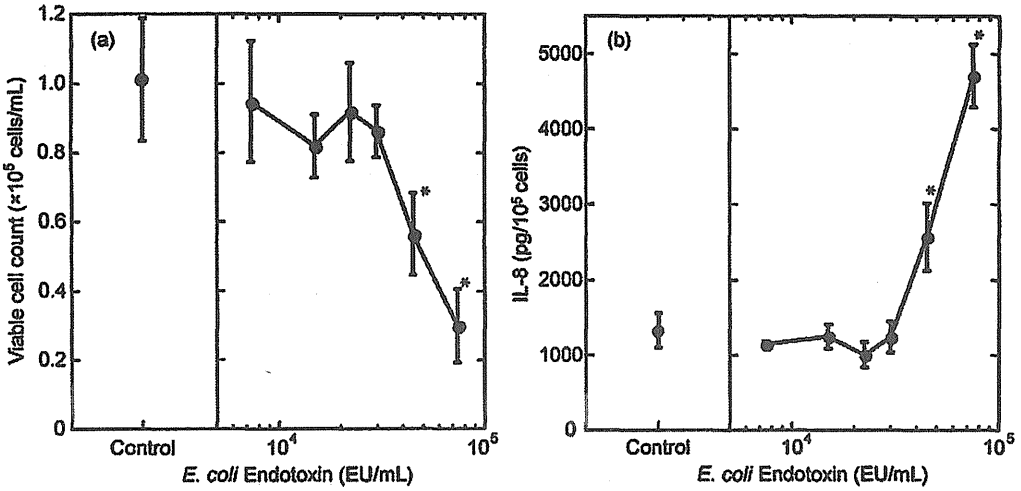

Fig. 1. Effects of lipopolysaccharides from *E. coli* O55:B5 on (a) cell viability and (b) IL-8 secretion of human keratinocytes. The data represent the mean ± the standard deviation of three independent experiments. Asterisks indicate significant difference in comparison to the cells without LPS stimulation.

LPS stimulation was observed with endotoxin stimulation above 45000 EU/mL. The cytokine levels at different LPS concentrations were compared in Figure 1(b) using the amounts of cytokine production per 10^5 viable cells. The IL-8 secretions were also increased significantly by stimulation with endotoxins above 45000 EU/mL. The decrease of cell counts, however, caused by the cytotoxic effect at a higher LPS stimulation contributed significantly to these increments. No TNF-α secretion was observed with LPS stimulation in NHEK.

Our previous results indicated that the endotoxin levels in water systems in Japan ranged from 10^0 (drinking water) to 10^4 (effluent from sewage treatment facility) EU/mL.[18] The NHEK cytokine secretions occurred only with stimulation at extremely high LPS concentrations compared to the endotoxin levels in aquatic environments.

CD14⁺ monocytes

The cell survivals of CD14$^+$ monocytes after stimulation with purified *E. coli* LPS for 6 or 24 h are shown in Figure 2. Although there were no large changes in cell counts with 6 h stimulation, the approximately 1.5-fold cell counts after 24 h stimulation were observed in all examined endotoxin levels (30 – 30,000 EU/mL).

The cytokine secretions, TNF-α and IL-8, are presented in Figure 2(b). In all of the following figures, the cytokine levels at different LPS concentrations were compared using the amounts of cytokine production per 10^6 viable cells. The IL-8 production after 6 h stimulation at 30 EU/mL was drastically increased to 30,000 pg/10^6 cells. The same level of IL-8 secretion even at 0.3 EU/mL was observed here (data not shown). At a higher range of endotoxins, the IL-8 stayed at around 20,000 pg/10^6 cells.

On the other hand, secreted IL-8 levels were decreased to 16,000 – 21,000 pg/10^6 cells after 24 hours stimulation. The TNF-α was also increased up to 4800 pg/10^6 cells by LPS stimulation at 30 EU/mL for 6 h, but further increased to more than 7800 pg/10^6 cells with LPS stimulation above 3000 EU/mL. After 24 h stimulation, the TNF-α were then decreased to less than 260 pg/10^6 cells at all LPS concentrations. These results suggested that cytokines were secreted within 6 h and the decomposition rate of each cytokine largely exceeded the newly secretion during the next 18 h.

As described previously, the endotoxin levels in aquatic environment were in the range of 10^0 – 10^4 EU/mL in Japan[18]. The LPS stimulation even at very low levels caused significant cytokine secretions, and no clear dose-dependent changes in cytokine secretions was obtained.

THP-1

The cell survivals of THP-1 after stimulation with purified *E. coli* LPS for 6 or 24 h are compared in Figure 3(a). The cell counts of THP-1 after 6 h stimulation showed no significant change in the examined endotoxin range of 7 – 70,000 EU/mL as well as CD14$^+$ monocytes, but the counts were increased significantly in a dose-dependent manner after 24 hours stimulation.

The IL-8 and TNF-α secretions after LPS stimulation are also presented in Figure 3 (b). The cytokine productions after 6 h LPS stimulation were increased dose-dependently in the examined endotoxin range, although the secreted levels were 890 and 640 pg/10^6 cells at maximum for IL-8 and TNF-α, respectively, which were much lower than the levels observed in CD14$^+$ monocytes. After 24 h stimula-

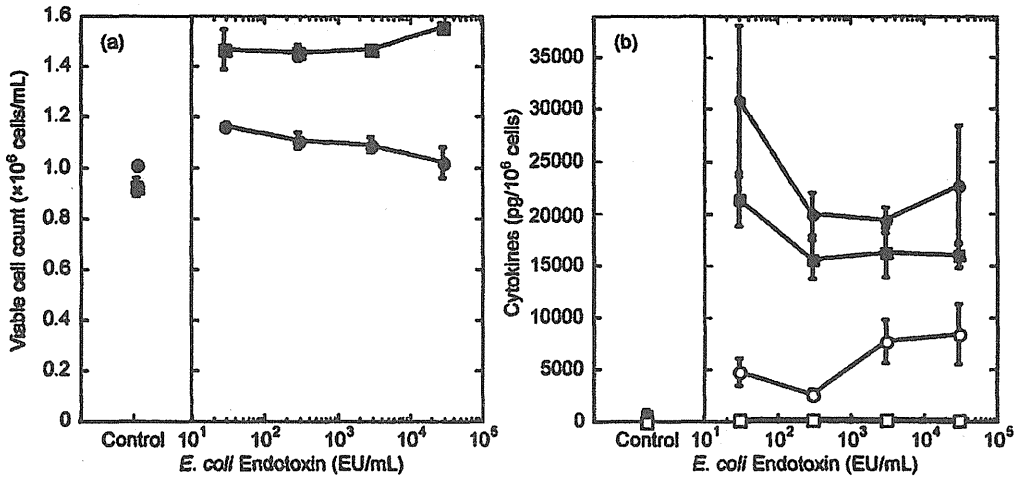

Fig. 2. Effects of lipopolysaccharides from *E. coli* O55:B5 on (a) cell viability and (b) cytokine secretion of $CD14^+$ monocytes. In (a), the viable cell count after 6-h stimulation (solid circle) and 24 h stimulation (solid square) are presented. In (b), the IL-8 after 6 h (solid circle) and 24 h (solid square) stimulation and the TNF-α after 6 h (open circle) and 24 h (open square) stimulation are also presented. The data represent the mean with the range of two independent experiments.

tion, TNF-α disappeared completely, while IL-8 production decreased, but was still detected in the culture medium.

The exposure routes of endotoxins via water in daily life include oral ingestion, inhalation, and dermal contact, and only inhalation route has been already identified as a health risk factor[19–21]. Among the examined cell lines in this investigation, NHEK is the only cells forming the boundary of the human body against endotoxin exposure via water in daily life. Therefore, the obtained inflammatory responses in NHEK could be more directly interpreted as toxicity on the skin via dermal contact to water. Our results, however, showed very poor inflammatory responses of NHEK with LPS stimulation.

On the other hand, the human blood cells revealed more sensitive responses to LPS stimulation at average levels in an

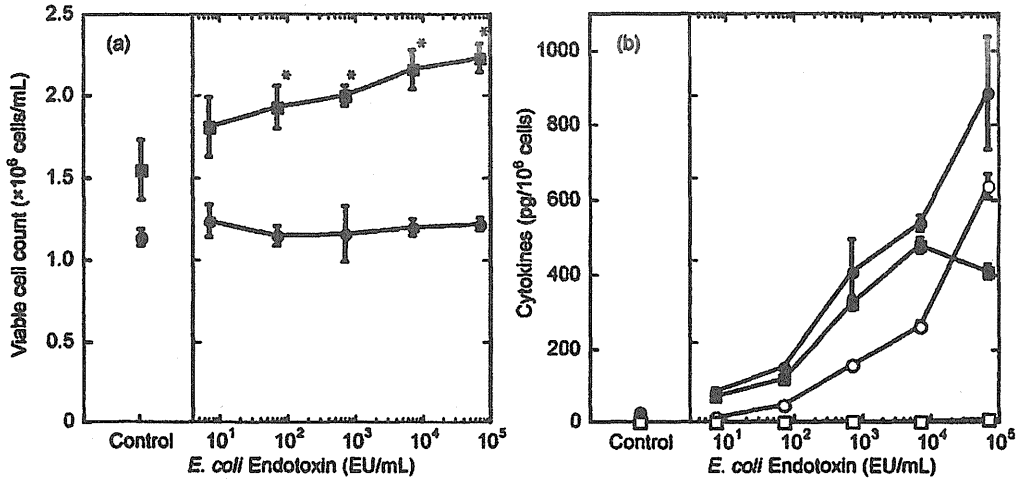

Fig. 3. Effects of lipopolysaccharides from *E. coli* O55:B5 on (a) cell viability and (b) cytokine secretion of THP-1. In (a), the viable cell count after 6 h stimulation (solid circle) and 24 h stimulation (solid square) are presented. In (b), the IL-8 after 6 h (solid circle) and 24 h (solid square) stimulation and the TNF-α after 6 h (open circle) and 24 h (open square) stimulation are also presented. The cell count data represent the mean ± the standard deviation of four independent experiments. The cytokine data represent the mean with the range of two independent assays. Asterisks indicate significant difference in comparison to the cells without LPS stimulation.

Table 1. Microbiological parameters of river water samples.

	Yodo River sample
Heterotrophic plate counts (CFU/mL)	4.1×10^3
Total bacterial cell counts (cells/mL)	3.9×10^5
Total Endotoxin (EU/mL)	4.4×10^2
Free Endotoxin (EU/mL)	2.4×10^2
Cell-bound endotoxin (EU/mL)	2.2×10^2

aquatic environment. By comparison of $CD14^+$ monocytes with THP-1, the dose-dependent responsiveness in THP-1 could cover a wide range of endotoxin levels generally observed in aquatic environments, while $CD14^+$ monocytes significantly responded to a trace level of endotoxin. Based on these results, THP-1 with 6-h LPS stimulation was chosen for the following assay to determine the inflammatory potencies caused by endotoxins in aquatic environments.

Obviously, there is a limitation to applying the obtained data using these cell lines to a toxicity assessment caused by LPS, because the blood cells are rarely exposed to them directly except during medical care or in wound tissues. The blood cells are usually exposed only to some portion of inhaled or ingested endotoxins because of various physical and chemical defenses in the human body. The actual inflammatory responses in blood cells, therefore, should be estimated based on the internal doses, but little information on internal LPS doses is currently available. This limitation of THP-1 assay system should be considered in a further toxicity assessment, however, the assay system enables us to detect and to compare the inflammatory potency in various water samples taken from aquatic environments or water treatment/reclamation processes, which were contaminated with endotoxins at different levels.

LPS extraction from indigenous bacterial cells in aquatic environment

Table 1 shows a summary of microbiological parameters in the river water sample taken in the downstream area of the Yodo River. It is a main river in the Kinki region in Japan, and is a main resource of drinking water in Osaka City, the second biggest city in Japan. The heterotrophic plate counts were 4.1×10^3 CFU/mL, and the total bacterial cell counts determined by DAPI staining were 3.9×10^5 cells/mL. This result indicates that only 1.1% of indigenous bacteria in the river water could be cultivated on R2A agar medium at 20 °C, which is the ordinary condition for detecting heterotrophic bacteria in aquatic environments. Additionally, this result suggested that such an unculturable fraction of the aquatic bacterial community could contribute to endotoxins in aquatic environments.

The total endotoxins determined by LAL assay were 440 EU/mL. The free endotoxins determined after centrifugation to remove cell-bound endotoxins were 240 EU/mL. The endotoxic activity per indigenous bacterial cell was calculated as 1.1×10^{-3} EU/cell in this sample. Our previous investigation showed that the endotoxin levels in the middle part of the river were greater than 1500 EU/mL, because there are many contamination sources of endotoxins, such as the inflow of effluent from several sewage treatment facilities located along the river [18]. However, the endotoxin contamination seemed to be partially mitigated less than 1000 EU/mL in the downstream of the Yodo River based on our two time samplings.

The changes of total endotoxin and weight in the sample during purification steps are shown in Table 2. After enrichment with ultrafiltration membrane, the total endotoxic activity increased by 78%. We have not yet identified the reason for the endotoxin increase, but new endotoxin synthesis with multiplication or release from existing bacteria might have occurred because it took a few days to concentrate over 20 L sample by ultrafiltration. During the purification steps, 72% and 54% of the endotoxic activities were removed by phenol extraction and RNase A treatment, respectively. In these two steps, the losses of dry weight overwhelmed the decrease in the total endotoxic activity, and consequently the endotoxic activity per dry weight increased.

Phenol extraction can destroy cell membranes and remove proteinaceous compounds contained in bacterial cells by denaturing. The RNase A treatment was then applied to the crude extract, because it was considered to still contain abundant RNA fractions. The degraded high molecular weight RNA could be removed by the following dialysis in this procedure. As a result, the purity of the LPS contained in the extract from indigenous bacterial cells could be increased. Finally, the purified LPS sample exhibited 1.29×10^6 EU/mg.

Inflammatory responses caused by LPS derived from indigenous bacteria in aquatic environment

The THP-1 cell viability after 6 h stimulation with purified LPS from the river water samples, called environmental LPS here, is presented in Figure 4(a). The cell viability was increased by 20% with LPS stimulation in all examined ranges. This result was inconsistent with the observation in THP-1 cells stimulated with the purified *E. coli* LPS for 6 h. There are two possible reasons for the difference. The first is that the remaining contaminant substances except LPS, which are hydrophilic high molecular weight organic compounds and could not be removed by phenol extraction or the following dialysis, could activate THP-1 cells. The second is that the variety of LPS structures in environmental bacteria could affect the cell viability. Unfortunately, there is no clear evidence to identify which factor could contribute more to the THP-1 activation at this stage.

The IL-8 and TNF-α secretions from THP-1 are compared in Figure 4(b), and both secretions were increased in a dose-dependent manner. It is of note that the environmental LPS induced stronger inflammatory responses in THP-1

Table 2. Purification of LPS from indigeneous bacterial cells in the river water sample.

	Total endotoxic activity (EU)	*Dry weight (mg)*	*Endotoxic activity per dry weight (EU/mg)*	*Relative purity* (%)*
River water	9.59×10^6	—	—	—
After ultrafiltration	1.71×10^7	20.3	8.40×10^5	100
After phenol extraction	4.78×10^6	4.4	1.09×10^6	130
After RNase A treatment	2.19×10^6	1.7	1.29×10^6	154
After sterilization	2.50×10^6	—	—	—

*Relative purity means how much endotoxic activity per dry weight was increased compared to the start sample after ultrafiltration.

than purified *E. coli* LPS at the doses corresponding to the same endotoxic activities determined by LAL assay. With regard to the overall range of endotoxins, more than 10-fold cytokine productions were observed in the culture medium of THP-1 with the environmental LPS stimulation.

The second noteworthy point is that TNF-α secretion was comparable to IL-8 secretion in THP-1 stimulated with the environmental LPS, whereas IL-8 secretion was superior to TNF-α secretion in all tested human cells stimulated with LPS from *E. coli* O55:B5 as shown in Figures 1 – 3. Thus, the stimulation of THP-1 with the environmental LPS induced completely different levels and patterns of cytokine secretions compared to *E. coli* LPS.

Previous researches investigated the secretions of various proinflammatory and immunoregulatory cytokines, such as IL-1β, IL-6, IL-8, IL-10, IL-12, interferon (IFN)-γ, and TNF-α, from human blood cells after stimulation with various bacterial LPS[6, 14]. Recently, Skovbjerg et al.[22] compared productions of several cytokines from PBMCs stimulated with gram-positive bacteria and gram-negative bacteria inactivated by UV irradiation, respectively. These researches proved that gram-negative bacteria more strongly induced IL-10, IL-8, and IL-6 than gram-positive bacteria, while gram-positive bacteria induced more IFN-γ, TNF-α, and IL-1β.

Their results suggested that the IL-8 and TNF-α secretions tested in our investigation might be consequences of different responsiveness to inflammatory substances derived from aquatic bacteria. Based on the above knowledge, the stronger IL-8 secretion could be attributed to the diversity of LPS structures and their inflammatory potency as a reflection of the diversity of indigenous bacteria in aquatic environments, while some part of the TNF-α elevation observed here might be caused by unidentified contaminated substances from gram-positive bacteria in the river water or other microorganisms.

In addition to the above information, several literatures would be helpful for interpreting our result. For example, it is known that peptidoglycan or lipoteichoic acid derived from gram-positive bacteria could be inducers

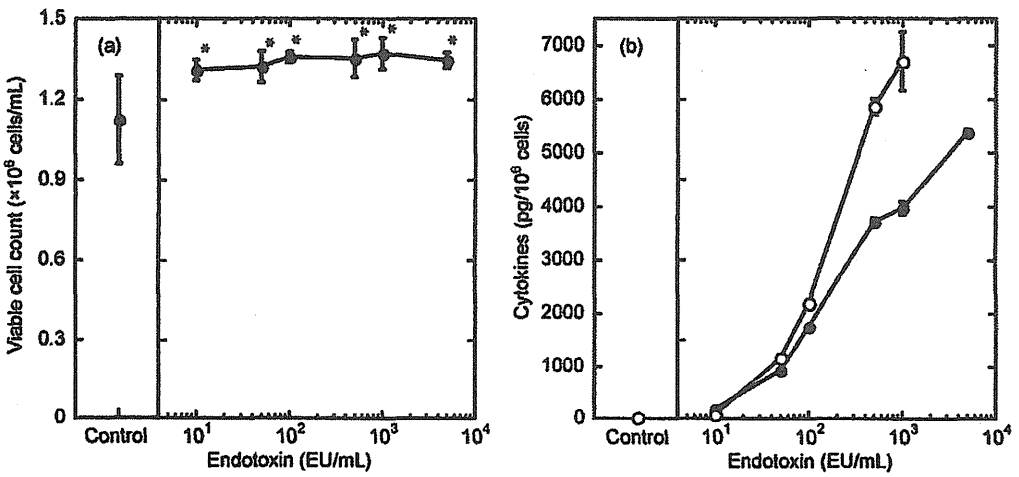

Fig. 4. Effects of lipopolysaccharides from indigenous bacteria in river water samples taken from the Yodo River on (a) cell viability and (b) cytokine secretion of THP-1. Two types of cytokines, IL-8 (solid circle) and TNF-α (open circle) were determined after 6-h stimulation. The cell count data represent the mean ± the standard deviation of 6 independent experiments. The cytokine data represent the mean with the range of two independent assays. Asterisks indicate significant difference in comparison to the cells without LPS stimulation.

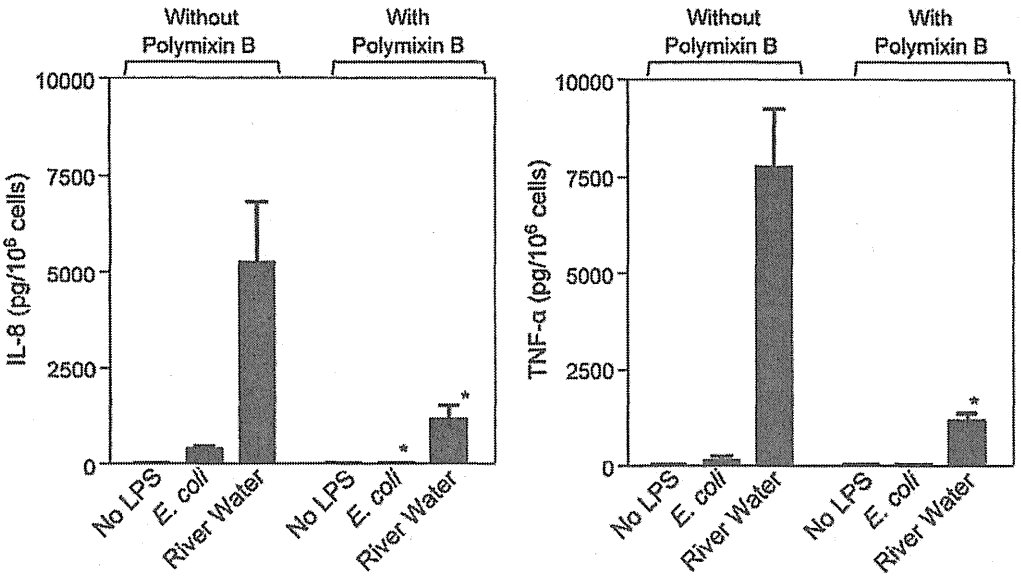

Fig. 5. Effects of Polymixin B addition as an inhibitor of endotoxin activity on cytokine secretion of THP-1. Two types of cytokines, IL-8 and TNF-α were determined after 6 h stimulation with LPS samples and pretreated LPS with Polymixin B, respectively. The cytokine data represent the mean with the standard deviation of four independent assays. Asterisks indicate significant difference in comparison to the cells without Polymixin B pretreatment.

of pyrogenic reactions in human blood cells. Nucleic acids could also cause cytokine secretion. The river water contains these bacterial substances as abundantly as LPS. However, it would be reasonable to conclude that there was little contribution of these contaminated substances to cytokine secretions. The following researches are evidences.

Nakagawa et al.[23] compared cytokine (IL-6, IL-1, TNF-α) inducibility with several pyrogens using a monocytic cell line, Mono Mac 6, and reported that the equivalent cytokine secretions were observed only after stimulation with 250-fold or higher doses of peptidoglycan and poly (I:C), which is a synthetic analog of double-stranded RNA, compared to endotoxin. Similarly, several researches proved that the equivalent cytokine secretions also required more than 100-fold amounts of lipoteichoic acid compared to LPS.[13, 17]

To clarify the contribution of contaminant substances in the prepared environmental LPS samples to the TNF-α elevation, the changes in mRNA expression levels of TLR-2, which can recognize lipoteichoic acid and peptidoglycan from gram-positive bacteria as ligands, after stimulation were examined. In THP-1, mRNA of TLR-2 was constitutively expressed without any stimulation, but no increase in the expression level after the stimulation was observed (data not shown).

On the other hand, the addition of Polymixin B as an inhibitor of endotoxin activity significantly, not completely, inhibited both cytokine secretions as shown in Figure 5. This result clearly indicated that the major contributor to cytokine elevations was LPS components in the environmental LPS sample. The higher secretion level of TNF-α than IL-8 by stimulation with the environmental LPS sample was also confirmed in this experiment.

Above all information strongly suggested that the contribution of contaminated substances from gram-positive bacteria to TNF-α elevation was not significant in our result. On the other hand, Erridge et al.[24] reported that *Acinetobacter* LPS could induce higher levels of TNF-α secretion than IL-8 in THP-1. They also proved that *Acinetobacter* LPS could induce stronger immunoreactions than *E. coli* LPS in THP-1. Thus, some bacteria existing ubiquitously in aquatic environment possibly induce stronger inflammatory responses including TNF-α elevation. The impact of various bacterial LPS in aquatic environment on inflammatory potencies or patterns of cytokine secretions should be further investigated to deeply understand immunoreactions in human cells caused by LPS in aquatic environment.

Our result also suggested a limitation of the LAL assay for detecting the toxicity changes caused by endotoxins to humans. Wichmann et al.[10] have already reported that filtered river water samples could induce cytokine production in healthy human blood cells. Their report also indicated that cytokine production in human blood cells could be a good indicator for LPS in river water. In addition to their findings, our results presented that cytokine secretion assay using THP-1 cells can reflect the differences of LPS derived from aquatic bacteria in their potency to induce inflammatory responses in humans.

Dehus et al.[6] reported that cytokine release from human whole blood did not correlate with LAL activity based on the results of whole blood samples stimulated with LPS from enteric bacteria and *Pseudomonas aeruginosa*, and their result also strongly supported our result. The bacterial community differs according to the locations or water types, such as river, lake, effluent from sewage treatment facilities, or inside water distribution pipes. Our proposed THP-1 cell culture assay combined with cytokine production could be a useful tool for covering a broad range of water types inducing human inflammatory responses by various bacterial communities in aquatic environments.

Conclusion

This investigation indicated that cytokine assay using THP-1 with stimulation for 6 h could be useful for detecting the changes in inflammatory potencies of bacterial LPSs. The environmental LPSs purified from a river water sample induced stronger cytokine secretions in THP-1 including TNF-α elevation than *E. coli* LPS. Our findings suggested not only that LAL assay has a limitation for detecting changes in toxicity of bacterial LPSs in aquatic environment, but also that indigenous bacteria in aquatic environment can cause strong immune responses in humans even though they exhibited relatively low endotoxic activities.

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[報　文]

ナノろ過膜処理を適用した浄水の細菌再増殖特性の評価

Characterization of Bacterial Regrowth in Nanofiltrated Drinking Water

大河内由美子*　　矢田祐次郎**
文　　亮太***　　伊藤　禎彦*

微生物学的安定性向上の一手法として，現行の高度浄水処理の後段にナノろ過(NF)処理を導入した場合の，浄水の微生物学的な水質評価を行なった。NF処理により92%と安定して高い細菌阻止率が得られたものの，処理水からは恒常的に従属栄養細菌が検出された。また，残留塩素なしの条件では，これらの顕著な再増殖が確認された。とくに，NF処理水中の再増殖細菌群は，同等の炭素量からより多くのバイオマスを生産することが判明した。また，NF処理水中の代表的な再増殖細菌種は，*Herbaspirillum*属，*Curvibacter*属，*Undibacterium*属であり，文献調査の結果からこれらは普遍的に水環境に存在し，かつ極貧栄養条件下での増殖に適した細菌種であることがわかった。

本研究の成果は，NF処理により同化可能有機炭素(AOC)を十分に除去しても，一度汚染が起これば残存する少量のAOCを基質として爆発的な再増殖が起こることを意味する。そのため，NF処理適用後にも浄水水質に応じて最小限の残留塩素を併用することが不可欠である。

Key words：ナノろ過処理，微生物学的安定性，細菌再増殖，水道システム

1. はじめに

日本の水道事業は，原水水質の状況に合わせてオゾン(O_3)－粒状活性炭(GAC)処理を軸とした高度浄水処理を導入し，科学的な観点から十分に安全な水を供給する努力を続けてきた。しかし，このように格段に水質が向上した水道水であっても，利用者からは必ずしも高い評価を得ているわけではない[1]。水道水に対する満足感を低下させる因子として，塩素との反応生成物であるカルキ臭に対する注目が集まっている。カルキ臭を抑制するためには，塩素消毒プロセスに先立つ前駆物質の除去と並んで，残留塩素濃度の最小化が有効なアプローチとなり得る。前者については，化学

＊　Yumiko OHKOUCHI, Sadahiko ITOH, 京都大学大学院地球環境学堂　博士(工学)　〒615-8540 京都市西京区京都大学桂C-Ⅰ, Dr. Eng., Graduate School of Global Environmental Studies, Kyoto University, C-Ⅰ Kyoto Daigaku Katsura, Nishikyo-ku, Kyoto-shi 615-8540 Japan (E-mail : yohkouchi@urban.env.kyoto-u.ac.jp)
＊＊　Yujiro YATA, 日立造船㈱
＊＊＊　Ryota BUN, バブコック日立㈱

酸化処理とイオン交換処理を組み合わせたプロセスにより，多種多様な無機・有機化合物により構成されるカルキ臭前駆物質を大幅に低減できることが報告されており[2]，従来の高度浄水処理の枠組みを超えた取組みが進められている。カルキ臭原因物質としてはトリクロラミンの他，有機クロラミンやN-クロロアルドイミン，アルデヒドなどの物質が報告されている[3)4)]が，水道水カルキ臭に対する寄与が約20%を占めるトリクロラミンの場合，低有離残留塩素濃度領域では生成されにくいことが明らかとなっている[3]。一方，現在の多くの水道システムでは，給水末端で0.3～0.4 mg/l前後の残留塩素を維持する[5]ことで細菌類の再増殖抑制に成功している。

上述のように残留塩素濃度とカルキ臭は密接に関連しており，過剰な塩素添加がカルキ臭上昇を招くと考えられる。たとえば，促進酸化－GAC－陽イオン交換処理を行なった水に対して，24時間後の遊離残留塩素濃度が0.05～0.08mg/lとなるよう塩素処理を行なうことで，15℃における臭気強度を60TON超から10TON以下へと大幅低減可能であることがわかっている[6]。これらの情報を踏まえると，給水栓で検出される残留塩素濃度を必要最小限，たとえば0.05mg/l程度に低減することにより，同時にカルキ臭抑制も達成できると考えられる。

しかし，残留塩素濃度の低減は浄水の微生物学的安全性の悪化に直結するため，同時に微生物学的に安定な浄水水質の確保が必要となる。筆者らは，現在主流のO_3－GAC処理を軸とした高度浄水処理プロセスを対象に，微生物学的安定性の一つの指標として細菌再増殖の基質となる同化可能有機炭素(AOC)の除去特性の評価[7]に取組むとともに，残留塩素濃度を最小化した水道システムの微生物学的安定性維持に求められるAOC条件を提示した[8]。また，現行の処理プロセスでは季節変動が大きく不十分なAOC処理能強化を目的として，物理化学的処理の一種であるナノろ過(NF)膜処理の適用による浄水の微生物学的安定性の向上を検討した結果，NF膜処理プロセスを現行の高度処理プロセス後段に増設した場合，浄水のAOC濃度を半減できるものの処理性は大きく変動し，微生物学的に安定な水質条件の継続的な達成は難しいこと，また，残留塩素なしの条件ではNF処理水であっても容易に細菌類が再増殖するため，極微量の残留塩素維持が必要であることも明らかにした[9]。

このように，本来有機物除去能のみならず微生物バリアーとしても高い性能を発揮すると期待されるNF膜であるが，微生物学的に安定な水質を継続的に達成するためには，NF処理水の細菌再増殖ポテンシャルを把握したうえで，再増殖抑制に必要な残留塩素レベルを決めることが重要である。以上の背景を踏まえて，本研究ではNF処理による細菌阻止能ならびに処理水の細菌再増殖特性の評価に取組み，微生物学的に安定な水供給の達成に向けた基礎情報の収集を行なう。

2. 実験方法

2.1 実験施設概要

淀川表流水を原水とするA浄水場実験処理施設で稼働中のNF膜処理ユニット(表1)を対象とした。なお，使用した膜はメーカーによると逆浸透膜に分類されているが，運転はむしろNF膜に適用される圧力範囲で行なっていることから，本稿ではNF膜として取り扱うこととした。本実験処理施設の設計処理能は50t/d であり，実施設のO_3－GAC処理水を通水して膜処理を行なった。運転期間中，膜ファウリングにより膜間差圧の上昇がみられた場合には，薬品洗浄(クエン酸洗浄＋カ性ソーダ洗浄)を実施した。

2.2 採水方法

NF処理の細菌阻止性能把握を目的とした採水は，2010年5～12月，2011年8～12月の期間に行なった。採水バルブを開けて数分間放水した後に，NF膜供給水(GAC処理水)およびNF処理水の各

表1 使用した膜処理ユニットの概要

膜形式	スパイラル型NF膜(超低圧RO膜)
サイズ	φ99.5mm×L 1,016mm
本数	3本(2本-1本の1パス2段式)
型式	ESNA1-4040(日東電工㈱製)
除塩率	90%
膜材質	芳香族ポリアミド樹脂

試料を有機物フリーの採水瓶に採取して密栓し，4℃で運搬した。再増殖細菌の16S rRNA系統解析を目的とした試料は，2010年12月，2011年1月，8月，9月に，それぞれ同様の方法で採取した。

2.3 NF処理による細菌阻止性能

NF処理前後で採取した試料の，従属栄養細菌数(HPC)の測定を行なった。必要に応じて試料を希釈後，R2A寒天培地(日水製薬㈱製)を用いて20℃で7日間の培養により形成された集落数をカウントした。また，一部の試料については，孔径0.20 μmのフィルター上に試料中の細菌を捕集した後に，4' 6-Diamidino-2-phenylindole(DAPI，㈱同仁化学研究所製)を添加して室温で10分間染色し，蛍光顕微鏡を用いて染色細胞を計数することにより全菌数を測定した。

2.4 再増殖性評価試験

再増殖性の評価は，回分培養系で2通りの試験方法により行なった。いずれの系においても塩素処理は行なっていない。試験方法(1)では，採取した試料をそのまま密栓し，遮光条件・20℃で培養し，経時的に試料を採取して再増殖HPCを測定した。この方法は，NF処理による水質変化，すなわち炭素のみならず窒素やリン，微量元素などの水質変化，ならびに微生物量と種の変化が再増殖性に与える影響を，総合的に評価する手法とみなすことができる。一方，試験方法(2)では，各試料採取後，いったん加熱殺菌により残存細菌を不活化した後に，窒素・リンを補給したうえで0～200 μgC/lの濃度で酢酸ナトリウムを添加し，さらに，あらかじめ再増殖させておいたGAC処理水・NF処理水におのおの由来する菌体を植種した。試験方法(1)と同様の条件で培養し，再増殖HPCの測定を行なった。この方法では，おもにNF処理により変化した微生物種が再増殖性に及ぼす影響評価が可能となる。

2.5 再増殖細菌を対象とした16S rRNA系統解析

各試料1l採取後，6倍濃縮R2A液体培地を1/5容積添加し，24時間培養してから再増殖細菌を孔径0.20 μmの滅菌済みメンブレンフィルター上に集菌した。リン酸緩衝液で洗菌後，Ultra Clean® Water DNA Isolation Kit(MO BIO Laboratories社製)を用いて混合DNA試料を調製した。これらの混合DNA試料を鋳型として，バクテリア用ユニバーサルプライマー対(27F/5'-AGAGTTTGATCMTGGCTCAG-3'，1492R/5'-TACGGYTACCTTGTTACGACTT-3')[10]を用いて，16S rRNA領域を対象としてPCRを行なった。

得られたPCR断片をアガロースゲル電気泳動により分離した後，該当バンドを切り出し，Nucleospin Gel and PCR Clean-up(タカラバイオ㈱製)を用いて標的産物の精製を行なった。さらに，pCR4-TOPOベクターを用いたTAクローニング(Invitrogen，Life Technologies社製)により得たクローン株を対象として，1試料につき96クローンをピックアップしてシーケンス解析を行なった。シーケンス解析は，タカラバイオ㈱ドラゴンジェノミクスセンターに依頼した。十分な長さが得られた塩基配列に対してblastnデータベースを用いた相同性検索を行ない，99%以上かつ最も高い相同性を示す微生物種を最近縁種として決定した。

3. 結果と考察

3.1 NF処理による細菌阻止性能

NF処理前後におけるHPCの変化を，図1に示す。期間中，一時的にNF処理水のHPCが劇的に上昇したが，採水前にバルブを全開にしてしばらく放水することで通常レベルに戻ったことから，二次側配管での滞留水が影響したと考えられる。この異常値を除いたHPCの平均除去率は92±7%と高くかつ安定しているものの，NF処理後も平均12CFU/ml(濃度範囲：2～32CFU/ml)のHPCが恒常的に検出される状況であった。

本来，膜孔径と比較するとはるかに大きいサイズである細菌類が膜処理水から検出される原因としては，膜エレメントの構造に起因する一次側からの漏出と並んで，二次側の汚染に由来する可能性が指摘できる。Owenらは，パイロットスケール装置を用いて*Bacillus subtilis*の芽胞添加実験を行なった結果，中空糸型MF膜を使用した場合には5.6～5.9logの胞子除去率が得られたのに対して，平均膜孔径が2オーダー小さいスパイラル型

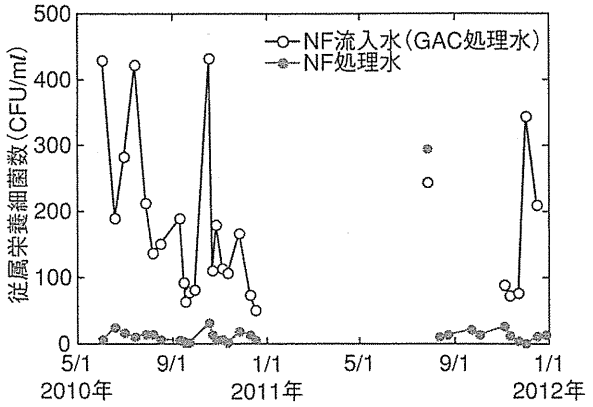

図1　ナノろ過処理前後における従属栄養細菌数の経時変化

NF膜を使用した場合でも2.2～4.5logの阻止率にとどまることを報告し，袋状かつ原水/透過水スペーサーが挿入されたスパイラル型膜の構造に起因すると指摘した[11]。この知見に基づくと，本研究で用いたスパイラル型膜構造から細菌が漏出する可能性は十分に考えられるものの，現時点ではHPC検出の原因は特定できておらず，今後詳細な検討が待たれるところである。なお，NF処理水の全菌数をDAPI染色により調べた結果，約3,000cells/m*l*が残存しており，HPCより2オーダー程度多い“R2A培地で検出できない細菌”がNF処理水に存在することも明らかとなった。

3.2　NF処理水の微生物再増殖性評価

試験方法(1)で得られた，AOC濃度の異なる試料における最大再増殖HPCの結果を図2に示す。NF処理によりAOCが低減されているにも関わらず，より多量の再増殖HPCが検出されている傾向がわかる。また，NF処理水では，試料のAOC濃度に比例して最大再増殖量が増大する傾向が弱いながらも確認された一方で，GAC処理水では両者の間に明確な関係は確認できなかった。GAC処理水には直接的にはAOCとして計測されない中～高分子量の生物分解性有機物類が多量に残存しており，AOCのみならずこれらの画分も再増殖に影響を及ぼした可能性が高い。

続いて，試験方法(2)で得られた，酢酸添加濃度と最大再増殖HPCの関係を図3に示す。試験方法(1)で得られた結果と同様に，酢酸ナトリウム濃度が同等の場合，NF処理水で得られた最大再増殖量はGAC処理水のそれを大きく上回った。前述のように，試験方法(2)では窒素やリン，ミネラル分などを試料水に添加しているため，同一炭素濃度での最大再増殖量の違いはNF菌体とGAC菌体の増殖性の違いを表わしているとみなせる。添加した酢酸ナトリウム濃度と各最大再増殖量の間には，線形関係が認められた。得られた回帰式の傾きに基づくと，GAC菌体のバイオマス収率は1 μgCのAOC当たり0.54細胞であるの

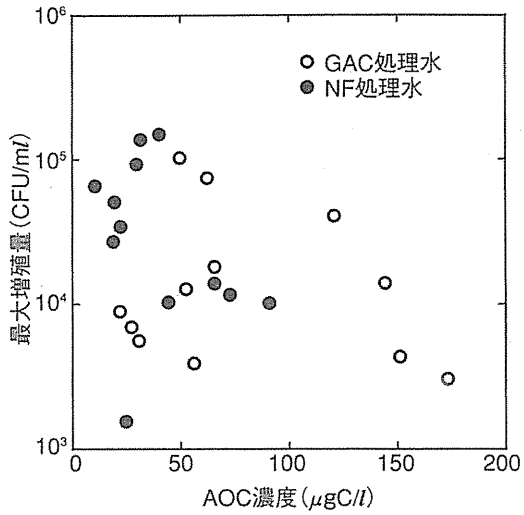

図2　試料水AOC濃度と最大再増殖量の関係：試験方法(1)の場合

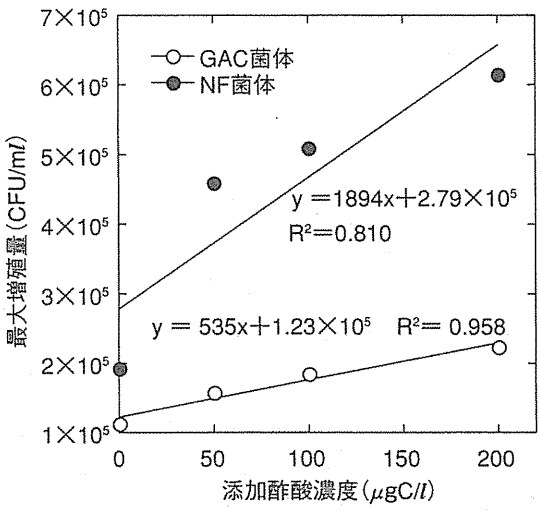

図3　添加酢酸濃度とGAC菌体/NF菌体の最大再増殖量との関係：試験方法(2)の場合

に対して，NF菌体のバイオマス収率は1.9細胞と3倍以上高いバイオマスへの変換率と推定される。すなわち，低いAOC濃度であるNF処理水試料には，微量に残存する生物分解性有機物を利用して多量のバイオマスを生産可能な微生物種が存在すると判断される。

この結果は，NF処理により細菌類および生物分解性有機炭素の大部分を除去しても，処理水中に細菌がわずかに漏出あるいは混入すれば，結果として爆発的な再増殖につながることを示唆する。実のところ，既往研究においてもNF処理水が高い細菌再増殖ポテンシャルを有する現象は複数の研究者により繰り返し確認されてきた。たとえば，Parkらは逆浸透(RO)膜処理水を連続通水して生物膜形成を調べた結果，RO透過水では細菌細胞がほとんどなくかつ低栄養状態であるにも関わらず，残留消毒剤なしの場合には生物膜が蓄積されることを示した[12]。また，Liikanenらも種々のNF膜の水質改善効果を比較した結果，供給水と比較してNF処理によりAOCやMicrobially available phosphateが低減された膜透過水で，従属栄養細菌の増殖倍率がより大きいことを報告した[13]。その理由として，膜処理により，

1) 微生物増殖阻害因子，たとえばアルミニウム塩が除去された[13]，
2) 微生物細胞当たりの利用可能なAOC量が増大した[14]，

などの，いくつかの因子関与の可能性が指摘されてきた。

しかし，ここには示していないが，筆者らが塩化アルミニウムを添加して細菌再増殖に及ぼす影響を調べた結果，0～100 μgAl/lのアルミニウム塩の濃度範囲では細菌再増殖抑制効果はみられないことを確認している[15]。むしろ本研究で得られた結果は，膜処理が再増殖細菌種の変化をより直接的に引き起こしている可能性を明確に示している。

3.3 再増殖細菌を対象とした16S rRNA系統解析

そこで，NF処理前後における再増殖細菌種の変化を明らかにするとともに，とくにNF処理水中に出現するバイオマス収率の高い再増殖細菌種の推定を試みた。まず，各試料で再増殖した細菌種を，16S rRNA配列に基づいて綱レベルで比較した結果を図4に示す。GAC処理水ではガンマプロテオバクテリアおよびバチルス綱が優占しており，ベータプロテオバクテリアが占める割合は小さかったのに対して，NF処理後の試料ではいずれも綱レベルでの多様性が減少し，ベータプロテオバクテリア綱が優占種となっていることがわかる。

また，低水温期試料の再増殖微生物を，種レベルで比較した結果を表2に示す。12月採水時の水温は約13℃であったが，1月採水時には水温は10℃以下へと低下し，それに伴いGAC処理水の再増殖細菌の優占種も*Bacillus*属から*Pseudomonas*属へと大きく変化していた。一方，NF処理水では，12月および1月の各採水試料における優占再増殖細菌種は，それぞれ*Herbaspirillum*属，*Curvibacter*属であった。このように1カ月という比較的短期

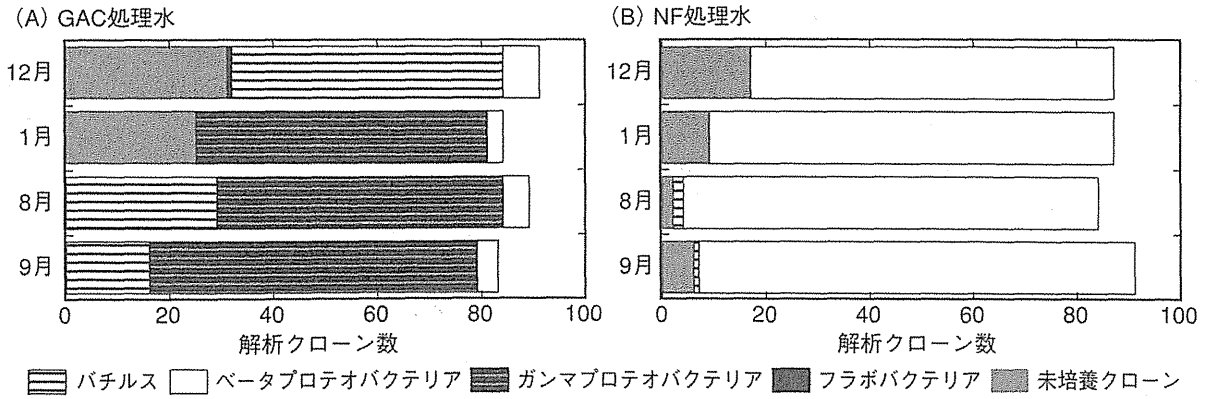

図4　16S rRNA系統解析による再増殖微生物種の綱分類結果

間に大きく再増殖細菌種が変化するという結果からも，水温や水質といった環境条件の変化に応答して，GAC上あるいはNF処理システム二次側配管内の細菌叢が変化したことがわかる。もちろんこの結果は，GAC処理水・NF処理水に存在する細菌種の存在割合をそのまま反映しているわけではなく，少量の有機物を添加した24時間の培養を経ることにより，存在割合の多い細菌種がさらに優先的に増殖し，存在割合の差を増幅した可能性がある点に注意が必要である。また，NF処理前後で1月採水試料を比較すると，検出割合は小さいものの，GAC処理水で検出された*Curvibacter*属がNF処理水では優占種となっており，NF膜エレメントのスパイラル型構造等に由来して漏出した細菌がNF処理水で再増殖した可能性も指摘できる。

同様に，高水温期の再増殖細菌群を種レベルで比較した結果を表3に示す。GAC処理水ではともに*Aeromonas*属，次いで*Bacillus*属が再増殖細菌の大きな割合を占めていた。一方，NF処理水で再増殖が確認された主要な細菌種は，8月採水試料では*Undibacterium*属，9月採水試料では上述の12月採水試料と同様に*Herbaspirillum*属であった。また，8月採水試料をNF処理前後で比較すると，やはりGAC処理水に少数ながら存在して

表2　16S rRNA配列解析結果に基づいた低水温期試料において優占的に再増殖した細菌種の多様性

最近縁種	GAC処理水	
	12月	1月
Bacillus cereus	29(31.9)	—
Bacillus sp.	22(24.2)	—
Bacillus thuringiensis	1(1.1)	—
Pseudomonas fluorescens	—	24(28.6)
Pseudomonas sp.	—	32(38.1)
Acidovorax sp.	2(2.2)	—
Flavobacterium sp.	1(1.1)	—
Curvibacter sp.	—	1(1.2)
ベータプロテオバクテリア	5(5.5)	2(2.4)
未培養クローン	31(34.1)	25(29.8)
計	91	84

最近縁種	NF処理水	
	12月	1月
Herbaspirillum sp.	43(49.4)	1(1.1)
Curvibacter sp.	—	40(46.0)
Curvibacter gracilis	—	33(37.9)
Ralstonia sp.	1(1.1)	1(1.1)
Oxalobacteraceae bacterium	—	1(1.1)
ベータプロテオバクテリア	26(29.9)	2(2.3)
未培養クローン	17(19.5)	9(10.3)
計	87	87

括弧内の数値は存在割合(%)を表わす

表3　16S rRNA配列解析結果に基づいた高水温期試料において優占的に再増殖した細菌種の多様性

最近縁種	GAC処理水	
	8月	9月
Aeromonas hydrophila	24(27.0)	3(3.6)
Aeromonas allosaccharophila	16(18.0)	1(1.2)
Aeromonas jandaei	—	25(30.1)
Aeromonas encheleia	1(1.1)	—
Aeromonas sp.	14(15.7)	34(41.0)
Undibacterium sp.	2(2.2)	4(4.8)
Vogesella sp.	3(3.4)	—
Bacillus cereus	23(25.8)	9(10.8)
Bacillus thuringiensis	1(1.1)	—
Bacillus sp.	5(5.6)	7(8.4)
計	89	83

最近縁種	NF処理水	
	8月	9月
Undibacterium sp.	79(94.0)	—
Herbaspirillum sp.	—	44(47.3)
Aquabacterium sp.	1(1.2)	1(1.1)
Cupriavidus metallidurans	—	1(1.1)
Bacillus subtilis	1(1.2)	—
Bacillus megaterium	1(1.2)	—
Bacillus sp.	—	1(1.1)
Paenibacillus alginolyticus	—	2(2.2)
ベータプロテオバクテリア	—	38(40.9)
未培養クローン	2(2.4)	6(6.5)
計	84	93

括弧内の数値は存在割合(%)を表わす

いた*Undibacterium*属がNF処理水の優占再増殖細菌となっており，なんらかの理由で膜処理プロセスから漏出してHPC再増殖の原因となっていることが示唆される。

ここで，NF処理水で優占的な再増殖が確認された*Herbaspirillum*属，*Curvibacter*属，*Undibacterium*属の特徴に触れておく。これらはいずれもベータプロテオバクテリアに分類され，好気または微好気条件下で増殖する。トータルで最も高い検出率となった*Herbaspirillum*属については，BergらがR2A培地や有機炭素を含有しない低栄養細菌分離用培地を用いた場合に，少数ながらも種々の環境水試料から分離されることを報告している[16]。また，窒素固定能を有する種が多いこと，浄水[17]や血液透析システム[18]といった非常に有機炭素濃度が低い試料水からも分離されること，が報告されている。一方，*Curvibacter*属は井戸水[19]やボトルウォーター[20]，*Undibacterium*属も飲料水や精製水からの分離例[21]がそれぞれ報告されており，いずれの細菌種も普遍的に環境水中に存在しており，かつ極貧栄養条件下での増殖に適した細菌種であるとみなされる。つまり，NF処理水のようにきわめて低濃度の有機炭素が残存する条件下では，この極貧栄養条件下であっても生育可能という特性を最大限に活かして，他の低～中栄養細菌種との増殖競合に打ち勝ち，高い収率でバイオマスを生産すると推測される。

一方，より多様性が確認されたGAC処理水における再増殖細菌群のなかには，ヒトに対する病原性も一部の種で報告されている*Aeromonas*属や*Bacillus*属[22]が優占種となるケースも確認された。NF処理水で優占種となった*Herbaspirillum*属についても，リンパ芽球性白血病患者の血液培養サンプル[23][24]や嚢胞性線維症の患者の痰サンプル[25]から分離されており，これらの菌株が日和見感染症を引き起こす可能性も指摘されている。繰り返しになるが，本研究で得られた結果がそのまま各処理水に存在する細菌種の存在割合を表わしているわけではないものの，適切な濃度の残留塩素が維持されないなど，一度再増殖を許す環境に置かれると，これらの病原細菌が再増殖してくると予想される。このように，前節でも述べた通りNF処理を行なったとしても，なんらかの理由で細菌が混入すると爆発的な再増殖につながる可能性が高い点に加えて，水系感染症予防の観点からも，浄水水質に応じて必要最小限の残留塩素を維持することにより，水道システム内における再増殖を抑制する必要があることが再確認された。

4. まとめ

最後に，本研究で得られた結果をまとめる。NF処理による従属栄養細菌の除去は92%と安定して高いものの，恒常的に処理水からHPCが検出される状況であり，かつ残留塩素なしの条件のNF処理水では顕著な細菌再増殖が確認された。NF処理水における再増殖細菌群はGAC処理水のそれと比較して，同等の炭素量から3倍程度多くのバイオマスを生産するためと考えられる。NF処理水で再増殖が確認された微生物種について16S rRNA系統解析を行なった結果，*Herbaspirillum*属，*Curvibacter*属，*Undibacterium*属が代表的なものであり，文献調査の結果からこれらの細菌は普遍的に水環境に存在するのみならず，極貧栄養条件下での増殖に適した種であることがわかった。

本研究で得られた結果は，NF処理により仮に十分なAOC除去が可能になったとしても，一度汚染が起これば少量の有機炭素を基質とした爆発的なHPC再増殖につながることを意味する。汚染の原因が膜漏出にあるのか，あるいは二次側配管内の再増殖にあるのかは現時点では不明であるが，いずれにせよNF処理適用後にも浄水水質に応じた最小限の残留塩素の併用は不可欠であることが確認された。

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Effect of the Ratio of Illness to Infection of *Campylobacter* on the Uncertainty of DALYs in Drinking Water

Sadahiko ITOH

Department of Environmental Engineering, Kyoto University, C-1, Kyotodaigaku Katsura, Nishikyo, Kyoto 615-8540, Japan

ABSTRACT

The yearly risk of *Campylobacter* infection was estimated for a treatment plant in the Netherlands. The median and mean values for the overall removal efficacy of the four treatment steps at the plant were estimated to be 7.46 $\log_{10}$ and 6.22 $\log_{10}$, respectively. The mean yearly risk of infection was estimated to be 1.68×10^{-3} infection/person/yr. The uncertainty analysis demonstrated that the following items had large impacts on the yearly risk of infection: the ratio of *Campylobacter* to *E. coli* in the source water, the method of pairing the concentration data before and after treatment, and the variation in the removal efficacy of slow sand filtration depending on the water temperature. Based on these results, the important components that are required to improve the accuracy of the estimates were identified. Disability Adjusted Life Years (DALYs) and costs-of-illness in the distribution area were estimated. 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.

Keywords: *Campylobacter*, disability adjusted life years, quantitative microbial risk assessment

INTRODUCTION

Since the 1980s, Quantitative Microbial Risk Assessment (QMRA) has been used to quantify the microbial safety of drinking water (Haas *et al.*, 1999; Medema *et al.*, 2006). The microbial exposure or dose is calculated based on the pathogen concentration in the drinking water and the consumption of unboiled drinking water. The risk of infection is calculated based on the chance of ingesting pathogens and developing an infection from this exposure (dose-response relation). In many studies, the variability of each element, such as pathogen concentration in the source water and the removal and inactivation efficacy of the water treatment steps, is described by a Probability Density Function (PDF). The yearly risk of infection is quantitatively estimated by Monte Carlo simulation. To date, the methodology of QMRA has been developed and improved by several studies.

Drinking water is supplied without chlorination in the Netherlands. The Dutch legislation requires that several microbes are assessed, notably enteric viruses, *Cryptosporidium*, *Giardia* and other relevant pathogens (Smeets *et al.*, 2009). Although *Campylobacter* is not specified in this list, this pathogen is considered to be one of the most important bacteria that cause waterborne diseases in many European countries (Medema *et al.*, 2006). The removal and inactivation efficacy required by the water treatment plants was estimated based on the monitored concentrations of pathogens in raw water and the acceptable risk level (10^{-4} infection/person/yr) in the Netherlands (Smeets *et al.*, 2009). These results suggest that the required log reduction for

Address correspondence to Sadahiko Itoh, Department of Environmental Engineering, Kyoto University, Email: itoh@urban.env.kyoto-u.ac.jp
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Campylobacter is the largest among the four index pathogens listed above. To date, however, the accuracy of estimating the risk of *Campylobacter* infection via drinking water and the adverse health outcomes has not been clarified.

First, many techniques that were developed by previous QMRA studies were applied in this study, and the yearly risk of *Campylobacter* infection was estimated in a case study. Second, Disability Adjusted Life Years (DALYs) as adverse health outcomes and costs-of-illness were approximated. Thus, one of the aims of this study is to perform a complete QMRA. Third, uncertainty analyses of the estimates were conducted. As a result, the variable that has the greatest impact on decreasing the yearly risk of infection was identified, and components or variables that can help improve the accuracy of the estimates were determined. In addition, the uncertainty analyses of DALYs were conducted to find important factors that affect the DALYs.

METHODS

Case description

The Weesperkarspel treatment plant of Waternet (water cycle company for Amsterdam and the surrounding areas) located in the western Netherlands was used as a case study. With a production average of 115,000 m^3/day, the plant supplies drinking water to the eastern part of Amsterdam and the suburbs. The source water for this plant is abstracted from a polder (the 5.4 km^2 Bethune Polder) that is a land reclaimed for agriculture and stock raising. The water is pumped out as surface water from the neighboring watercourses and is mainly seepage water flowing through a good permeable underground of the polder. When water demand is high, such as in the summer, water can also be abstracted from the nearby Amsterdam-Rhine Canal (ARK-water). The volume of ARK-water is 5% of the total annual produced volume. The Amsterdam-Rhine Canal water comes from the Rhine River, and is more polluted than the water from the Bethune Polder (Hijnen *et al.*, 2005).

Figure 1 shows the treatment process of the plant. The raw water is pre-treated by coagulation, stored in the lake water reservoir, and then undergoes rapid sand filtration (RSF) at the Loenderveen plant. The average time in the reservoir (123 ha, 6.9×10^6 m^3) is 89 days. After the RSF, the water is transported to the Weesperkarspel plant where it undergoes several treatment steps such as ozonation, softening, granular activated carbon (GAC) filtration and slow sand filtration (SSF).

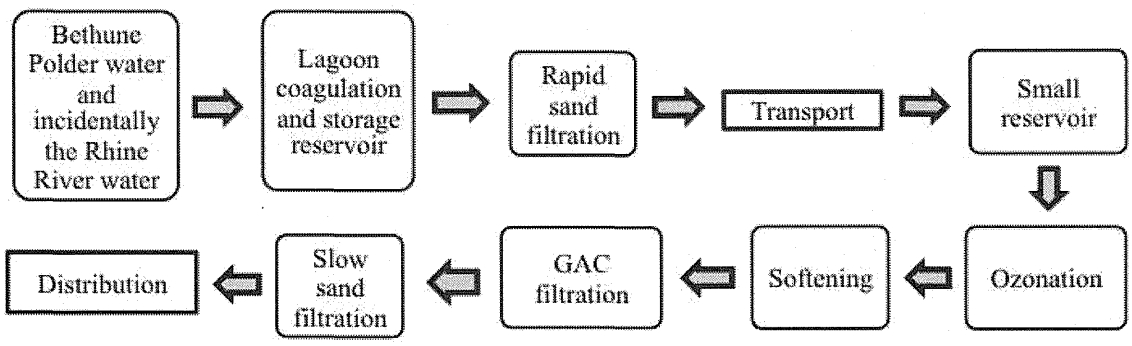

Fig. 1 - Treatment process of Weesperkarspel.

Pathogen and its indicator

Generally, it is not easy to measure the concentrations of pathogens in source water, treatment plant water or drinking water, and often there are not a large number of monitored concentrations. For pathogenic bacteria like *Campylobacter*, indicator bacteria like *E. coli* and enterococci have been proposed as process indicators to assess the elimination capacity of water treatment processes (Hijnen and Medema, 2010). For SSF, *E. coli* is a conservative surrogate for removal of *Campylobacter* as demonstrated by Hijnen *et al.* (2004) and Dullemont *et al.* (2006). For RSF, Hijnen *et al.* (1998) showed that *E. coli* is removed slightly better than environmental *Campylobacter* bacteria. It has been found that *E. coli* and *Campylobacter* can be similarly inactivated by ozonation (Smeets *et al.*, 2005). Based on these information, this study estimated the efficacy of removing and inactivating *E. coli* by the treatment plant. Then, the *E.coli* concentration was translated into a *Campylobacter* concentration using the ratio of *Campylobacter* to *E. coli* in the surface water.

Data on *E. coli* in the raw water from the Bethune Polder were provided by Waternet. The analyses included *E. coli* concentrations that were measured 46 times from April, 2002 to December, 2004.

Non-parametric validation of treatment efficacy

First, the method to determine the removal and inactivation efficacy of the water treatment process was selected. Pairing by date has been widely used to determine the reduction efficacy. In this method, influent and effluent samples taken on the same day are compared and the reduction efficacy is calculated for each pair. This assumes that samples before and after treatment are correlated in time. On the other hand, pairing by rank assumes that there is a complete correlation between the influent and effluent concentrations (lowest influent concentrations correlate to lowest effluent concentrations, etc.). To enable pairing by rank, the samples of the influent and effluent concentrations are sorted by concentration in descending order before determining the treatment efficacy. The other pairing method is the random method, which assumes no correlation by date or rank. In this method, the influent and effluent sample concentrations are paired randomly. Smeets *et al.* (2008) demonstrated that the date and random methods resulted in similar removal, while the rank method proved to be the best method to validate the treatment efficacy in a water treatment process in the Netherlands. However, the applicability of the rank method to different situations is not known. Therefore, the rank, date, and random methods were compared in this study.

The confidence interval for the monitored *E. coli* concentrations was determined by adapting a standard nonparametric bootstrapping procedure. Thus, bootstrap samples of *E. coli* concentrations were produced for the monitored water before and after each treatment step, such as coagulation-storage, RSF, and ozonation. The observed treatment efficacy was calculated from the bootstrap samples paired by date and rank. A difference of 89 days (average time in the reservoir) was given even when the samples were paired by date before and after coagulation-storage. By comparing the predicted concentrations to the monitored effluent concentrations, the accuracy of the rank, date, and random methods was compared.

Parametric treatment model

Coagulation-storage, RSF, ozonation and SSF are considered the main microbial barriers at the water treatment plant examined in the case study. Therefore, the removal and inactivation efficacy were estimated for each of these four steps. The *E. coli* concentrations after coagulation-storage were measured 91 times from January, 2002 to August, 2005. The *E. coli* concentrations after RSF and after ozonation were measured 556 and 326 times, respectively, from January, 2003 to December, 2004. Thirty of the 326 measurements in ozonated water were performed with large volume samples ranging from 10 L to 100 L.

For each step of coagulation-storage, RSF and ozonation, the parametric distributions, which fit the distributions of the treatment efficacy estimated by the rank method, were selected for the QMRA.

Water treated with SSF is the drinking water that is supplied to the city. The available *E. coli* data include concentrations monitored daily from January, 2003 to December, 2004 for each of two parallel lines. *E. coli* was detected only once (0.2 *E. coli*/100 mL) over a total of 1393 measurements, and all of the other samples were negative for *E. coli*. Therefore, the removal efficacy of SSF cannot be determined based only on the monitored data. In order to estimate the removal efficacy, a pilot-scale plant was dosed with *E. coli* in order to detect *E. coli* in the effluent (Dullemont *et al.*, 2006). A maximum value of 4.2 $\log_{10}$, mean elimination capacity (MEC) of 2.4 $\log_{10}$ and minimum value of 2.0 $\log_{10}$ were obtained when determining the removal efficacy six times under conditions where the water temperature was below 13°C. A triangular distribution with these parameters was constructed. In addition, the removal efficacy under conditions where the water temperature was above 13°C was determined three times. The impact of these results will be examined by an uncertainty analysis in this study.

The overall removal efficacy with the four treatment steps was calculated by Monte Carlo simulation, which was performed by drawing random values from each PDF that was given to the four steps. This procedure was repeated 100,000 times to achieve stable results. The *E. coli* concentration in the finished (treated) water can be estimated by multiplying the overall removal efficacy with the source water concentration by Monte Carlo simulation. Crystal Ball 7® (Oracle Corporation, USA) was used to select the parametric PDFs fitted to the variables and to perform the Monte Carlo simulation.

Risk calculation

Daily exposure (dose) (*E. coli*/day) was calculated by multiplying the estimated concentration in the treated water with the amount of unboiled drinking water consumed per day in the Netherlands. To account for the variability in water consumption within the population, a Poisson distribution with a mean value (rate in the Poisson model) of 0.706 glass/day was recommended to use with the QMRA (Mons *et al.*, 2007). Assuming that a glass contains 250 mL, the mean value is equivalent to 177 mL/day.

The *E. coli* dose (*E. coli*/day) was translated into the *Campylobacter* dose (*Campylobacter*/day) using the ratio of *Campylobacter* to *E. coli* (C/E ratio) in the

surface water. It is needed to use C/E ratio for the Bethune Polder water or the source water after the ARK-water was added to the Bethune Polder water. *Campylobacter*, however, is not a pathogen measured in the routine practice of water quality monitoring. There is very limited data of *Campylobacter* concentrations in the source water that can produce C/E ratios with corresponding *E. coli* concentrations (Hijnen *et al.*, 2005). On the other hand, there is a data set of concentrations of *E. coli* and *Campylobacter* in the Meuse River at the intake site of a water company in the Netherlands (Medema *et al.*, 2006). These data measured 22 times in 1994 were used to determine the C/E ratio in this study. After calculating the ratios from the concentrations of *E. coli* and *Campylobacter*, a PDF that fits the distribution of the C/E ratios was chosen.

The daily risk of infection P_d (infection/person/d) was calculated from the *Campylobacter* dose using a dose-response model. The dose-response relationship of *Campylobacter jejuni* presented by Tenuis *et al.* (2005) is a Beta-Poisson model where $\alpha = 0.024$ and $\beta = 0.011$. Although the Beta-Poisson approximation should retain the criteria of ≥ 1 and $\alpha \leq \beta$, the above α and β do not satisfy these criteria. Actually, when the aforementioned Beta-Poisson model was applied, it was noted that the Beta-Poisson model can exceed the maximum risk curve at low doses (Medema *et al.*, 2006). This means that the dose-response model predicts a theoretically impossible probability of infection. Therefore, the Beta-Poisson model is not appropriate for the calculation. Alternatively, the exact Beta-Poisson model can be approximated for low doses (< 0.1 organisms/L) by setting γ of the exponential model equal to the expected value of the Beta distribution (α / (α + β)), thus avoiding this complication. Consequently, the Beta-Poisson model was approximated by the exponential model ($P_d = 1 - \exp(-0.686 \times D)$, D: dose) with $\gamma = 0.686$, which was used in this study. The effects of using the maximum risk curve or the Beta-Poisson model were examined by the uncertainty analysis.

The individual health risk is represented by the average yearly risk of infection. Under the assumptions of a binomial process, the yearly risk of one or more infections is calculated using the following equation (1).

$$P_y = 1 - (1 - P_d)^{365} \quad (1)$$

Monte Carlo simulation was performed by drawing random values from each PDF of the *E. coli* concentration in the source water, four treatment steps, water consumption, and the C/E ratio to calculate the yearly infection risk P_y. This simulation assumes that there are no correlations between the variables. Stable results were achieved with acceptable calculations 100,000 times.

Uncertainty analysis

It is natural that the estimated values of target variables and yearly risk of infection have large uncertainty. Uncertainty analyses were performed to examine the impact of using ARK-water, the impact of the removal efficacy of SSF under conditions with high water temperatures, the impact of the C/E ratio, the impact of dose-response models, and the impact of data pairing methods such as the rank and date methods. The uncertainty analyses of DALYs were also performed to find important factors that affect the DALYs and examine the impact of the ratio of illness to infection.

RESULTS AND DISCUSSION

Comparison of the data pairing methods

The *E. coli* concentrations in the treated water that were predicted by the date, rank and random methods were compared to the monitored *E. coli* concentrations. Figures 2(a) and 2(b) show examples of the predicted and monitored *E. coli* concentrations after coagulation and storage. The distributions of the concentrations were presented by CCDFs (Complementary Cumulative Distribution Functions) on a double log scale, which is well-suited for magnifying data from rare events (Smeets *et al.*, 2008). It was found that the pairing method by date resulted in an overestimation of effluent concentrations, indicating that the date method tends to assess the removal efficacy at a lower value. This reduced value is because the date method often yielded a low removal efficacy and could even predict "negative removal". "Negative removal" would imply that microbes were occasionally "produced" by the treatment, which is unlikely. However, the rank method did not allow for negative removal. The rank method provided an appropriate estimate of the removal efficacy for Monte Carlo simulation since the monitored concentrations in Fig. 2(a) were consistent with the predicted concentrations. The random and the date method resulted in a similar estimate of the removal efficacy (data are not shown). Consequently, the rank method was used in the following analyses.

Application of distribution type

PDFs were selected to describe the distributions of the *E. coli* concentrations in the source water, the removal and inactivation efficacy by coagulation-storage, RSF, ozonation and SSF, and the C/E ratio. In general, extreme events can dominate the average health risk. Therefore, the PDF should fit the extremes (tail) of the observed variations. From the point of emphasizing the fit to rare events, the results of the Anderson-Darling test were more emphasized than the results of the chi-square test and the Kolmogorov-Smirnov test when selecting a distribution type. The selected PDFs

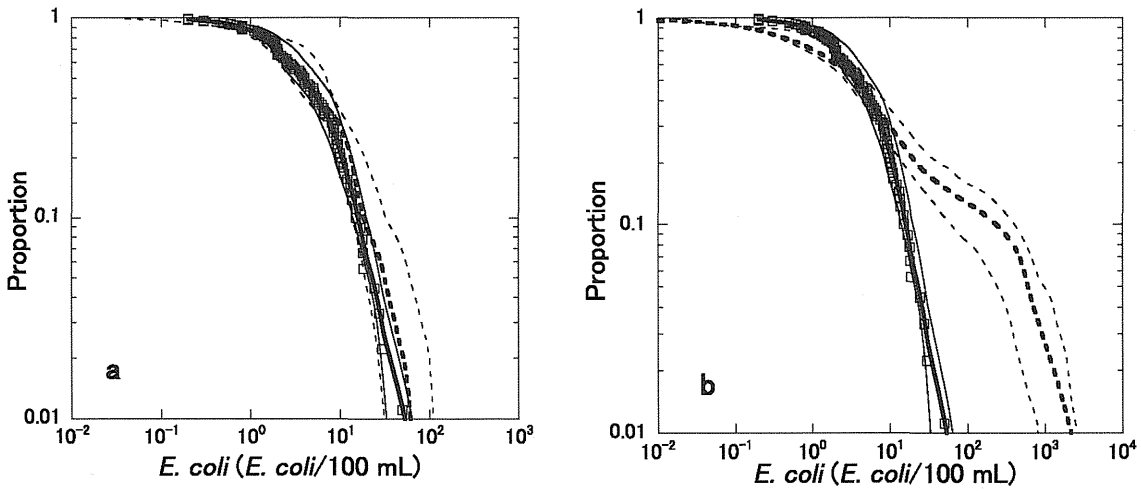

Fig. 2 - *E. coli* concentration after coagulation and storage calculated with the non-parametric model validated by the rank (a) and date (b) methods. Calculated concentration by the rank and date methods (dashed) are compared to monitored concentrations (line and markers). Median concentration (thick) and 95% CI (fine) are shown.