008). Based on these data, the maximum C/E ratio was set to 1 (100%).

The results of calculating the yearly risk of infection are summarized in Table 3. When the C/E ratio was set at 0.001 (0.1%), the mean value was estimated to be a very low at 6.59×10^{-5} infection/person/yr. On the other hand, when the C/E ratio was set at 1 (100%), the mean value increased to 2.53×10^{-2} infection/person/yr. Therefore, it is clear that C/E ratio significantly affects the estimated yearly risk of infection. The C/E ratio used for the base model is the value measured in the Meuse River, and not the value for the actual source water. The data of *Campylobacter* concentrations in the Bethune Polder water or the source water after the ARK-water was added to the Bethune Polder water were not available for this study. It should be noted that a strategic monitoring of *Campylobacter* concentration in the source water that can be used to perform the QMRA is necessary. It could help improve the accuracy of the risk calculation model.

Impact of the dose-response model

The maximum risk curve was applied instead of the Exponential model. The maximum risk curve is calculated when the probability that an ingested organism will pass the host's defense mechanisms and find a site suitable for colonisation is maximized and assumed equal to 1. The results of calculating the yearly risk of infection are shown in Table 3. The mean value slightly increased to 2.30×10^{-3} infection/person/yr. Since the maximum risk curve is given by the exponential dose-response function with r = 1, the daily probability of infection P_d is described as $P_d = 1 - \exp(-D)$. This can be approximated by $P_d \approx D$ at low doses. On the other hand, the Exponential model is approximated by $P_d = 1 - \exp(-\gamma D) \approx \gamma D = 0.686 \times D$ at low doses. It can be verified that the ratio between the mean value of 1.68×10^{-3} infection/person/yr by the base model and the mean value of 2.30×10^{-3} infection/person/yr by the maximum risk

curve is close to 0.686. Thus, the maximum risk curve is also an important tool for the uncertainty analysis, providing the upper limit for the possible infection response.

It was described in **Risk calculation** that it is not appropriate to use the Beta-Poisson model (Tenuis *et al.*, 2005). As a trial, the yearly risk of infection was calculated using the Beta-Poisson model. As shown in Table 3, the estimated mean value was 4.24×10^{-3} infection/person/yr, which is larger than the mean value of 2.30×10^{-3} infection/person/yr by the maximum risk curve. Therefore, it was confirmed that the Beta-Poisson model is not appropriate for these analyses.

Comparison between the rank method and date method

It was confirmed that it was appropriate to use the rank method rather than the date and random methods for pairing the microbe concentrations before and after treatment. The removal and inactivation efficacy by coagulation-storage, RSF and ozonation was estimated by the date method for reference. The median value of the overall removal efficacy was 6.18 $\log_{10}$, which was smaller than the median value of 7.46 $\log_{10}$ obtained by the rank method by 1.28 $\log_{10}$. Moreover, the mean value was 2.16 $\log_{10}$ and was much smaller than the mean value of 6.22 $\log_{10}$ by the rank method. This is because a low removal efficacy was often found with the date method, and even "negative removal" could occur. The results of calculating the yearly risk of infection are shown in Table 3. The mean value was estimated to be 3.18×10^{-2} infection/person/yr and was 19 times larger than that obtained by the rank method. Therefore, it is necessary to select an appropriate data pairing method before performing a QMRA.

Summary of the uncertainty analyses

The removal efficacy by SSF increases during seasons with high water temperature. When incorporating this increased removal efficacy, the yearly risk of infection slightly decreased. It is recommended that the influence of water temperature on removal efficacy is investigated for the treatment steps.

It was found that the C/E ratio significantly affects the yearly risk of infection. The C/E ratio used for the base model is the value measured in the Meuse River and not that of the treatment plant source water. It is highly important to determine the C/E ratio in the source water in order to improve the accuracy of the QMRA. In addition, it is preferable to directly monitor the *Campylobacter* concentration, when possible.

With respect to data pairing methods before and after treatment, the estimated yearly risk of infection for the rank method and date method differed 19 times. Therefore, it should be emphasized that it is necessary to select an appropriate data pairing method before performing the QMRA.

Disease burden and costs-of-illness

Estimation of DALYs and costs-of-illness

QMRA primarily focuses on estimating the risk of infection. Waterborne diseases, however, differ in nature, severity and duration. Therefore, it is necessary to use a metric that measures the overall health burden of waterborne diseases, such as the DALYs (Havelaar and Melse, 2003). DALYs are the sum of Years of Life Lost (YLL) by

premature mortality and Years Lived with a Disability (YLD), weighted with a factor between 0 and 1 for the illness severity. Since the health outcomes associated with *Campylobacter* infection are not only diarrhoeal illness but also critical health effects, such as Guillain-Barré syndrome or reactive arthritis, estimations using DALYs are meaningful and important. Havelaar and Melse (2003) determined the DALYs associated with *Campylobacter* infection in developed countries. Since YLD was estimated to be 3.2 and YLL was estimated to be 1.4 per 1000 cases of gastroenteritis, the DALYs were calculated as follows.

$$3.2 + 1.4 = 4.6 \text{ DALYs} \quad (2)$$

The drinking water produced in the Weesperkarspel treatment plant is supplied mainly to the eastern part of the city of Amsterdam and the suburbs. The service population is estimated to be approximately 360,000 persons based on the volume produced (de Moel *et al.*, 2006). Since the mean yearly risk of infection was calculated to be 1.68×10^{-3} infection/person/yr, the number of persons infected yearly in the supply area is:

$$1.68 \times 10^{-3} \times 360{,}000 = 605 \text{ persons} \quad (3)$$

Although infection is necessary to cause disease, not all infections induce symptoms. The process of developing an illness due to an infection is a dynamic phenomenon (Havelaar *et al.*, 2009). The ratio of illness to infection is not simple and can be affected by protective immunity, pathogen dose, area, country, and so on. Setting the ratio of illness to infection is discussed in ***Uncertainty analysis of DALYs***. For this estimation, a conservative ratio of 1 is assumed. In this case, the disease burden based on DALYs would be calculated using the expected number of infections per year for the population multiplied by the DALY contribution per infection:

$$605 \times 4.6 / 1{,}000 = 2.78 \text{ DALYs} \quad (4)$$

The cost of one case of gastroenteritis by *Campylobacter* was estimated to approximately 370 € (if the case is not discounted), which is the sum of direct health care costs, direct non-health care costs, and indirect non-health care costs (Kemmeren *et al.*, 2006). Using this estimate, the costs-of-illness in the distribution area per year are calculated as follows:

$$605 \times 370 \text{ €} = 220{,}000 \text{ €} \quad (5)$$

The DALYs and costs-of-illness demonstrated here were calculated based on values given tentatively and assumptions because of insufficient information. Factors affecting the accuracy of estimated DALYs are severity weights and durations for different outcomes of *Campylobacter* infection for calculating YLD, the probability to develop severe diseases such as reactive arthritis (ReA) after infection, the case-fatality ratio for calculating YLL, and so on (Havelaar and Melse, 2003). A scenario set to estimate the DALYs and costs-of-illness also has assumptions because of the absence of information with respect to use of medical services. For example, only patients visiting a general practitioner would be at risk for ReA (Kemmeren *et al.*, 2006). To improve the accuracy of the estimates, more research is needed, and data collection should focus

on quantitative data. The estimates shown here can be updated if new information becomes available.

Uncertainty analysis of DALYs

The result of calculating the DALYs by the Monte Carlo simulation is shown in Table 2. The mean and median were estimated to be 2.71 and 6.37×10^{-5} year, respectively. The ratio of illness to infection was set at 1 as a conservative value. As discussed above, however, it seems this ratio has a large uncertainty.

Dose-response models applied in microbial risk assessment are established based on some evidence such as that obtained by volunteer experiments. As most dose-response models reach a maximum probability of illness of 1, it is also assumed that the ratio of illness to infection is 1 (Havelaar *et al.*, 2009). These infectious disease models do not usually distinguish between asymptomatic and symptomatic infections.

The best fitting parameter estimates of the unconditional model for illness given dose show that 69% of subjects exposed to a single colony forming unit would become ill (Tenuis *et al.*, 2005), although the second outbreak occurred among schoolchildren during a visit to a daily farm was analyzed. A volunteer experiment conducted by Black *et al.* (1988) demonstrated that the ratio of diarrhoeal illness to infection ranged from 0% to 60%. In developed countries, it has been reported that approximately one-third of all infected patients develop watery diarrhoea or more severe health outcomes (Havelaar and Melse, 2003). A risk assessment study conducted in the Netherlands gave an estimate of more than 9 million infections and approximately 3 million clinical cases of campylobacteriosis per year (Evers *et al.*, 2008). These studies are the examples supporting relatively high ratios of illness to infection.

An exposure, however, would frequently result in asymptomatic infections. It was reported that healthy carriers outnumbered diseased persons by a factor of approximately 80, or in other words, only 1.2% of all excreters of *Campylobacter* are actually experiencing an episode of illness (Tompkins *et al.*, 1999). Similar calculations for the Netherlands (de Wit *et al.*, 2001) suggest the ratio of asymptomatic to symptomatic shedders is 120 : 1.

There are also reported conflicts between estimates of infection with *Campylobacter* from theoretical and empirical studies (Havelaar *et al.*, 2009). As described above, a risk assessment gave an estimate of more than 9 million infections and approximately 3 million clinical cases per year in the Netherlands (Evers *et al.*, 2008). In contrast, a population-based study from the same country indicated that in 1999 the incidence of acute *Campylobacter*-associated gastroenteritis was only 80,000 cases (Mangen *et al.*, 2005). In the latter case, only 1 out of 100 of infections leads to symptoms of campylobacteriosis. Thus, there is an approximately 40-fold difference between the risk-based and the epidemiological estimates of disease incidence. Normal risk assessment models do not take the influence of acquired protective immunity into account. *Campylobacter* infection, however, can develop protective immunity. As a consequence, not every individual in the population is fully susceptible to illness from a *Campylobacter* infection. A high level of exposure would frequently result in asymptomatic infections. A large difference shown above could be explained mainly by

the impact of protective immunity that contributes to reduce the risk of infection and increase the rate of asymptomatic infections. This is accord with the high prevalence of antibodies detected by sero-surveillance (Havelaar *et al.*, 2009).

Based on these information, the minimum ratio of illness to infection can be set at 0.01. A mean obtained by the Monte Carlo simulation was 2.76×10^{-2}, which was 98 times smaller than that obtained in the base case (2.71 shown in Table 2).

DALYs can be basically calculated from the yearly risk of infection shown in Table 3 using the formulas (3) and (4). As a result of comparing the impacts of factors shown in Table 3, it was found that the ratio of illness to infection is the most important factor that affects the DALYs. Currently, both 0.01 and 1 given in estimating DALYs are the ratios having some evidence that can be found in literatures as discussed above. It should be noted that it is important to estimate the ratio of illness to infection in a target area or country to decrease the uncertainty of the DALYs.

CONCLUSIONS

The yearly risk of infection of *Campylobacter* was estimated for a treatment plant in the Netherlands as a model case. First, it was confirmed that it was appropriate to use the rank method rather than the date and random methods for pairing the microbe concentration data before and after the treatment steps. Next, the median and mean overall removal efficacy by the four treatment steps at the plant was estimated to be 7.46 $\log_{10}$ and 6.22 $\log_{10}$, respectively. The mean value of the yearly risk of infection was estimated to be 1.68×10^{-3} infection/person/yr. From the sensitivity analysis, it was noted that it is most effective to stably inactivate organisms by ozonation to stably produce safe drinking water at the water treatment plant.

The uncertainty analysis demonstrated that the factors with large impacts on the yearly risk of infection were the C/E ratio in the source water, the method of pairing the microbe concentration data, and the variation in the removal efficacy of SSF depending on the water temperature. From the obtained results, several important components or variables were identified that can help improve the accuracy of the QMRA estimates, and data collections where the priority should be given were suggested.

Based on the yearly risk of *Campylobacter* infection at the treatment plant, DALYs and costs-of-illness in the distribution area per year were estimated to be 2.78 DALYs and 220,000 €, respectively. The uncertainty analyses of DALYs showed that the ratio of illness to infection is the most important factor that affects the DALYs. It should be noted that it is important to estimate the ratio of illness to infection to decrease the uncertainty of the DALYs.

ACKNOWLEDGEMENT

The author would like to thank Dr. Gertjan Medema and Dr. Partick Smeets from KWR Watercycle Research Institute in the Netherlands for their instruction in performing QMRA.

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銅を用いた水中の微生物の不活化技術の現状と課題

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目的　近年注目を集めている銅を用いた水中の微生物の不活化技術の現状および課題を明らかとする。

方法　国内外の学術雑誌等に掲載された文献情報を基に，銅を用いた微生物の不活化技術の歴史，不活化機構，不活化効果が確認されている微生物，水中の微生物の不活化技術について整理した。

結果　銅を用いた微生物の不活化技術は古くから利用されていたが，1930年代より抗生物質の利用が広まったことから，銅を用いた不活化技術は使用されなくなった。一方で，近年は抗生物質耐性菌の存在が問題視されており，抗生物質に代わる微生物の制御アプローチの1つとして，銅を用いた微生物の不活化技術が再認識され始めている。不活化機構については，その詳細はいまだ明らかとなっていないものの，銅イオン自体の毒性と銅表面に生成される活性酸素による強力な酸化作用によって不活化が起こると推測されている。*Legionella pneumophila*, *Salmonella enterica*, *Mycobacterium tuberculosis* 等の公衆衛生上問題となる多くの病原微生物に対して不活化効果が確認されている。建物内の給水管を中心に多くの水関連設備において，近年銅を用いた不活化技術の導入が検討されており，人への健康影響がほとんど発生しないと推測される水道水質基準を満たす濃度範囲であっても，水中の微生物を不活化可能であることが一部の研究でわかってきた。一方で，不活化効果が短期間に留まることも多く，効果を長期間持続させる技術を開発することが今後の課題であるといえる。また，銅管は残留塩素の低減や消毒副生成の生成にも影響を及ぼしていると報告されており，このようなリスクと不活化効果というベネフィットのアセスメントが今後必要であろう。

結論　銅を用いた水中の微生物の不活化技術には，実用上の課題は残るものの，その有用性は十分に明らかとなっており，病院施設の給水設備等での利用が今後期待される。

Key words：銅，消毒，抗菌性，活性酸素，水衛生

Ⅰ　緒　　言

水の安全性を鑑みた上で，最も重要な要素の1つとして微生物の管理が挙げられる。人体に重大な健康影響を及ぼす病原微生物の制御は，水衛生の歴史の中でも常に最も重要な課題であった。我が国をはじめとする先進国においては，公衆衛生の普及に伴い水系感染症の発生は劇的に減少したが，2010年11月にスウェーデンの Östersund 市で水道経由のクリプトスポリジウムの集団感染症が発生し，約10,000人の推定患者が発生する等[1]，依然として水系感染症が散発している現状にある。

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水の微生物学的安全性を確保する上で最も重要な要素は，水中の微生物の不活化(消毒)技術である。我が国の水道システムにおいては，水道法で義務付けられているように，塩素による微生物の不活化によって，微生物学的に安全な飲料水を供給しているが，クリプトスポリジウムやジアルジア等，塩素に耐性のある微生物も存在しており，塩素消毒以外の様々な不活化技術が提案されている。本稿ではその中でも近年注目を集めている銅を利用した不活化技術について述べる。

Ⅱ　方　　法

銅を用いた微生物の不活化技術については，ドアノブ等の銅固体表面に存在する微生物の不活化効果に関する総説論文が過去に発表されているが[2]，水

中の微生物の不活化効果については報告されていない。そこで本稿では，国内外の学術雑誌等に掲載された文献情報を基に，銅を用いた水中の微生物の不活化技術の現状と課題について整理した。また，銅を用いた微生物の不活化技術の歴史，不活化機構，不活化効果が確認されている微生物の種類についても詳解する。

国内外の文献情報の検索には，㈱科学技術振興機構が運営するJ Dream II（JST文献検索サービス）およびエルゼビア社が運営するScopus（文献検索ツール）を用いた。J Dream IIは，科学技術や医学・薬学関係の国内文献および一部の海外文献を網羅的に検索可能な日本最大級の科学技術文献データベースである。またScopusは，世界の5,000以上の出版社から出版される20,500以上の科学技術・医学等のタイトルを網羅する世界最大級の抄録・引用文献データベースであり，海外文献の網羅的検索に適している。さらに，一般のインターネット検索等によって学術文献以外の情報も収集した。銅（Copper），不活化（Inactivation），消毒（Disinfection），水（Water），水道（Water supply），飲料水（Drinking water）等のキーワードを組み合わせて検索を用い，ヒットした文献の題名・抄録内容から，銅を用いた微生物の不活化技術に関連していると思われる文献を選別した。これらの検索により，学術論文24報，報告書・学会講演集等のその他の文献9件を収集し，それらの情報を整理した。

Ⅲ　結果および考察

1.　銅を用いた微生物の不活化技術の歴史

銅を用いた微生物の不活化技術の歴史は古く，紀元前2,600～2,200年頃には，エジプトにおいて胸部裂傷や飲料水の消毒に利用されており，ギリシャやローマ等でも同様に火傷の処置等に使用されていたと記録されている[3]。19世紀になると，銅工業の従事者がコレラに対する免疫を有していることがフランスで確認されたことから，医学での有効性が認識され，19世紀から20世紀初頭に掛けて，結核や梅毒等の幅広い疾病の処置に無機銅の製剤が使用された[3]。1930年代より，抗生物質の利用が広まったことから，銅を用いた消毒技術は使用されなくなったが，一方で，抗生物質耐性菌の存在が問題視されており，抗生物質に代わる微生物の制御アプローチの1つとして，銅を用いた微生物の不活化技術が再認識され始めている[2]。近年では，ドアノブ[4,5]やシャワーヘッド等の給水装置[6]，貯水瓶[7]等にも抗菌材料として銅の利用が検討されており，幅広い分野での利用が期待されている。米国環境保護庁（EPA）は2008年に銅合金を正式に抗菌材料として登録しており，安全性についても評価している[8]。また硫酸銅は藻類の制御に有効であることが知られており，湖沼や貯水池等では殺藻剤として100年以上前から現在に至るまで世界各地で使用されている[9]。

2.　銅による微生物の不活化機構

銅による微生物の不活化機構の詳細はいまだに明らかとなっていないのが現状であるが，いくつかの不活化機構・ルートが推測されており，本稿では代表的なものを紹介する。

銅イオンから各種の反応を経て活性酸素が発生することが知られており，活性酸素による強力な酸化作用によって微生物の不活化が起こると考えられている。はじめに銅板等から溶出した2価の銅イオンは，次式の通り，システインやグルタチオン等のチオール（スルフドリル）基と反応し，1価の銅イオンを生成する。そして1価の銅イオンは酸素と反応し，2価の銅イオンへと戻るとともに，過酸化水素を生成する。さらに生成された過酸化水素はフェントン反応と類似した反応(式3)によって1価の銅イオンと反応し，2価の銅イオンを生成するとともに，より強力な酸化作用を持つヒドロキシラジカルを生成する。これらの反応によって生成されたヒドロキシラジカルがたんぱく質や脂質を酸化すること等によって，細胞分子に損傷を与えると考えられている[2]。

$$Cu^{2+} + 2RSH \longrightarrow 2Cu^{+} + RSSR + 2H^{+} \quad (1)$$

$$2Cu^{+} + 2H^{+} + O_2 \longrightarrow 2Cu^{2+} + H_2O_2 \quad (2)$$

$$Cu^{+} + 2H_2O_2 \longrightarrow Cu^{2+} + OH^{-} + {\bullet}OH \quad (3)$$

*RSH：チオール；RSSR：ジスルフィド

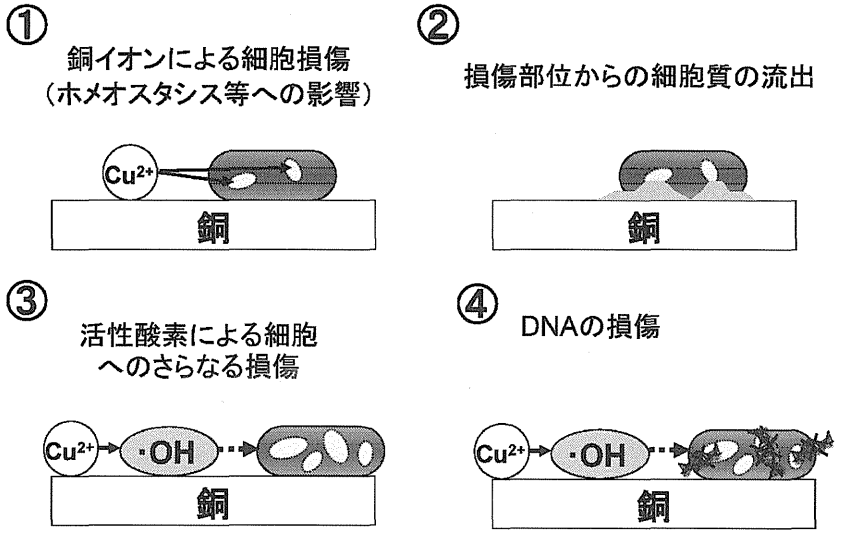
また，銅イオンは微生物の恒常性（ホメオスタシス）に影響を与えることが知られており，この作用も銅を用いた微生物の不活化に関与していると考えられている。このことは，実際に*copA*, *cueO*遺伝子欠損株を用いた実験で実証されており，とくに細胞内損傷の一次機構として重要であると報告されている[10,11]。

これらの情報を踏まえてGrassらは，図1に示す作用順序を提案している[2]。はじめに銅イオン自体の毒性によって細胞の損傷が起こり，損傷部位から細胞質が流出し，その後，銅イオンによって生成されたヒドロキシラジカル等の活性酸素が細胞へさらなる損傷を与える。そして最終的にDNAまで損傷されると推測されている。

3.　銅による不活化効果が確認されている微生物種

表1に示すとおり，これまでに数多くの病原微生物に対して，銅を用いた不活化技術が有効であるこ

図1　銅による微生物の不活化機構・順序の概念図
（文献2を一部改変）



とが確認されている。大部分が，水中の微生物ではなく，銅・銅合金の固体表面に付着させた微生物の不活化効果を調べたものであるが，病原細菌だけでなく，ウイルスや原虫にまで不活化効果があることが明らかとなっている。メチシリン耐性黄色ブドウ球菌（MRSA）等の抗生物質耐性菌にも有効であることは，極めて重要な特徴であり，1節でも述べた通り，銅を用いた不活化技術が近年注目を集めるきっかけとなっている。また，公衆浴場等で増殖し，レジオネラ肺炎を引き起こす *Legionella pneumophila*，細菌性食中毒の主な原因となっている *Salmonella enterica*，結核の原因菌である *Mycobacterium tuberculosis* 等，公衆衛生上問題となっている多くの微生物に不活化効果が認められている点も重要である。なお，水中の微生物の不活化については，4節以降で詳しく解説する。

4.　銅管における水中の微生物の不活化

銅管は，我が国では1923年に大阪医科大学付属病院で給湯用に使用されたのが初めといわれ，1932年には東京市水道局が水道用銅管を採用し，水道用にも使用されるようになった。銅管は一般的に耐食性がよく，温水や水に対して腐食やサビの発生はほとんどみられない。また，コンクリートや土壌に対する耐食性もよく，漏水の発生も少なく，軽量で切断曲げ加工継手による接合の施工性がよい等の特長を持っている[26]。表2に示すとおり，我が国においては，銅管は給湯等の用途で用いられているものの，その利用範囲は限られている[27]。一方，一部の欧米諸国では主要な給・配水用配管として広く用いられている[28]。

笹原らは銅の抗菌作用に着目し，給水用銅管における *Legionella pneumophila* に対する殺菌効果を検討している[20]。銅管では，塩化ビニル管等と異なり，管路表面に *Legionella* の増殖に関与していると考えられているバイオフィルムの形成が確認されず，*Legionella* の不活化効果も確認された。新品の銅管に，*Legionella pneumophila* を含む試験水を充填し，ゆっくり浸透した結果，6時間で99.98％（3.67 $\log_{10}$）の不活化が確認されている。一方，6か月使用した（エージングした）銅管においては，不活化率が1/30程度まで減少することが報告されている。van der Kooij らが実施した同様の調査においても，通水当初においては，ステンレス管等に比べ，銅管内の *Legionella pneumophila* の濃度が有意に低くなることが報告されているが[29]，通水2年後にはその差があまりみられなくなっており，長期間不活化効果を持続させるための工夫を行うことが今後の課題の一つであるといえる。同様に，笹原らは給水用銅管における *Cryptosporidium parvum* のオーシストの不活化についても検討しており，24時間で95.0％（1.3 $\log_{10}$）の不活化率を得ている[25]。この検討では，銅イオン自体の毒性効果は認められなかったことから，銅管表面で発生した活性酸素によって不活化が起こっていると推測された。一方，これらの不活化実験における水中の銅イオン濃度は2～4 mg/Lであったが，この濃度は我が国における水道水質基準値（銅およびその化合物として1 mg/L）よりも高い値であり，より低濃度で不活化に効果があるかどうか今後調査する必要があるといえる。水中の銅は高濃度となると，洗濯物等を変色させる性質を持っており，さらに消化管への急性影響が疑われていることから，WHO でも飲料水水質ガイドライン値として

表1　銅による不活化作用が確認された微生物（文献2を基に情報を追加して作成）

微生物種	反　応	文献番号
細菌		
Acinetobacter baumannii	銅固体表面	12
Acinetobacter baumannii	水中	13
Acinetobacter johnsonii	銅固体表面	14
Brachybacterium conglomeratum	銅固体表面	14
Campylobacter jejuni	銅固体表面	15
Clostridium difficile	銅固体表面	16
EMRSA（流行性メチシリン耐性黄色ブドウ球菌）	銅固体表面	17
Escherichia coli（大腸菌）	銅固体表面	18
Enterococcus hirae（腸内連鎖球菌）	銅固体表面	19
Klebsiella pneumoniae（肺炎桿菌）	銅固体表面	12
Legionella pneumophila	水中	20
Listeria monocytogenes	銅固体表面	21
MRSA（メチシリン耐性黄色ブドウ球菌）	銅固体表面	17
Mycobacterium tuberculosis(結核菌)	銅固体表面	12
Pantoea stewartii	銅固体表面	14
Pseudomonas aeruginosa（緑膿菌）	銅固体表面	12
Pseudomonas aeruginosa（緑膿菌）	水中	13
Pseudomonas oleovorans	銅固体表面	14
Salmonella enterica	銅固体表面	15
Salmonella typhi	水中	7
Salmonella typhimurium	水中	7
Staphylococcus warnerii	銅固体表面	14
Stenotrophomonas maltophilia	水中	13
Vibrio cholerae（コレラ菌）	水中	7
真菌		
Candida albicans	銅固体表面	12
Aspergillus flavus	銅固体表面	22
Aspergillus fumigatus	銅固体表面	22
Aspergillus niger	銅固体表面	22
Fusarium culmonium	銅固体表面	22
Fusarium oxysporum	銅固体表面	22
Fusarium solani	銅固体表面	22
Penicillium crysogenum	銅固体表面	22
Saccharomyces cerevisiae	銅固体表面	23
ウイルス		
Influenza A virus（H1N1）	銅固体表面	24
原虫		
Cryptosporidium parvum	水中	25

設定している（2 mg/L）[30]。また，銅管は残留塩素の低減や消毒副生成物として問題視されているハロ酢酸類の生成にも影響を及ぼしていると報告されており[31]，このようなリスクと不活化というベネフィットのアセスメントが今後必要であろう。

小林らはカワヒバリガイの増殖抑制にも銅管が有効であると報告している[32]。カワヒバリガイはイガイ科に属する比較的小型の淡水棲二枚貝であり，管壁に大量に増殖すると，管路の閉塞等の利水障害を引き起こすことが知られているが[33]，銅配管では，広く用いられているステンレス製配管に比べてカワヒバリガイの増殖が著しく減少することを示している。

5.　銅を利用したその他の水中の微生物の不活化技術

銅およびその合金は，近年，管路以外にも様々な水関連の用途で使用されており，本稿でもその一部を紹介する。

Huangらは病院内における水系感染症を防止するためのオンサイト消毒技術として銅イオン発生装置に着目し，*Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*を対象に不活化実験を行っている[13]。その結果，0.1～0.8 mg Cu^{2+}/Lという我が国の水道水質基準を満たす濃度範囲において，いずれの濃度でも*P. aeruginosa*を1.5時間以内に99.999％（5 $\log_{10}$）以上不活化できることを示している。*S. maltophilia*に関しては，0.2～0.8 mg/Lの範囲で6時間以内に99.999％（5 $\log_{10}$）以上の不活化率が得られ，また*A. baumannii*に関しても，0.4～0.8 mg/Lの範囲で24時間以内に99.999％（5 $\log_{10}$）以上の不活化率が得られている。また，*P. aeruginosa*および*A. baumannii*に関しては，銀イオンを併用することで不活化の相乗効果が得られることも報告している。なお，銅・銀イオン発生装置は欧米の300以上の病院で使用実績があることも記載されている。

乾らはシャワーヘッド内部に金属銅を溶射したものと通常のシャワーヘッドでバイオフィルム形成能の違いを調査している[6]。その結果，金属銅を溶射したシャワーヘッドでは，通常のものと比べ，バイオフィルを形成する従属栄養細菌数が1/260～1/40に減少することを示しており，シャワーヘッドへの銅利用の有効性が明らかとなっている。

Sharanらは，銅製の瓶（容量：12 L）を用いて水を保存した際に，水系感染を引き起こす*Salmonella typhi*, *Salmonella typhimurium*, *Vibrio cholerae*を不活化可能かどうか検討している[7]。その結果，短時間では効果が低いが，24時間以上保存することで十分な不活化効果があることが明らかとなった。

Ⅳ　結　　論

我が国においては，19世紀後半から20世紀にかけて公害問題のさきがけである足尾銅山鉱毒事件が発

表2　病院建築の衛生設備配管における最多使用管材（建築設備技術者協会調べ；文献27を基に作成）

配管系	1991年	1996年	2001年	2006年	2011年
上水管	塩ビライニング鋼管（VA, VB）				
	65.8	56.4	57.3	49.0	48.8
雑用水管	塩ビライニング鋼管（VA, VB）				
	66.3	66.3	55.7	49.8	48.8
給湯管	銅管			ステンレス管	
	53.0	43.3	26.6	47.7(19.6%†)	48.9(7.4%†)
汚水管	—	塩ビ管		耐火二層管	
		25.0	37.3	32.2	42.5
雑排水管	—	塩ビ管		耐火二層管	
		24.7	34.2	30.5	41.1
通気管	—	塩ビ管	塩ビ管	二層/塩ビ	二層管
		43.0	35.7	33.3	50.0

* 表中の数字は最多使用管材の使用割合（%）を示す。
* 給湯管以外は，主要な管材ではないため，銅管の使用割合の集計データなし。
† 銅管の使用割合（%）

生したため，銅に対しては有毒性のイメージが先行し，銅の利用が敬遠されることもあるが，人への健康影響がほとんど発生しないと推測される水道水質基準を満たす濃度範囲であっても，水中の微生物を不活化可能であることが一部の研究で明らかとなっている。不活化効果の持続性や残留塩素の低減，消毒副生成物の生成等の課題は存在するものの，公衆衛生上問題となっている多くの病原微生物の不活化に効果があることがわかってきており，病院施設の給水設備等での利用が今後期待される。

本検討は，厚生労働科学研究費補助金健康安全・危機管理対策総合研究事業「水道の浄水処理および配水過程における微生物リスク評価を用いた水質管理手法に関する研究」および国立保健医療科学院調査研事業（基盤的研究費）「平常時及び緊急時の施設等の水・衛生管理に関する研究」の一環として行われた。

（受付　2012.10. 2
採用　2013. 6. 6）

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Current situation and problems associated with inactivation of microorganisms in water using copper

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Key words : copper, disinfection, antimicrobial property, reactive oxygen species (ROS), water sanitation

Objectives The current situation and problems associated with inactivation of microorganisms in water using copper were elucidated.

Methods A literature review was conducted regarding the history and mechanisms of inactivation technology using copper, the variety of microorganisms shown to be inactivated by these methods in previous experiments, and the efficacy of such technologies for the inactivation of microorganisms in water.

Results The use of copper for inactivation of microorganisms has a long history. Although the use of copper was discontinued temporarily owing to the advent of antibiotics in the 1930s, the occurrence of antibiotic-resistant bacteria has resulted in the need for different approaches to control pathogenic microorganisms. One such alternative is the use of copper. Although the mechanisms underlying the efficacy of copper inactivation technology have not yet been elucidated in detail, it has been suggested that pathogenic bacteria are inactivated due to the toxicity of copper ions and strong oxidation effects of reactive oxygen species. Copper inactivation technology is effective against many pathogenic microorganisms that pose a risk to public health, such as *Legionella pneumophila*, *Salmonella enterica*, and *Mycobacterium tuberculosis*. In recent years, copper inactivation technology has been used in various water-related devices, especially water supply pipes in buildings. Previous studies have demonstrated that microorganisms can be sufficiently inactivated by copper even at concentrations below that specified in the Water Quality Standard for Drinking Water. However, some previous studies have indicated that the inactivation effect of copper is short-lived. Therefore, the development of techniques to maintain a long-term inactivation effect is a key concern. In addition, it has been reported that the use of copper pipes triggers chlorine decay and results in the formation of chlorine disinfection byproducts. Hence, further studies should aim at assessing the risks and benefits associated with the use of copper.

Conclusion Although the practical issues regarding copper inactivation technology are persistent, this method has been demonstrated to be efficacious. Therefore, this technology could be expected to be used in many devices such as water supply systems in hospitals in the near future.

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Quantitative Analysis of the Inactivation Mechanisms of *Escherichia coli* by a Newly Developed Method Using Propidium Monoazide

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ABSTRACT

The present study investigates a newly developed method using propidium monoazide (PMA) to detect damage on the outer membrane of bacteria. In order to verify this method, *Escherichia coli* were disinfected by ultraviolet, chlorine and sawdust treatments assuming a composting toilet. The inactivation mechanisms were investigated by multiple detection methods focused on which parts and/or functions were damaged. The differences in detection principles among three kinds of growth media and the polymerase chain reaction (PCR) method were used as methods to investigate the damage caused by disinfection. In addition, damage to the outer membrane was distinguished using PMA as pretreatment following PCR or conventional cultivation media, Tryptic Soy Agar (TSA), called PMA-PCR and PMA-TSA, respectively. As a result, it was indicated that chlorination caused outer membrane damage, and that ultraviolet treatment did not. Sawdust treatment at high temperature damaged the outer membranes effectively. It was confirmed that PMA-TSA, a newly developed method, could detect damage on the outer membrane of *Escherichia coli* more sensitively and quantitatively than PMA-PCR.

Keywords: *Escherichia coli*, inactivation mechanism, propidium monoazide

INTRODUCTION

The percentage of the world population using improved drinking water sources reached 87% in 2008, while in the WHO African Region, the percentage was only 61%, with just 34% of the population using improved sanitation facilities. These situations cause many deaths by water-related diseases (WHO, 2010). Even in cities that have introduced sewage systems, the regulation of drinking water quality is necessary. Therefore, water disinfection and improved water environments are urgently required. Several kinds of disinfection methods can be utilized, but it is important to understand the inactivation mechanisms of these methods, and to use appropriate detection methods without false positive or false negative detection in order to assume the disinfection effect. Many research studies have used *Escherichia coli* (*E. coli*) as a model of pathogenic bacteria. However, it is unreasonable to apply the disinfection effect for *E. coli* to pathogenic bacteria if the inactivation mechanisms are unclear. Ultraviolet (UV) disinfection and chlorination have been investigated, and the inactivation mechanisms in water treatment and several environments have been researched as well. It was considered that UV caused microorganism nucleic acid damage (Cho *et al.*, 2010), and that chlorination mainly damaged the outer membrane (Virto *et al.*, 2005). These researches clarified the inactivation mechanisms, but did not reveal the disinfection effect quantitatively. In this study, we tried to analyze the disinfection mechanisms quantitatively. Propidium monoazide (PMA) has been applied to detect DNA from live

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Received April 15, 2013, Accepted August 3, 2013.

cells only, combined with the polymerase chain reaction (PCR) method (Nocker *et al.*, 2006, 2007; Kobayashi *et al.*, 2010; Taskin *et al.*, 2011; Yang *et al.*, 2011), which is called PMA-PCR. Propidium monoazide is a photoreactive DNA-intercalating dye that can penetrate cells with compromised membranes, but not those with intact membranes. The penetrating PMA can bind to DNA, and exposure to bright visible light makes a covalent link between PMA and DNA, which inhibits PCR amplification. Therefore, if *E. coli* could not be detected after PMA treatment, damage to the outer membrane was assumed. However, care must be taken about the possibility of detection when using PCR, because the detection target region for PCR is a small part of the whole DNA. If the DNA has damage or makes covalent links to PMA outside of the target region, it can be detected and the inactivation rate cannot be evaluated.

In previous studies, we investigated the inactivation mechanisms of *E. coli* in a composting toilet where sawdust was used as a matrix, as a model of pathogenic bacteria using three different media with different detection principles, focusing on which parts and/or functions were damaged (Kazama and Otaki, 2011). However, this method could not clearly distinguish metabolic function damage from nucleic acid damage, or outer membrane damage from enzyme activity damage. Therefore, we investigated the inactivation mechanisms of *E. coli* by a new method using a conventional cultivating medium (Tryptic Soy Agar - TSA) with PMA called PMA-TSA for both the estimation of damage and evaluation of the inactivation rate (Kazama and Otaki, 2012). The results indicated that PMA-TSA could estimate the outer membrane damage and evaluate the inactivation rate of *E. coli*.

In this research, PMA-TSA was applied to *E. coli* after sawdust treatment and also UV treatment or chlorination in order to verify PMA-TSA. To investigate the inactivation mechanisms by each treatment, the results obtained from the multiple detection methods were compared. Studies were carried out simultaneously using these methods, with three kinds of media and PCR used to investigate the damage. In addition, PMA-PCR and PMA-TSA were used to distinguish outer membrane damage.

MATERIALS AND METHODS

Microorganisms

Microorganisms were obtained from NBRC (National Institute of Technology and Evaluation Biological Resource Center, Japan). *Escherichia coli* (NBRC3301) was used as the model microorganism of pathogenic bacteria.

For UV or chlorine treatment, *E. coli* was incubated at 37°C for 1 night on TSA (Difco, USA), after which an aliquot of *E. coli* colonies was dissolved in PBS (phosphate buffered saline).

For sawdust treatment, TSB (Tryptic Soy Broth, Difco) was used as a growth medium for *E. coli*. *Escherichia coli* cultures were incubated in a shaking water bath at 37°C for 3 – 4 hr, and an aliquot of solution was injected into the sawdust.

In UV and chlorination experiments, a nutrient solution like TSB should not be included in the *E. coli* solution because of the interfering UV or chlorination disinfection

efficiency. The colony formed on TSA was picked up and directly suspended in the PSB solution to make the *E. coli* solution. Nutrient contamination could be ignored in sawdust treatment, because the sawdust contains more nutrients than that of a TSB droplet. Therefore, a TSB solution containing high *E. coli* concentration was used for propagation.

Disinfection methods

UV treatment

A 20-mL *E. coli* solution (approximately 10^5 CFU/mL in PBS) in a petri dish (diameter: 5.2 cm, height: 1.2 cm, Eiken Co., Japan) was irradiated by a low pressure UV lamp (STANLEY infection lamp GL 20W, Toshiba Co., Japan) and agitated using a magnetic stirrer. The intensity of UV was 0.3 mW/cm^2, and samples were taken at several irradiation times. Ultraviolet experiments were performed in dark conditions, and every experiment was replicated three times.

Chlorine (Cl) treatment

A 40-μL volume of sodium hypochlorite solution (12% sodium hypochlorite, Kishida Chemical Co., Japan) in a 40-mL volume of distilled water was used as a Cl stock solution.

A 3% sodium thiosulfate (Kishida Chemical Co.) solution was used to stop the chlorination.

A 60 – 300 μL volume of Cl stock solution was added into approximately 35-mL PBS (pH7) to get a Cl working solution with a free Cl concentration of 0.20 – 1.25 mg/L. A 20-mL volume of *E. coli* solution (approximately 10^5 CFU/mL in PBS) was added to a 20-mL volume of Cl working solution in a petri dish (diameter: 8.8 cm, height: 1.4 cm, Eiken Co.) and agitated using a magnetic stirrer. This means the initial free Cl concentration was half of the working Cl solution. A 10-mL aliquot was taken and neutralized by a 100 μL 3% sodium thiosulfate solution at several retention times. The initial free Cl concentration was measured by the DPD (N,N-diethyl-*p*-phenylenediamine) colorimetric method using a portable colorimeter (DR/890, HACH, USA), and every experiment was replicated five times.

Sawdust treatment

In this study, sawdust was obtained from an actual operating composting toilet that had been used for 80 days at a household in Chichibu City (Saitama Prefecture, Japan). The schematic diagram and specifications of this composting toilet were described in the previous report (Kazama and Otaki, 2011).

Escherichia coli was inactivated under two different conditions in sawdust that had been used in the composting toilet. The temperature was set at 37°C or 50°C and the water content was 50%, both similar to the actual operating conditions (37°C with 50% water content) with the highest temperature of 50°C (Kazama and Otaki, 2010, 2011). At first, the water content was removed by heating at 105°C for 24 hours, and then adjusted to 50% with distilled water. No indigenous *E. coli* was detected by TSA and PCR. Ten grams of sawdust was transferred to a sterilized glass bottle with a cotton plug and kept at 37°C or 50°C in an incubator. A 1-mL volume of *E. coli* (about 10^9 CFU/mL) stock