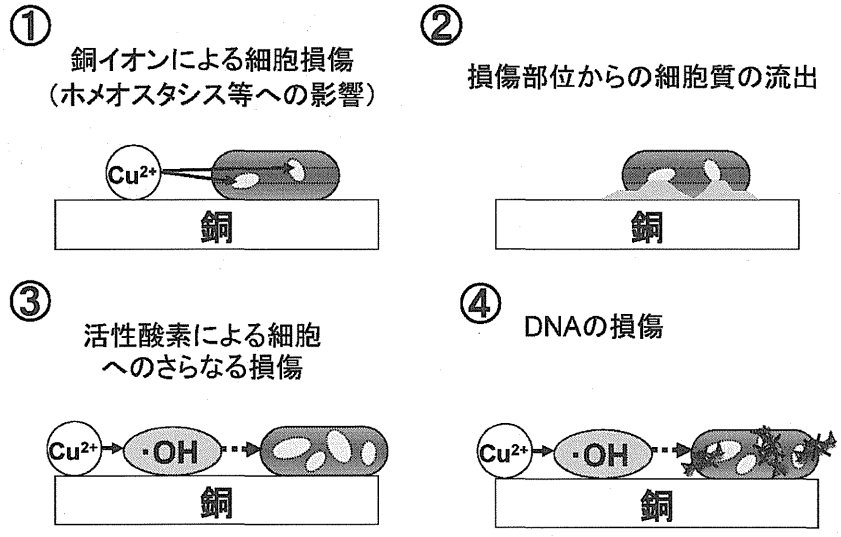
図1　銅による微生物の不活化機構・順序の概念図
（文献2を一部改変）



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。

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文　献

1) Baldursson S, Karanis P. Waterborne transmission of protozoan parasites: review of worldwide outbreaks - an update 2004-2010. Water Res 2011; 45(20): 6603-6614.
2) Grass G, Rensing C, Solioz M. Metallic copper as an antimicrobial surface. Appl Environ Microbiol 2011; 77(5): 1541-1547.
3) Dollwet HHA, Sorenson JRJ. Historic uses of copper compounds in medicine. Trace Elem Med 1985; 2(2): 80-87.
4) Mikolay A, Huggett S, Tikana L, et al. Survival of bacteria on metallic copper surfaces in a hospital trial. Appl Microbiol Biotechnol 2010; 87(5): 1875-1879.
5) 斎藤晴夫．銅の抗菌作用を住宅設備に活かす．住まいと電化 2008; 20(2008 March): 47-49.
6) 乾香代子，菊野理津子，小澤智子，他．従属栄養細菌によるバイオフィルム形成に及ぼす環境要因の検討．全国水道研究発表会講演集 2011; 62: 658-659.
7) Sharan R, Chhibber S, Reed RH. Inactivation and sub-lethal injury of salmonella typhi, salmonella typhimurium and vibrio cholerae in copper water storage vessels. BMC Infect Dis 2011; 11: 204.
8) United States Environmental Protection Agency. EPA Registers Copper-containing Alloy Products. 2008. http://www.epa.gov/pesticides/factsheets/copper-alloy-products.htm（2012年6月26日アクセス可能）
9) Haughey MA, Anderson MA, Whitney RD, et al. Forms and fate of Cu in a source drinking water reservoir following $CuSO_4$ treatment. Water Res 2000; 34(13): 3440-3452.
10) Macomber L, Rensing C, Imlay JA. Intracellular copper does not catalyze the formation of oxidative DNA damage in Escherichia coli. J Bacteriol 2007; 189(5): 1616-1626.
11) Macomber L, Imlay JA. The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. Proc Natl Acad Sci USA 2009; 106(20): 8344-8349.
12) Mehtar S, Wiid I, Todorov SD. The antimicrobial ac-

tivity of copper and copper alloys against nosocomial pathogens and Mycobacterium tuberculosis isolated from healthcare facilities in the Western Cape: an in-vitro study. J Hosp Infect 2008; 68(1): 45-51.
13) Huang HI, Shih HY, Lee CM, et al. In vitro efficacy of copper and silver ions in eradicating Pseudomonas aeruginosa, Stenotrophomonas maltophilia and Acinetobacter baumannii: implications for on-site disinfection for hospital infection control. Water Res 2008; 42(1-2): 73-80.
14) Santo CE, Morais PV, Grass G. Isolation and characterization of bacteria resistant to metallic copper surfaces. Appl Environ Microbiol 2010; 76(5): 1341-1348.
15) Faundez G, Troncoso M, Navarrete P, et al. Antimicrobial activity of copper surfaces against suspensions of Salmonella enterica and Campylobacter jejuni. BMC Microbiol 2004; 4: 19.
16) Weaver L, Michels HT, Keevil CW. Survival of Clostridium difficile on copper and steel: futuristic options for hospital hygiene. J Hosp Infect 2008; 68(2): 145-151.
17) Noyce JO, Michels H, Keevil CW. Potential use of copper surfaces to reduce survival of epidemic meticillin-resistant Staphylococcus aureus in the healthcare environment. J Hosp Infect 2006; 63(3): 289-297.
18) Noyce JO, Michels H, Keevil CW. Use of copper cast alloys to control Escherichia coli O157 cross-contamination during food processing. Appl Environ Microbiol 2006; 72(6): 4239-4244.
19) Molteni C, Abicht HK, Solioz M. Killing of bacteria by copper surfaces involves dissolved copper. Appl Environ Microbiol 2010; 76(12): 4099-4101.
20) 笹原武志，菊野理津子，山田 豊，他．給水用銅管によるLegionella殺菌効果に及ぼすバイオフィルム形成の影響．用水と廃水 2007; 49(2): 132-141.
21) Wilks SA, Michels HT, Keevil CW. Survival of Listeria monocytogenes Scott A on metal surfaces: implications for cross-contamination. Int J Food Microbiol 2006; 111(2): 93-98.
22) Weaver L, Michels HT, Keevil CW. Potential for preventing spread of fungi in air-conditioning systems constructed using copper instead of aluminium. Lett Appl Microbiol 2010; 50(1): 18-23.
23) Quaranta D, Krans T, Espirito Santo C, et al. Mechanisms of contact-mediated killing of yeast cells on dry metallic copper surfaces. Appl Environ Microbiol 2011; 77(2): 416-426.
24) Noyce JO, Michels H, Keevil CW. Inactivation of influenza A virus on copper versus stainless steel surfaces. Appl Environ Microbiol 2007; 73(8): 2748-2750.
25) 笹原武志，菊野理津子，中村 健，他．銅管によるCryptosporidium parvumオーシストの感染性不活化．感染症学雑誌 2006; 80(4): 377-382.
26) 日本水道協会，編．水道用語辞典(第2版)．東京：日本水道協会，2003; 558.
27) 中澤敏行，渋谷紘子．病院建築における竣工設備データ 共通事項．志賀一鑑，編．2011建築設備情報年鑑．東京：建築設備技術者協会，2011; 56-75.
28) 横井弘明．銅管と衛生．長橋 捷，監修，株式会社ピー・アール・オー，編．新・銅と衛生．東京：日本銅センター，2004; 30-40.
29) van der Kooij D, Veenendaal HR, Scheffer WJ. Biofilm formation and multiplication of Legionella in a model warm water system with pipes of copper, stainless steel and cross-linked polyethylene. Water Res 2005; 39(13): 2789-2798.
30) World Health Organization. Guidelines for Drinking-water Quality (4th ed). Geneva: World Health Organization, 2011; 340-342. http://whqlibdoc.who.int/publications/2011/9789241548151_eng.pdf (2013年7月6日アクセス可能)
31) Zhang H, Andrews SA. Catalysis of copper corrosion products on chlorine decay and HAA formation in simulated distribution systems. Water Res 2012; 46(8): 2665-2673.
32) 小林卓也，中野大助，坂口 勇，他．銅によるカワヒバリガイ付着抑制作用に関する研究．日本鉱業振興会助成研究成果報告書 2011; 2011: 81-84.
33) 日本水道協会，編．生物障害を起こさないための浄水処理の手引き．東京：日本水道協会，2006; 54-55.

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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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

solution was added, and the sawdust was agitated for 1 minute. An aliquot of sawdust was sampled and the target microorganism extracted (see below). The temperature of the sawdust was maintained in an incubator. The concentration of microorganisms in the sawdust was measured by extraction at several time periods. Each experiment was replicated twice.

In order to measure the concentration of *E. coli* in the composting toilet sawdust, microorganisms were extracted from the sawdust using a 3% (w/v) beef extraction solution (Otaki *et al.*, 2002). The beef extract (MP Biomedicals, Japan) was dissolved in deionized water, adjusted to pH9.5 with NaOH, and then sterilized. A weighed sample of sawdust (0.4 g) was added to a 20-mL volume of extraction solution and agitated for 3 minutes to extract the microorganisms. It was reported that the percentage recoveries of *E. coli* obtained using this method were 70 to 100% (Otaki *et al.*, 2002). In the present study, the recoveries of *E. coli* were confirmed by measuring the concentrations of *E. coli* in the stock solution and initial concentration in sawdust at 0 hr in every experiment. They were approximately 70% and stable in every experiment.

A phosphate buffer solution was used to dilute the extract to a suitable concentration for measuring microorganisms. For PCR and PMA cross-linking (see below), 10-fold diluted extraction solutions were used as samples in order to reduce the pH effect and materials from the extraction solutions.

Measurement of *E. coli* using three kinds of media

The concentrations of *E. coli* in the samples were measured in triplicate by the double agar layer method using TSA (Difco) and DESO (Desoxycholate Agar, Eiken Chemical Co., Japan), and C-EC (Compact Dry EC, Nissui Pharmaceutical Co., Japan). Compact Dry EC contained X-Gluc and Magenta-GAL.

Measurement of *E. coli* using PCR

Two hundred μL of samples with or without PMA cross-linking (see below) were centrifuged at 14,000 rpm for 5 min, and the supernatants were changed to PBS before DNA extraction. Deoxyribonucleic acid was extracted using a QIAamp DNA mini kit (Qiagen, USA). For real-time PCR, the 25-μL reaction mixture contained 5 μL of sample, 12.5 μL of 2×TaqMan Gene Expression Master Mix (Applied Biosystems, USA), forward and reverse primer (with a final concentration of 1 μM, Invitrogen), TaqMan MGB Probe with a final concentration of 200 nM and distilled water. The PCR cycling conditions were 2 min at 50°C and 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C using an ABI 7300 Real-Time PCR System (Applied Biosystems). The PCR target region was the *tnaA* gene started at 464, which generated specific amplicons of 136 base pairs. The primer and the probe were 5'-GGG GCG GTG ACG CAG-3' (forward), 5'-CCT GGT GAG TCG GAA TGG TG-3' (reverse), and 5'-FAM-CGA TGA TGC GCG GCG-MGB-3', respectively (Bernasconi *et al.*, 2007). Every sample was measured in triplicate.

Investigation of the PMA cross-linking condition

Propidium monoazide was dissolved in distilled water at 10 mg/mL as the stock solution, and stored at –20°C in the dark. The stock solution was appropriately diluted for PMA cross-linking in every experiment. The PMA solution was added to the sample

and mixed. This was incubated in the dark, and then exposed to light using a 650 W halogen light source (tungsten halogen lamps 650 W, 100 V; Selecon Pacific MSR575W, Philips, Netherlands). The sample tubes were laid horizontally in cool water to avoid excessive heating.

At first, we investigated the appropriate PMA cross-linking condition (concentration of PMA solution, incubation time and light exposure time) for both PMA-PCR and PMA-TSA. In this study, the maximum concentrations of *E. coli* samples for PMA cross-linking were approximately 10^5 CFU/mL. Therefore, we confirmed that the condition made links between PMA and DNA, and that no DNA was detected by PCR. For the confirmation, the naked DNA of 10^5 CFU/mL *E. coli* (in PBS) extracted using a QIAamp DNA mini kit (Qiagen) was measured by PCR after PMA cross-linking. In addition, in order to confirm that the condition did not inactivate intact *E. coli*, 1.1×10^5 CFU/mL and 9.5×10^2 CFU/mL of *E. coli* after PMA cross-linking were measured by the double agar layer method using TSA (Difco).

Investigation of PMA cross-linking for *E. coli* after UV treatment

Escherichia coli after UV treatment can photoreactivate with exposure to visible light. A wavelength around 380 nm was reported to cause photoreactivation (Takebe, 1983; Kamiko and Ohgaki, 1989), and the wavelength range of the halogen light used in this study covered it. Figure 1 shows the transparent spectrum of yellow cellophane (110500, TOYO Co., Japan). The yellow cellophane absorbs wavelengths around 380 nm and is considered to inhibit photoreactivation. Therefore, tubes containing UV-treated (see *UV treatment*) samples were covered with this yellow cellophane and exposed to light. In order to confirm the effect, the *E. coli* concentrations of samples with or without yellow cellophane were measured by the double agar layer method using TSA (Difco). However, there is a possibility that the yellow cellophane may inhibit PMA cross-linking. Therefore, PMA cross-linking with yellow cellophane was carried out for DNA extracted from 200 μL of 10^5 CFU/mL *E. coli* solution.

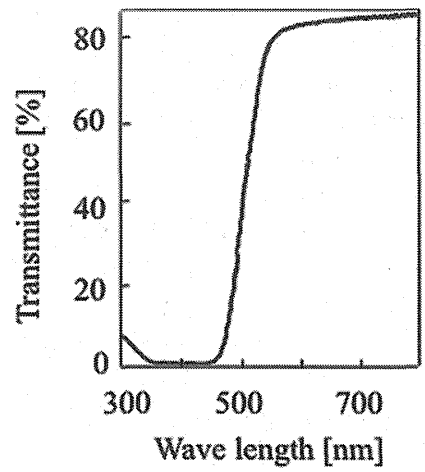

Fig. 1 - Transparent spectrum of yellow cellophane.

Estimation of the damage to *E. coli*

We simultaneously used six different methods, three different kinds of media, TSA media with PMA cross-linking (PMA-TSA), PCR, and PCR with PMA cross-linking (PMA-PCR).

According to the detection principles of the three kinds of media, which were TSA, DESO and C-EC, damage to *E. coli* can be assumed (Kubo and Otaki, 2005, 2006; Wang and Otaki, 2007; Mizozoe *et al.*, 2010; Kazama and Otaki, 2011, 2012). *Escherichia coli* is capable of metabolizing proteins and growth can be detected by TSA. Therefore, when *E. coli* could not be detected by TSA, it was assumed that its nucleic acid and/or metabolic function were damaged. Desoxycholate Agar, a selective agar, can detect *E. coli* that can grow by metabolizing lactose in the presence of desoxycholic acid. Gram-positive bacteria are unable to grow in the presence of desoxycholic acid because they lack an outer membrane and their growth is inhibited by its surface-active effects. Therefore, when *E. coli* cannot be detected by DESO, this indicates that its outer membrane and/or nucleic acid and/or metabolic function have been damaged. Compact Dry EC, also a selective agar, can detect *E. coli* that can produce ß-glucuronidase, which is the enzyme involved in the metabolism of peptone, pyruvic acid, and lactose. Therefore, when *E. coli* cannot be detected by C-EC, it is assumed that its enzyme activity and/or nucleic acid and/or metabolic function have been damaged. By comparing the detected concentration on each medium, the damage to *E. coli* could be estimated, as shown in Table 1.

In this research, PMA-TSA was used as a newly developed method to confirm the outer membrane damage. When *E. coli* cannot be detected by PMA-TSA, it is assumed that its outer membrane and/or nucleic acid and/or metabolic function have been damaged. Therefore, if *E. coli* can be detected by TSA, but not by PMA-TSA, it indicates that only its outer membrane has been damaged.

However, investigation by the three kinds of media and PMA-TSA could not distinguish whether the DNA was damaged. Therefore, the distinction of DNA damage was investigated by PCR. Polymerase chain reaction was also used with PMA-PCR to confirm whether the outer membranes were damaged or not. By comparing the detected concentration by each method, the damage to *E. coli* could be estimated, as shown in Table 2.

Table 1 - Estimated parts of *E. coli* damaged according to the detection differences among the three kinds of media (Kazama and Otaki, 2012).

Detections by media*				Estimated parts
TSA	DESO	C-EC		which were damaged
×	×	×	→	Nucleic acid and/or metabolism (and also enzyme activity and outer membrane)
○	○	×	→	Enzyme activity
○	×	○	→	Outer membrane
○	×	×	→	Enzyme activity and outer membrane

*○, detected; ×, not detected.

Table 2 - Estimated parts of *E. coli* damaged according to the detection differences among the methods (Kazama and Otaki, 2012).

Detection by methods*			Estimated parts which were damaged
TSA	PMA-TSA		
×	×	→	Nucleic acid and/or metabolism (and also enzyme activity and outer membrane)
○	×	→	Outer membrane

Detection by methods*			Estimated parts which were damaged
PCR	PMA-PCR		
×	×	→	Nucleic acid (and also outer membrane)
○	×	→	Outer membrane

*○, detected; ×, not detected.

RESULTS AND DISCUSSION

Investigation of the PMA cross-linking condition

As a result of our investigation, it was proper to add 1 μL of 1 mg/mL PMA solution to 200 μL of sample, incubate the sample mixed PMA for 3 min in the dark, and then expose it to a light source at a distance of 15 cm for 5 min.

DNA extracted from 10^5 CFU/mL *E. coli* was not detected by PCR after PMA cross-linking, and the concentrations of intact *E. coli* after PMA cross-linking were not significantly decreased (initial concentration: 1.1×10^5 CFU/mL or 9.5×10^2 CFU/mL, concentration after PMA cross-linking: 1.1×10^5 CFU/mL or 8.6×10^2 CFU/mL). Therefore, it was assumed that this PMA cross-linking condition was sufficient to inactivate *E. coli* with compromised membranes, but not *E. coli* with intact membranes.

PMA cross-linking for *E. coli* after UV treatment

According to the above result, UV-treated *E. coli* with or without yellow cellophane were placed about 15 cm from the light source and exposed to light for 5 min. They photoreactivated in the condition without yellow cellophane (Fig. 2(a)), but did not photoreactivate with it (Fig. 2(b)).

For the inhibition of yellow cellophane for PMA cross-linking, 2.5% of *E. coli* (10^5 CFU/mL) could be detected by PCR after PMA cross-linking with yellow cellophane. It was considered that the wavelength of 464 nm was the most effective for PMA-cross linking (Biotium Inc., 2010), and this yellow cellophane absorbed it. The light exposure time was set longer (7 or 10 min), but it was not effective for PMA cross-linking. Therefore, the same PMA cross-linking condition (retention time: 3 min, light exposure: 5 min) but with yellow cellophane was used for both PMA-TSA and PMA-PCR after UV treatment. If UV treatment causes outer membrane damage, we can see an approximate 2-log reduction using this condition.

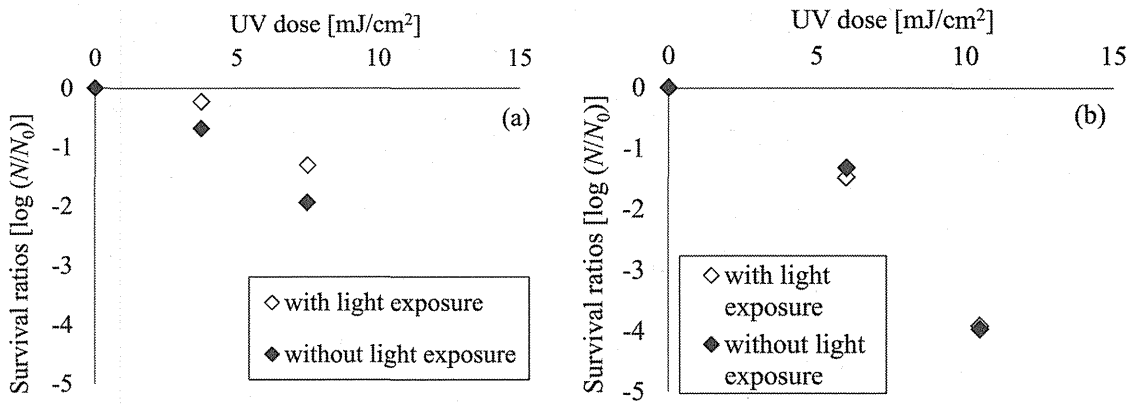

Fig. 2 - Change of the survival ratios of *E. coli* treated by UV with or without light exposure.
(a) without yellow cellophane; (b) with yellow cellophane.

Results of the disinfection experiments

Figure 3 shows changes in the survival ratios of *E. coli* by UV treatment, Cl treatment, and sawdust treatment as determined by three kinds of media, PMA-TSA, PCR and PMA-PCR. For the results of Cl treatment, the Ct (chlorine concentration × exposure time) values were different in every experiment and therefore all data were plotted.

Damage to E. coli by UV treatment

In Fig. 3(a), no significant differences were observed in the inactivation rate by any of the three media. Therefore, inactivation was assumed to be mainly due to damage to nucleic acids and/or metabolic functions because *E. coli* could not be detected by any of the three media, as shown in Table 1. If nucleic acids were damaged, *E. coli* could not be detected by both PCR and PMA-PCR, as shown in Table 2. However, no significant decrease was observed with the results under this UV dose condition. Therefore, it could not clarify that *E. coli* caused damage to DNA, and not to the outer membrane. When comparing the inactivation rates by TSA and PMA-TSA, no significant differences were observed. This indicated that *E. coli* did not damage the outer membrane. And these methods could evaluate the inactivation rate by UV treatment, though PCR and PMA-PCR could not.

Damage to E. coli by chlorine treatment

In Fig. 3(b), the inactivation rate by TSA was lower than the rates for the other two media, which indicated that chlorine treatment caused more significant damage to enzyme activity and/or outer membrane than to nucleic acids and/or metabolic functions, as shown in Table 1. Differences were also observed when the inactivation rate by TSA was compared to that by PMA-TSA. Therefore, chlorine treatment resulted primarily in damage to the outer membrane as shown in Table 2. *Escherichia coli* with damage to the outer membrane had the potential to grow because they could be detected by TSA. However, no significant difference was observed between the inactivation rate less than C × T 1.8 by PCR and the inactivation rate by PMA-PCR, which indicated that *E. coli* had no outer membrane damage. It was considered that because chlorine damage to the outer membrane was slight (Cho *et al.*, 2010), cell penetration of PMA was insufficient

to inhibit amplification of the target gene. However, PMA could bind to other gene areas and inhibit *E. coli* from growing on TSA. Then, a difference was observed between the inactivation rate by TSA and that by PMA-TSA. In short, using PCR and PMA-PCR could show outer membrane damage, but the inactivation rates could not be evaluated. By using TSA and PMA-TSA, it was considered possible to distinguish *E. coli* with damaged to the outer membrane sensitively and quantitatively.

Damage to E. coli by sawdust treatment

According to the results, at 37°C with 50% water content (Fig. 3(c-1)), inactivation was assumed to be mainly due to damage to metabolic functions and/or nucleic acids. At 50°C with 50% water content (Fig. 3(c-2)), the increase in temperature was assumed to cause damage to metabolic functions and/or nucleic acids because the inactivation rate by each medium was significantly higher compared with the rate at 37°C with 50% water content. However, the inactivation rate by TSA was lower than the rates for the other two media and PMA-TSA, and a difference was also observed when the inactivation rate by PCR was compared with that by PMA-PCR, which indicated that a temperature increase caused more significant damage to the outer membrane than to the metabolic functions. In short, high temperature conditions resulted primarily in damage to the outer membrane, and *E. coli* had the potential to grow.

In addition, for *E. coli* with only outer membrane damage (not slight), the difference in the survival ratio between TSA and DESO should be similar to that between PCR and PMA-PCR, as the difference between TSA and DESO was similar to that between

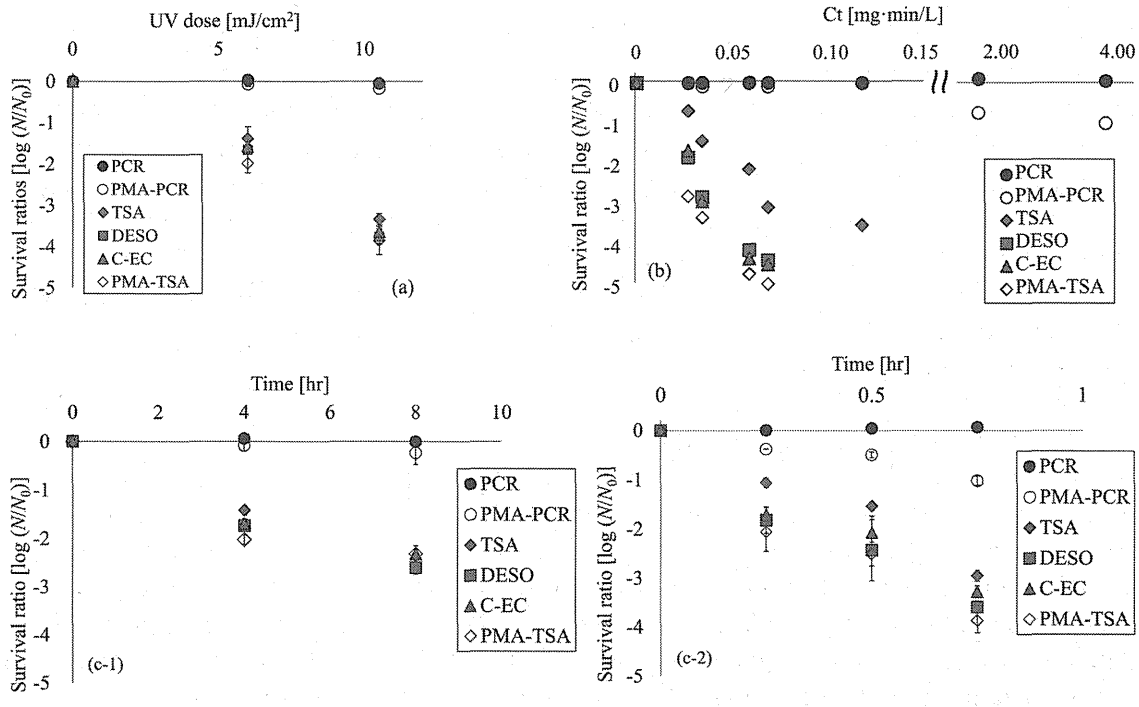

Fig. 3 - Change in the survival ratios of *E. coli* by UV, Cl and sawdust treatment.
(a) UV treatment; (b) Cl treatment; (c-1) sawdust treatment at 37°C with 50% water content; (c-2) sawdust treatment at 50°C with 50% water content.
*Error bars show 95% confidence.

PMA-TSA and TSA. These differences were compared using the results in Fig. 3(c-2). The difference between TSA and DESO was calculated by the inactivation rate constant of DESO divided by that of TSA. The difference between PCR and PMA-PCR employed the inactivation rate constant of PMA-PCR because the survival ratio of PCR did not change. They were respectively 1.34 and 1.25, and very close values. This result indicated that these methods could distinguish damage (not slight) to the outer membrane of *E. coli*.

CONCLUSIONS

The inactivation mechanisms of *E. coli* by UV treatment, chlorination and sawdust treatment were investigated, and the results were compared with other detection methods. As for the results, the damaged parts of *E. coli* could be estimated approximately by three different media, and *E. coli* with damage to each part could be detected quantitatively. However, this method could not distinguish the outer membrane damage from enzyme activity damage. The method comparing PCR to PMA-PCR might reveal whether the outer membrane of *E. coli* was damaged, however, it was difficult to evaluate the kinetics of the damage reaction rate. The disinfection effect should be assessed quantitatively because *E. coli* with outer membrane damage has the potential to grow. This research investigated a newly developed method using PMA combined with TSA to distinguish the damage to the outer membrane of *E. coli*. The results by TSA and PMA-TSA indicate that UV treatment did not cause *E. coli* outer membrane damage, while chlorination and high temperature sawdust treatment did. This method could distinguish outer membrane damaged *E. coli* that has the potential to grow, and evaluate the inactivation rate by PMA cross-linking using 1 μg of PMA for 10^4 CFU of *E. coli*. In addition, by using yellow cellophane it was considered that *E. coli* with outer membrane damage could be distinguished even though *E. coli* that can photoreactivate existed together. The method comparing TSA to PMA-TSA could distinguish *E. coli* with damage to the outer membrane sensitively and quantitatively.

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REFERENCES

Bernasconi C., Volponi G., Picozzi C. and Foschino R. (2007) Use of the *tna* operon as a new molecular target for *Escherichia coli* detection. *Applied and Environmental Microbiology*, **73**(19), 6321-6325.

Biotium Inc. (2010) PMA Product Information. Biotium, Hayward, CA, USA.

Cho M., Kim J., Kim J. Y., Yoon J. and Kim J. H. (2010) Mechanisms of *Escherichia coli* inactivation by several disinfectants. *Water Research*, **44**(11), 3410-3418.

Kamiko N. and Ohgaki S. (1989) RNA coliphage Qß as a bioindicator of the ultraviolet disinfection efficiency. *Water Science and Technology*, **21**(3), 227-231.

Kazama S. and Otaki M. (2010) Fate of indicator bacteria for pathogenic viruses in composting toilet. *Journal of JSCE, Division G (Environmental Systems and*

Engineering), **66**(4), 179-186. (in Japanese)

Kazama S. and Otaki M. (2011) Mechanisms for the inactivation of bacteria and viruses in sawdust used in composting toilet. *Journal of Water and Environment Technology*, **9**(1), 53-66.

Kazama S. and Otaki M. (2012) Inactivation mechanisms of *E. coli* in the sawdust used in composting toilet. *Journal of Water and Environment Technology*, **10**(4), 363-374.

Kobayashi H., Oethinger M., Tuohy M. J., Hall G. S. and Bauer T. W. (2010) Distinction between intact and antibiotic-inactivated bacteria by real-ime PCR after treatment with propidium monoazide. *Journal of Orthopaedic research*, **28**(9), 1245-1251.

Kubo K. and Otaki M. (2005) Study of the damages on *E. coli* by disinfection. In: Proc. the 42nd Environmental Engineering Forum, Tottori, Japan, pp.75-78. (in Japanese)

Kubo K. and Otaki M. (2006) Analysis of the damage to *E. coli* in a composting toilet. In: Proc. the 40th Japan Society on Water Environment Annual Conference, Sendai, Japan, p.227. (in Japanese)

Mizozoe M., Sano M., Said B. M. and Otaki M. (2010) Quantitative analysis of damage mechanisms of *E. coli* and *Pseudomonas aeruginosa* by disinfection. In: Proc. the 44th Japan Society on Water Environment Annual Conference, Fukuoka, Japan, p.302. (in Japanese)

Nocker A., Cheung C. Y. and Camper A. K. (2006) Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *Journal of Microbiological Methods,* **67**(2), 310-320.

Nocker A., Sossa-Fernandez P., Burr M. D. and Camper A. K. (2007) Use of propidium monoazide for live/dead distinction in microbial ecology. *Applied and Environmental Microbiology*, **73**(16), 5111-5117.

Otaki M., Nakagawa N. and Ito Y. (2002) The fate of pathogen in composting toilet and risk assessment. In: Proc. the 57th Japan Society of Civil Engineers Annual Conference, Sapporo, Japan, pp.561-562. (in Japanese)

Takebe K. (1983) DNA repair. University of Tokyo Press, Tokyo, Japan. (in Japanese)

Taskin B., Gozen A. G. and Duran M. (2011) Selective quantification of viable *Escherichia coli* bacteria in biosolids by quantitative PCR with propidium monoazide modification. *Applied and Environmental Microbiology*, **77**(13), 4329-4335.

Virto R., Mañas P., Álvarez I., Condon S. and Raso J. (2005) Membrane damage and microbial inactivation by chlorine in the absence and presence of a Chlorine-demanding substrate. *Applied and Environmental Microbiology*, **71**(9), 5022-5028.

Wang X. and Otaki M. (2007) Estimation of inactivation mechanism on *E. coli* by Ozonation. In: Proc. the 17th Japan Ozone Association Annual Conference, Kochi, Japan, pp.75-78. (in Japanese)

WHO (2010) Health-related millennium development goals. In: World Health Statistics, World Health Organization, Geneva, Switzerland.

Yang X., Badoni M. and Gill C. O. (2011) Use of propidium monoazide and quantitative PCR for differentiation of viable *Escherichia coli* from *E. coli* killed by mild or pasteurizing heat treatments. *Food Microbiology*, **28**(8), 1478-1482.

(8－22)浄水処理ユニットプロセスにおける微生物除去･不活化能の定量化法

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伊藤　禎彦(京都大学)

1. はじめに

飲料水の微生物的安全性を管理する手法として定量的微生物リスク評価（QMRA）がある。QMRAでは、対象微生物の除去性や非加熱飲水量等、各パラメータの持つ変動を数学モデルで表現した上で、モンテカルロシミュレーションを用いて感染リスクを区間推定する。その第一段階では、水中微生物の濃度データを蓄積しつつ、各処理ユニットにおける除去・不活化能を計算し、その変動性を含めて確率分布として記述する必要がある。本文は、幾つかのテクニックを活用して、浄水処理のユニットプロセスにおける除去・不活化能を定量化する手法を示したものである。

2. 方　法

1) 評価対象データとその特徴：淀川を原水とする浄水場における凝集・沈殿処理プロセスをとりあげた。原水の *E. coli* 濃度は2009年11月から2012年12月までに65回、凝集･沈殿処理後の *E. coli* 濃度は2009年1月から2012年12月までに27回測定された値を用いた。原水中 *E. coli* は10 mL—1 mL—0.1 mL—0.01 mLの4段階5本MPN法で測定した。凝集･沈殿処理水中 *E. coli* の濃度は低く、検出し易くするためには接種水量を増大する必要がある。ここでは操作性を考慮し、500 mL—50 mL—5 mLの3段階5本MPN法とした。なお、凝集・沈殿処理水の27回の測定のうち、0データは14回であった。このように評価対象データセットは、原水・処理水でデータ数が異なる、処理水に多くの0データを含むという特徴を有する。本文では、これらの情報全てを有効に活用する方法を示す。

2) 検出限界以下データに対する濃度補間：一般に、流入水あるいは流出水の微生物濃度が0であれば、除去・不活化率を計算できない。測定値だけを用いて計算した場合、除去・不活化能を過小評価する可能性がある。周ら[1]は、微生物濃度がポアソン分布に従うことを利用して、検出限界以下にデータ補間する方法を提案した。ここでは、0データの割合が大きく、その影響が大きいと考えられる凝集・沈殿処理後水に対して、周らの方法を適用した。試料水量500mLのとき、検出限界は0.036 *E. coli*/100mL(試料水量10mLで、対応する検出限界1.8 *E. coli*/100mLのケースもあり)なので、測定値が0であった試料水の *E. coli* 平均濃度はすべて0.036 *E. coli*/100mL以下と仮定した。検出限界0.036 *E. coli*/100mLのとき、試料水量500mLにおいて0個の *E. coli* を含む試料を見つける確率は、ポアソン分布から0.965と求められる。すなわち、*E. coli* の平均濃度が検出限界以下のとき、試料水量500mLにおいて0個の *E. coli* を含む試料を見つける確率は0.965から1までの範囲となる。したがって、0.965～1間の確率値に乱数を発生させて、対応する *E. coli* の平均濃度をポアソン分布式によって算出し、これを補間値とした。この操作により、0～0.036 *E. coli*/100mLの区間内で0 *E. coli*/100mLの側に偏在した補間データセットが得られる。

3) データのペアリング方法とブートストラップサンプリング：処理ユニットの除去・不活化能は通常デイト法によって評価される。この方法では同じ日(または同時刻)に採取された流入水と流出水の測定値をペアリングし、除去・不活化率を計算する。デイト法に対して、ランク法と呼ばれる方法では、流入水濃度が高ければ流出水濃度も高く、流入水濃度が低ければ流出水濃度も低いという仮定を設ける。このほかランダム法もあり、この場合、流入水濃度と流出水濃度のデータセットから値をランダムに取り出してペアリングする。ここでは、これら3種類のペアリング方法を比較した。また、データセットの信頼限界を調べるため、ブートストラップサンプリングを組み合わせた。はじめに、凝集・沈殿処理前後の *E. coli* 濃度のデータセットに対して1000回のブートストラップサンプリングを行った。つぎに、各ブートストラップデータセットに対して3種類の方法（デイト法、ランク法、ランダム法）でペアリングを行い、除去率を計算した。得られた除去率を流入水濃度に乗じて流出水濃度を計算し、流出水濃度の計算値と実測値を比較した。

3. 結果及び考察

凝集・沈殿処理水の補間前と補間後の *E. coli* 濃度のヒストグラムを図 1 に示す。また、流出水濃度の計算値と実測値の比較結果を図 2 に示す。相補累積分布 (Complementary Cumulative Distribution Function; CCDF)として両対数紙上にプロットしたもので、リスク計算上重要な正規確率が小さい領域を拡大して表示することができる。95%信頼区間とともに示しており、この図によりシミュレーションの妥当性を検証することができる。ここで示された計算値を用いて、この後の QMRA を進めることになる。さて、デイト法の場合、全体的に除去能が低く評価される傾向があることがわかる。ここでは、全体的な適合性からみて、ランク法の方が望ましいと判断した。一方、ランダム法はデイト法とよく似た結果を与えた。凝集・沈殿処理の除去能評価結果と適合させた分布形を図 3 に示す。正規分布が適合し、除去 $\log_{10}$ 数の平均値は 2.64、標準偏差は 0.49 であった。

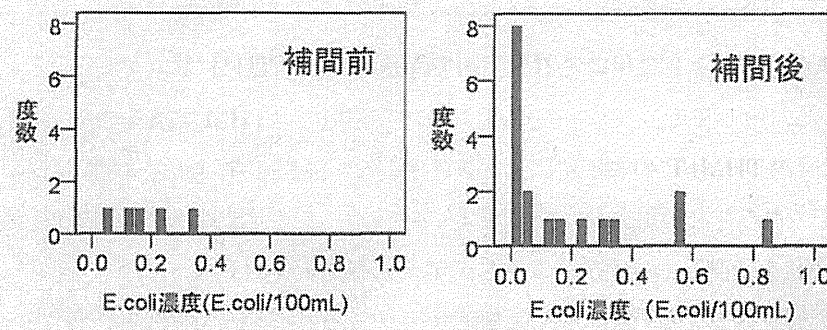

図 1　凝集・沈殿処理水における補間前後の濃度分布

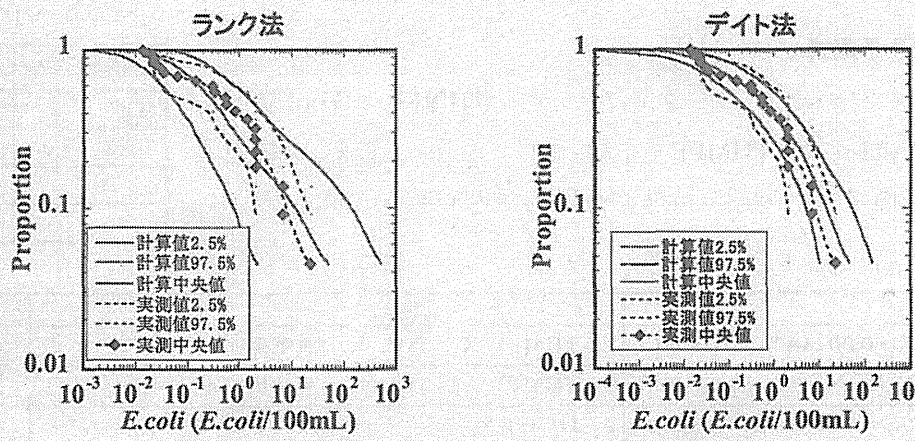

図 2　ランク法とデイト法の結果

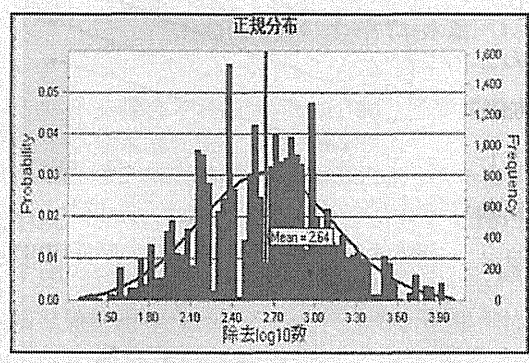

図 3　凝集・沈殿処理の除去能分布

4.　おわりに

原水・処理水でデータ数が異なり、かつ処理水に多くの 0 データを含むデータセットに対して、検出限界以下データに対する濃度補間、ペアリング法としてのランク法の採用、ブートストラップサンプリングという 3 つのテクニックを用いて、微生物除去能を評価する方法を示した。この方法は 0 データの割合が非常に高い場合には適用できず、除去・不活化能を定量化するためには微生物の注入実験を行う必要がある。本稿では凝集・沈殿処理の除去能の定量化を扱ったが、浄水処理プロセス全体の QMRA を行い、*Campylobacter* を指標としてリスク評価を行う手法に関する検討も行っている [2)]。

謝辞:原水 *E. coli* データの一部を提供いただいた大阪市水道局、および *E. coli* 測定にご協力いただいた宋金姫氏に謝意を表する。

参考文献: 1) 周,伊藤：第 47 回環境工学研究フォーラム講演集,151-153,2010.　2) Zhou,L., Echigo,S., Ohkouchi, Y., Itoh, S.: J. Wat. Supply: Res. Technol.-AQUA, 2013. (印刷中)

(8－38)膜損傷性とコロニー形成能を指標とした給水末端から単離された従属栄養細菌の塩素耐性評価

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1. 研究の背景と目的

近年、より安全でおいしい水に対する需要が高まっている。遊離残留塩素濃度は国の水道法施行規則、水質管理目標設定項目によりそれぞれ0.1mg/L以上、1.0mg/L以下という範囲で定められているが、上限値に独自基準を設けるなど、残留塩素低減化の取り組みが各地で進められている。一方、残留塩素低減化によって水道水の微生物学的安定性が低下し、水道給配水系における細菌再増殖のリスクが高まることも懸念される。適切な残留塩素の維持レベルを検討する上で、実際に給配水系で再増殖する細菌群の塩素耐性を知ることは重要であるが、こうした知見は限られているのが現状である。そこで、本研究では、実際の給水末端から単離・同定した複数の従属栄養細菌(Heterotrophic Plate Count: HPC)（水質管理目標設定項目）を対象として、それらの塩素耐性を膜損傷性とコロニー形成能に着目して評価することを目的とした。

2. 実験方法

従属栄養細菌の単離を目的として、東京都内10か所の給水末端から水道水を採水した。試料をR2A培地で培養後、HPCとして検出された細菌を単離した。二重標識T-RFLPにより異なる断片長を持つものを選択、16S rRNA遺伝子の全長を決定し、RDP classifierによる系統解析を行った。これらに、同化性有機炭素測定に用いられる *Pseudomonas fluorescens* P17株(ATCC49642)、*Aquaspirillum* sp. NOX株(ATCC49643)の2株と *E.coli*(IFO3301)を加え、以下の試験を行った。

初期生菌数を1.0×10^6 cells/mLに調整したpH7のリン酸緩衝液に次亜塩素酸ナトリウムを添加し、室温にて遊離塩素濃度を約0.1, 0.4,1.0mg/Lに維持した。所定の時間間隔で試料を採取し、直ちにチオ硫酸ナトリウムで塩素を中和した。採取した試料を、全菌の核酸を染色するSYBR Green I（Invitrogen社）と膜損傷している死菌の核酸のみを染色するPropidium Iodide（Wako）で暗所、37℃にて10分間染色することで、SYBR Green Iでのみ染色されたものを膜損傷性で評価した生菌とし、フローサイトメーター（Flow cytometer : FCM）（Accuri C6, BD社）で計数した。本機器の定量下限値は1.0×10^3 cells/mLであり、初期生菌数と比較し3logまでの不活化を評価した。また、塩素耐性をコロニー形成能でも評価するため、R2A培地に試料を塗布し、20℃にて7日間培養後、コロニー計数を行った。

3. 結果及び考察

従属栄養細菌の単離：本研究では、都内10か所の給水末端から単離、選別した従属栄養細菌15株（HPC1-15株）を塩素耐性試験に用いた（表1）。これらは単離した計190株について二重標識T-RFLPによるスクリーニングの結果、19種類のOTUに分類された株のうち、増殖性が安定していた株である。系統解析の結果、19株は14の属に分類された。15株の門レベルでの内訳は、*Proteobacteia* が9株と最も多く、次いで *Actinobacteria* 門（3株）、*Firmicutes* 門（2株）、*Bacteroidetes* 門（1株）であった。

塩素耐性試験：塩素耐性試験の結果、膜損傷性で評価した生菌数についてほとんどの株で生残割合の対

表1 従属栄養細菌株の単離結果

Name	Classification Information		
	Phylum	Genus	Identity
HPC1	*Firmicutes*	*Exiguobacterium*	100%
HPC2	*Proteobacteria*	*Sphingopyxis*	100%
HPC3	*Actinobacteria*	*Mycobacterium*	100%
HPC4	*Proteobacteria*	*Roseomonas*	100%
HPC5	*Proteobacteria*	*Burkholderia*	100%
HPC6	*Bacteroidetes*	*Sediminibacterium*	100%
HPC7	*Firmicutes*	*Staphylococcus*	100%
HPC8	*Proteobacteria*	*Brevundimonas*	100%
HPC9	*Proteobacteria*	*Brevundimonas*	100%
HPC10	*Proteobacteria*	*Methylobacterium*	100%
HPC11	*Proteobacteria*	*Aquabacterium*	100%
HPC12	*Actinobacteria*	*Mycobacterium*	100%
HPC13	*Proteobacteria*	*Lysobacter*	100%
HPC14	*Actinobacteria*	*Mycobacterium*	100%
HPC15	*Proteobacteria*	*Sphingomonas*	100%

数値が CT 値に比例しており、Chick-Watson 式に従うことが確認された。

$$\text{Chick-Watson 式}：-\log_{10}(N/N_0) = kCT$$

N_0: 初期生菌数[cells/mL], N: 時間 T における生菌数[cells/mL],
C: 消毒剤濃度[mg/L], T: 接触時間[min], k: 不活化速度定数[L/mg/min]

不活化速度定数 k が遊離塩素濃度に依存しなかったことから、3 段階の遊離塩素濃度での試験結果をまとめたものから k を求めた（図 1）。HPC10 株（*Methylobacterium* 属近縁）と HPC12 株（*Mycobacterium* 属近縁）の不活化速度定数がそれぞれ 0.015, 0.019 L/mg/min と、他の株と比べて著しく高い塩素耐性を有していることが確認された。最も塩素耐性が低かったのは *E. coli* であり、不活化速度定数は 8.0 L/mg/min であった。また、P17 株、NOX 株の不活化速度定数はそれぞれ 4.8, 6.1 L/mg/min であり、単離株と比較すると塩素耐性が低いことが確認された。本研究で用いた単離株は塩素処理をされた水道水から単離されているため、塩素耐性の低い菌は事前に不活化しており、相対的に塩素耐性の高い細菌群であったことが推察される。一方、単離株の中では HPC6 株（*Sediminibacterium* 属近縁）の塩素耐性が最も低く、不活化速度定数は 5.1 L/mg/min であった。このような株が単離された理由として、給水栓付近での滞留による塩素の消失により再増殖に適した環境となり、不活化された細菌が回復、再増殖したことが考えられる。HPC12 株と同様に *Mycobacterium* 属に近縁な株である HPC3, 14 株も高い塩素耐性を有していたが、不活化速度定数はそれぞれ 0.13, 0.19 L/mg min であり、HPC12 株と比べておよそ 1 オーダーの差があった。このことから種レベルで塩素耐性が異なることが確認された。

コロニー形成能による評価の結果、多くの菌は CT 値が最小の 0.025mg min/L 程度でもコロニー形成が確認されなかった。コロニー形成の有無は膜損傷評価による結果と関連せず、HPC15 株（*Sphingomonas* 属近縁）のように膜損傷評価で塩素耐性が低いと判断された株にもかかわらずコロニー形成が確認された例もあった。コロニーが確認された株の一例として、HPC12 株と HPC15 株についての結果を図 2 に示す。HPC12,15 株のコロニー形成能で評価した不活化速度定数は、それぞれ 0.069, 16 L/mg min であった。一方、膜損傷性で評価した場合は、それぞれ 0.019, 4.0 L/mg min であった。他の全ての株においてもコロニー形成能により評価した不活化速度定数の方が大きな値を示した。これは、コロニー形成は不可能であるが、膜損傷はしていないというレベルで不活化された細菌が存在することを示しており、膜損傷性による評価はより確実なレベルで細菌の不活化を判断可能な評価方法であると考えられる。

4. 結論

給水末端から単離した従属栄養細菌の塩素耐性を評価した。その結果、従属栄養細菌として包括的に評価される細菌群中には幅広い塩素耐性を持つ菌が存在することが確認された。膜損傷性による不活化の評価は、コロニー形成能による評価と比べて、再増殖リスクをより安全側で評価できる方法であることが確認された。

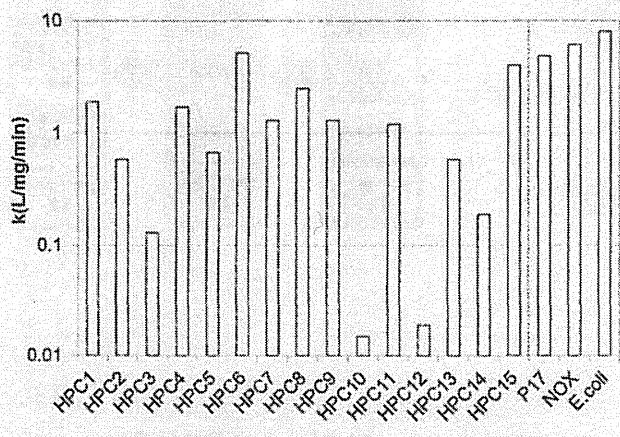

図 1 各菌株の膜損傷性評価による不活化速度定数

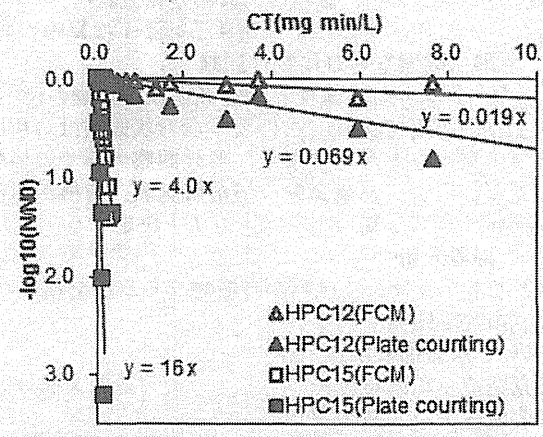

図 2 膜損傷性とコロニー形成能で評価した塩素による不活化の比較（HPC12, 15 株）