分担研究報告書５

配水過程における再増殖微生物の増殖特性解析

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「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」

平成23年度分担研究報告書
配水過程における再増殖微生物の増殖特性解析

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研究要旨

水道給配水系における細菌再増殖現象を評価する手法の検討と、給水栓の滞留・放流前後に伴う細菌再増殖現象の解析を行った。水道水中の低濃度細菌群集からのDNA抽出方法を比較検討した結果、ポリカーボネートフィルターによる菌体捕捉と、ビーズ法を用いた市販キットによるDNA抽出の組み合わせの回収率が高いことが明らかになった。また、HPC単離株の分類手法として、二重標識T-RFLPの適用可能性を検討し、実際に水道水から単離した株の分類に有用であることを確認した。実際の室内給水栓を対象として、滞留・放流に伴う細菌再増殖現象を観察した結果、滞留に伴って遊離残留塩素の低下、水温の上昇が進み、全菌数及びHPCが上昇することを明らかにした。しかし、2L放水後には滞留前のレベルに速やかに戻ることから、細菌再増殖は局所的な場所で発生していることが推測された。また、滞留時間と全菌数の増加との関係から、再増殖する細菌群の平均倍加時間は、2.8hであることを示した。滞留・放流に伴う細菌群集構造の解析を行い、再増殖した細菌に由来すると推察されるフラグメントの抽出を行った。

A. 研究目的

残留塩素の低減化が進められる中、水道給配水系における細菌再増殖リスクが高まることが懸念されている。残留塩素低減化を達成しつつ、生物学的に安定な水道水質の維持を達成するためには、細菌再増殖を適切に監視、制御する手法の確立が求められている。そのためには、再増殖する細菌の生理・生態を理解することが欠かせないが、我が国では水道水中の細菌再増殖や細菌現存量を、一般細菌に加えて「従属栄養細菌（Heterotrophic Plate Count: HPC）」指標（平成20年4月から水道水質管理目標値に追加：暫定目標値2000CFU/mL）という包括的な指標で評価しているのが現状である。しかし、HPCの指標性や位置付けについては、十分な知見が蓄積されていない。

そこで、本研究では、以下の項目に関する研究を進め、再増殖細菌の生理・生態特性に基づいた細菌再増殖の評価手法・制御策の提案を行うことを目的とする。今年度は主に①と②に関する研究を展開した。

① 実際に再増殖する細菌群の網羅的把握
② 従属栄養細菌の多様性の評価
③ 従属栄養細菌の再増殖特性の解析（基質利用性、増殖速度、塩素耐性）

④ 再増殖特性の高い従属栄養細菌を用いた同化性有機炭素（Assimilable Organic Carbon：AOC）測定手法の開発

B. 研究方法

1. 水道水中の低濃度細菌群集からのDNA抽出方法の検討

Pseudomonas fluorescens P17株（ATCC49642）を用いて、下記の①－③に示した異なる濃縮、抽出方法によるDNA回収率の比較を行った。①と②については、P17原液（4.6×10^7 cells/mL）1mlをリン酸緩衝液1Lに希釈し、全量をろ過処理した後、DNA抽出を行った。③については、原液1mLを遠心分離し、回収した菌体をDNA抽出試料とした。

① カートリッジ式フィルターSterivex™-GP 0.22μm Filter Unit(Millipore)による濃縮→PowerWater® Sterivex™ DNA Isolation Kit(MO BIO)による抽出

② Isopore membrane filter 0.22μm(Isopore)による濃縮→FastDNA for soil(Q-Biogene)による抽出

③ フィルターによる濃縮工程を省略し、遠心分離による菌体濃縮→FastDNA for soil (Q-Biogene)による抽出

それぞれの濃縮・抽出処理によって得られたDNA抽出液を用い、定量PCRによって16S rRNA遺伝子コピー数をTaqMan PCR法により定量した。なお、定量した16S rRNA遺伝子コピー数については、P17株の16S rRNA遺伝子コピー数(5)を用いて菌数に換算した。

2. HPC単離株のOTU分類手法の検討

水道水から単離したHPC単離株（黄色：Y-1, Y-5, 白：W-8, W-12, 赤：R-13, R14の計6株）の16S rRNA遺伝子の分析方法として、二重標識によるTerminal Restriction Fragment Length Polymorphism（T-RFLP）の適用を試みた。プライマーには、細菌の16S rRNA遺伝子を標的とした27Fと907rを用い、それぞれのプライマーの5'末端をFAM、HEXで別々に標識した。PCR増幅後、制限酵素 *Hha*Iによる消化を行い、キャピラリーシーケンサーABI3100-Avant（Applied Biosystems）によるフラグメント分析を行った。

3. 給水栓における細菌再増殖現象と滞留時間との関係

2011年12月12日から16日にわたって、東京大学実験室内の給水栓を対象として採水を行った。初めに、5L/minで5分間の放水を行った後、10Lを採水した。次いで、給水栓を24時間使用停止とし、給水栓付近の水道水を滞留させた。その後、水道水を5L/minで放水し、2Lずつ連続的に分取しながら計20Lを採水した。以後、使用停止時間を24, 16, 8, 4時間と変化させ、同様の採水を繰り返した。採水した試料については、水温及び遊離塩素濃度を測定すると共に、全菌数及び従属栄養細菌数（Heterotrophic Plate Counts: HPC）を求めた。全菌数の測定については、試料中の微生物をSYBR Green I（Invitrogen）で染色した後、フローサイトメーター（Accuri C6, BD社）を用いて計数した。HPCは、R2A培地を用い、20℃で7日間培養し、形成されたコロニー数を計数した。また、放水直後0-2L

（計 2L）の試料と、放水後 10-20L（計 10L）の試料を対象として、T-RFLP 法による群集構造解析を行った。試料は Isopore membrane filter 0.22μm(Isopore)によるろ過を行って菌体を捕捉した後、FastDNA for soil (Q-Biogene)による抽出を行った。

C. 研究結果

1. 水道水中の低濃度細菌群集からの DNA 抽出方法の検討

水道水中の細菌数は非常に低いため、網羅的な群集構造解析のためには回収率の高い DNA 抽出方法を選択する必要がある。そこで、3 種類の濃縮・DNA 抽出方法の比較検討を行った。

定量 PCR による 16S rRNA 遺伝子コピー数（菌数換算）に基づく回収率の結果を表 1 に示す。その結果、回収率が最も高いのは、Isopore メンブレンで濃縮し、FastDNA for soil で抽出する②のケースで、回収率は 6.7%であった。濃縮工程を遠心分離として、同じ FastDNA for soil で抽出した③のケースでは、回収率は 4.8%であり、②の結果と大差は見られなかった。一方、これまでに海洋などで適用事例のある Sterivex を用いて濃縮し、Sterivex 専用の抽出キットである PowerWater® Sterivex™ DNA Isolation Kit を用いた場合の回収率はわずか 0.2%であり、①、②の条件と比較しても 1log 以上低い値であった。

2. HPC 単離株の OTU 分類手法の検討

FAM 標識したフォワードプライマーと、HEX 標識したリバースプライマーを用いて、水道から単離した株の 16S rRNA 遺伝子を T-RFLP で解析した。ここでは、東京大学実験室の水道水 200mL（5L/min で 5 分間放水後に採水）から単離した HPC（黄色の株：Y-1, Y-5、

表 1 リアルタイム PCR 測定結果（DNA 抽出方法の比較）

菌体回収	抽出 DNA1μL 当たりコピー数(copies/uL)	試料 1mL あたり平均コピー数 (copies/mL)	菌数換算 (cells/mL)	全菌数比(%)
Sterivex (0.22um) (PowerWater)	4.5×10^3 5.3×10^3 4.9×10^3	4.9×10^5	1.1×10^5	0.2
Isopore (0.22um) (FastDNA)	8.4×10^4 7.4×10^4 8.2×10^4	1.5×10^7	3.1×10^6	6.7
遠心分離 (FastDNA)	6.7×10^4 6.0×10^4 5.5×10^4	1.1×10^7	2.2×10^6	4.8

* 16S rRNA 遺伝子コピー数を 3.6 として計算

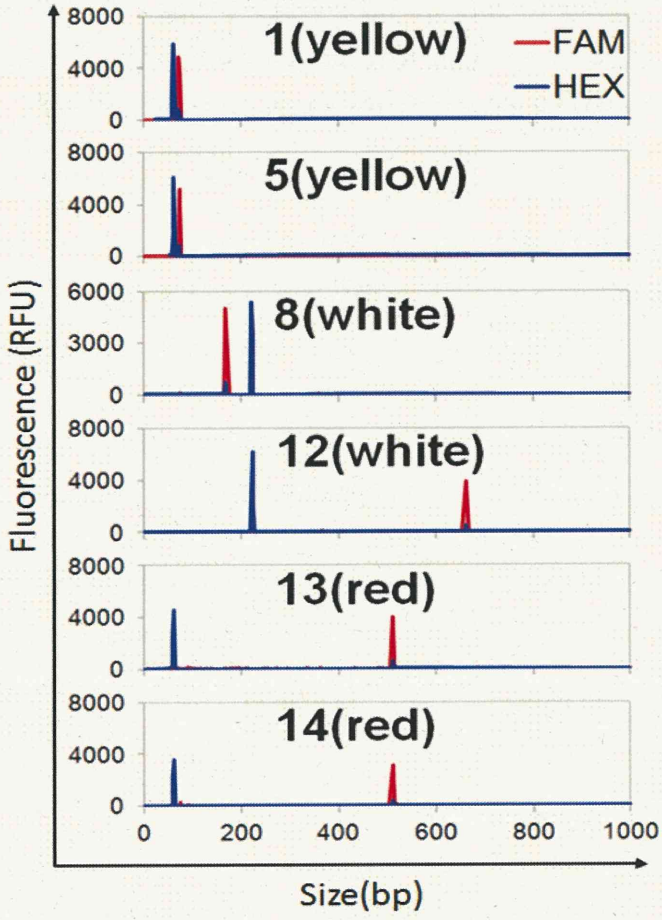

図 1　二重標識 T-RFLP による HPC 単離株の分析

白色の株：W-8, W-12、赤色の株：R-13, R-14）を対象とした。各色のコロニーについては、形状は類似しており、目による識別は不可能であった。

図 1 に T-RFLP の結果を示す。黄色（Y-1, Y-5）、赤色（R-13, R-14）の株では、それぞれ 5'末端と 3'末端の断片長が株間で共通しており、同じ OTU の株であることがわかる。一方、白色の株（W-8, W-12）については、HEX 標識した 3'末端の断片長は同じであるものの、FAM 標識した 5'末端の断片長は株間で異なった。このことから、見た目は同じであっても、OTU が異なる HPC が存在することが示された。

3.　給水栓における細菌再増殖現象と滞留時間との関係

図 2 に、異なる滞留時間とその後の放水に伴う給水栓における水質変化を示す。水温は、給水栓を 8-24 時間滞留させた前後で、15.2-18.0℃から 18.8-19.2℃まで上昇したが、4 時間の滞留による水温上昇は明確ではなかった。遊離塩素濃度は、滞留前には 0.27-0.36mg/L と十分な濃度が維持されていたが、24 時間の滞留により 0-0.07mg/L まで減少した。滞留時間が短くなるにつれ、減少幅は小さくなった。放水により水温の低下、遊離塩素濃度の

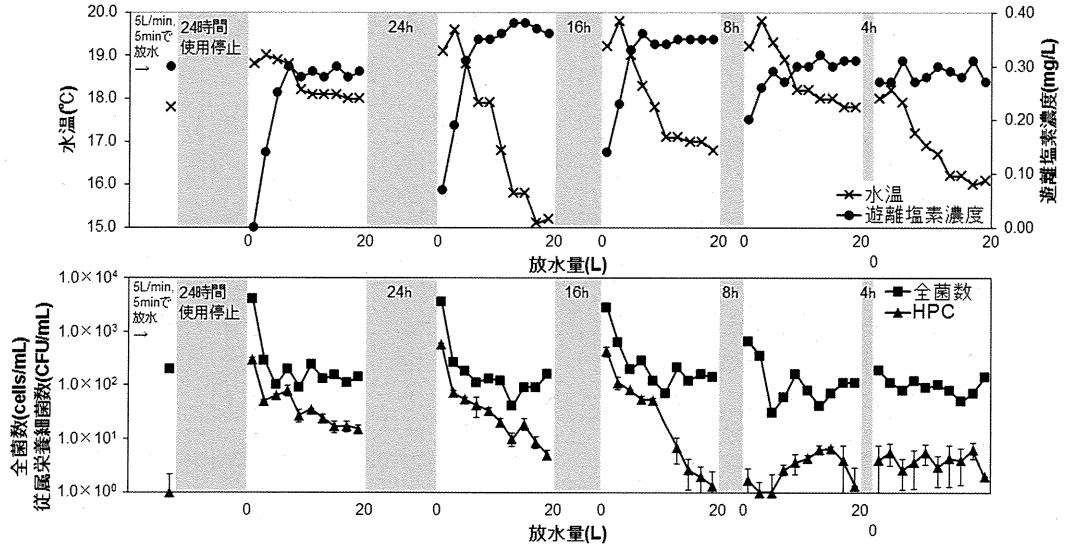

図 2 滞留及び放水に伴う給水栓水道水の水質変化

(A) 水温、遊離塩素濃度、(B) 全菌数、従属栄養細菌数（使用停止時間 16 時間後の 10-12L 放水分は欠測）

上昇が観察された。遊離塩素濃度は、6L 放水後には、いずれの場合も 0.25mg/L 以上に回復した。

全菌数は、実験開始時には 2.0×10^2 cells/mL であったが、24 時間滞留後には 3.9×10^3 cells/mL に増加し、滞留中に細菌再増殖が生じたことが明らかになった。放水を行うと、全菌数はただちに減少し、2L 放水後には滞留前のレベルに戻った。再度、給水栓を 24 時間滞留させた後の全菌数は、1 回目の滞留と同程度（3.6×10^3 cells/mL）まで増加し、給水栓における細菌再増殖現象には再現性が認められた。滞留時間を 16 時間にした場合も同程度の変化が確認されたが、8 時間の場合は 0.7 log の増加率にとどまり、24 時間の場合（平均 1.4 log）や 16 時間の場合（1.2 log）と比較して増加量が少なかった。4 時間の滞留による増加は認められなかった。一方、HPC も、全菌数と同様の変化を示した。実験開始時には 1 CFU/mL であったが、24 時間の滞留後には 302 CFU/mL まで増加した。放水を行うと漸減したが、20L 放水後でも 15 CFU/mL であった。再度、24 時間滞留させた時には 570 CFU/mL に増加しており、ほぼ同程度の増殖が観察された。滞留時間を 16 時間にした場合にも、滞留前後で 5CFU/mL から 425CFU/mL まで増加し、ほぼ同程度の増殖が観察された。滞留時間が 8 時間以下の場合には、顕著な増加は確認されなかった。

滞留時間 16-24 時間の試料を対象として細菌群集の T-RFLP 解析を行った結果を図 3 に示す。今回の系では、主に 52, 75, 332, 833bp などに共通のフラグメントが見られ、それらの相対強度が滞留後、放水後で変化する様子が確認された。一方、特定の試料にしかなく、前後で連続性のないフラグメントも見られた。主要なフラグメントである 52, 75, 332,

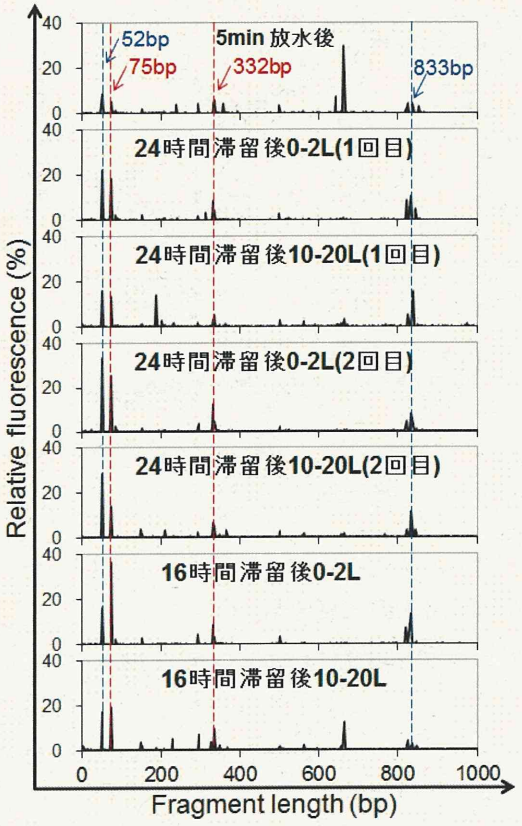

図 3 滞留・放水に伴う細菌群集構造の変化（24, 16 時間滞留）

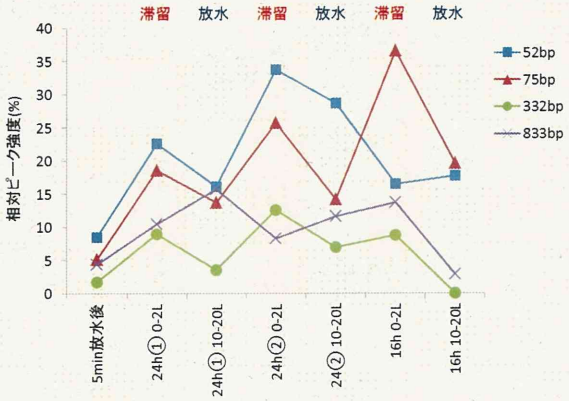

図 4 主要フラグメントの相対ピーク強度の変化

833bp の相対ピーク強度の増減の様子を図 4 に示す。特に、75, 332bp のフラグメントについては、相対ピーク強度が滞留後に上昇、放水後に減少する様子が確認された。

D. 考察

1. 水道水中の低濃度細菌群集からの DNA 抽出方法の検討

抽出方法の検討の結果、「Isopore メンブレンによる濃縮→FastDNA for soil による DNA 抽出」という②の条件が最も回収率が高く、フィルターによる濃縮の代わりに「遠心分離による濃縮→FastDNA for soil による DNA 抽出」という③の条件とほぼ同程度の回収率であった。遠心分離による菌体濃縮については、ロスがほぼないと考えられるため、DNA 抽出時に Isopore メンブレンから菌体が有効に剥離していることが推測される。一方、海洋などで使用実績のある「Sterivex による濃縮→PowerWater® Sterivex™ DNA Isolation Kit による DNA 抽出」という①の条件は、回収率が非常に低かった。Sterivex からの剥離、キットの回収率のいずれに原因があるかは今回の実験では明らかではないが、濃縮、DNA 抽出の方法で結果が大きく変化する可能性を示唆している。更なる回収率の向上を目指し、菌体濃縮方法、DNA 抽出方法の検討を継続する必要がある。

2. HPC 単離株の OTU 分類手法の検討

本研究では、HPC として単離される株の生理・生態特性を評価することを目的としており、どのような枠組みで HPC を分類するかということは大きな課題である。大量のコロニーを効率的に分類する上で T-RFLP は有効であるが、5'末端側の情報のみに依存しているため、情報の欠損も懸念される。その点、今回適用した二重標識 T-RFLP では、3'末端側の情報も加味することができるため、より高い精度で HPC の分類が可能になると期待できる。実際、見た目には同じ白色のコロニーである W-8, W-12 について、3'末端の断片長は同じであったが、5'末端側の断片長に差異が見られ、識別することができた。一方、黄色（Y-1, Y-5）、赤色（R-13, R-14）のコロニーについては、両末端の断片長が一致し、同じ OTU であることの信頼性が高まった。次年度以降、多くの HPC を単離、分類する手法として、活用する予定である。

3. 給水栓における細菌再増殖現象と滞留時間との関係

実際の水道給水栓を対象として、細菌再増殖の実態を観察した結果、滞留時間が 8-24 時間の場合には全菌数の上昇が、16-24 時間の場合には HPC の上昇が確認された。ただし、HPC の最大値は 570 CFU/ml であり、水質管理目標値 2000 CFU/mL よりも十分に低く、水質管理上の問題はない。細菌の再増殖条件としては、遊離残留塩素濃度の低下が第一に寄与していると考えられるが、滞留に伴う水温上昇も細菌の増殖活性を高めていることが推察された。新鮮な水道水中の全菌数としては、およそ 10^2 cells/ml 程度であるが、16 時間以上の滞留によって 10^3 cells/ml のオーダーにまで増加する傾向が明らかになった。ただし、2L 放水後には滞留前のレベルに戻ることも明らかになった。スイスにおける先行研究では、新鮮な水道水中の全菌数としておよそ 10^4 cells/ml という値が報告されている（Lautenschlager *et al.*, 2010）。我が国よりも 100 倍近く高いのは、残留消毒剤がないためである。家庭給水栓（10 か所）での滞留後（一晩）の全菌数は、平均して 1.1×10^5 cells/ml であり、本研究において 24 時間後の滞留後に観察された全菌数（$3.6\sim3.9\times10^3$ cells/ml）に比べても 30 倍近く高い値である。放水による影響については、非常に細かな調査をしており、滞留後 200mL 放水することで全菌数が滞留前のレベルに戻ることを示している。本研究では 2L 刻みでの採水のため、詳細な比較は困難であるが、スイスの事例同様、細菌再増殖が極めて局所的な場所で生じていることが推察される。

図 5 に滞留時間と滞留後の全菌数、HPC 濃度との関係を示した。ここでは便宜的に給水栓付近における回分条件を仮定して、細菌再増殖の特徴を考察する。図 5 を見ると、全菌数の場合は指数関数的な増殖傾向が見て取れる。一方、HPC の場合は、増殖が断続的であり、8 時間と 16 時間の間に大きなギャップがある。この間に HPC で検出されるような細菌群は対数増殖をした可能性が推察される。全菌数と HPC の増加傾向の差異から、HPC で検出される細菌群は、全細菌の中でも異なる増殖特性を有していることが考えられる。

図 6 に、対数変換した全菌数と滞留時間との関係を示す。対数増殖期に相当する滞留時間 4 時間から 16 時間までの間の傾きを取り、比増殖速度を計算すると 0.22(1/h)という値が

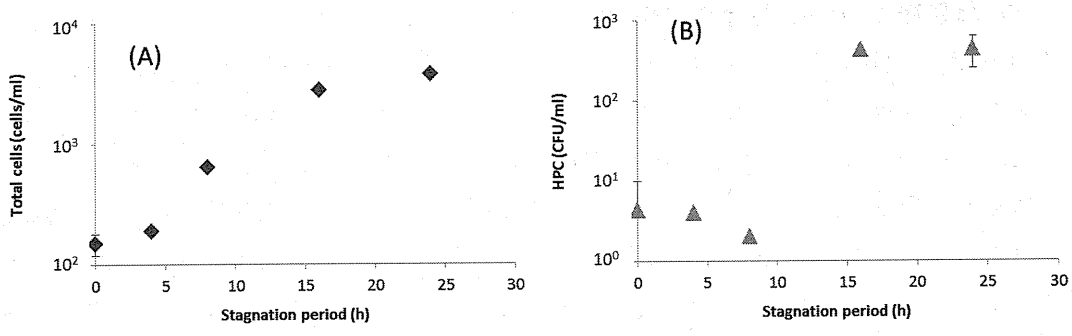

図 5 全菌数（A）・HPC（B）と給水栓滞留時間との関係

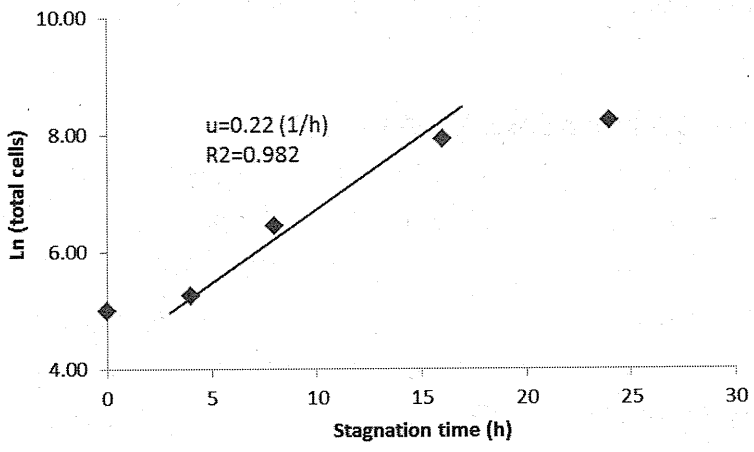

図 6 全菌数の対数変換値と給水栓滞留時間との関係

得られた。Lautenschlager らは（2010）、スイスの水道水中（回分条件）における細菌の増殖速度を 0.22(1/h)と報告しており、本研究の結果と完全に一致している。水道水中の再増殖細菌の特性を考える上で極めて興味深い知見である。なお、この比増殖速度の際の倍加時間は、約 2.8(h)である。

T-RFLP の解析の結果、相対ピーク強度に着目することで、特に 75, 332bp のフラグメントが、再増殖した細菌に由来する可能性が推察された。滞留後には全菌数が増加しているので、各フラグメントに相当する細菌の量も増加していると推察できるが、今回の結果だけでは量的な議論をすることには限界がある。今後は、これらのフラグメントのクローニングを行い、詳細な系統情報を得ると共に、特異的なプライマーを設計することで、水道水中におけるこれらの細菌群の動態を評価することを検討したい。

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E. 結論

今年度の研究の結果、以下の結論を得た。

① 水道水中の低濃度細菌群集からの DNA 抽出方法を比較検討した結果、アイソポアメンブレンで菌体を捕捉し、FastDNA for soil で DNA を抽出する手法の回収率が高いことが明らかになった。

② 二重標識 T-RFLP を適用することで、HPC 単離株の分類をより詳細に行うことができることが示された。

③ 給水栓の滞留・放流に伴う細菌再増殖現象を観察した結果、滞留に伴って遊離残留塩素の低下、水温の上昇が進み、全菌数及び HPC の上昇が認められた。しかし、2L 放水後には滞留前のレベルに速やかに戻ることから、細菌再増殖は局所的な場所で発生していることが推測された。全菌数の増加と滞留時間との関係から推測した結果、再増殖する細菌群の比増殖速度は 0.22(1/h)と見積もられた。また、滞留・放流に伴う細菌群集構造の解析から、再増殖細菌に由来すると推察されるフラグメントを抽出した。

F. 健康危険情報

該当なし

G. 研究発表

1. 論文発表

該当なし

2. 学会発表

該当なし

H. 知的財産権の出願・登録状況

該当なし

研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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研究成果の刊行物・別刷

Regulations and perspectives on disinfection by-products: importance of estimating overall toxicity

Sadahiko Itoh, Bruce A. Gordon, Philip Callan and Jamie Bartram

ABSTRACT

Chemical disinfection of drinking water results in the formation of disinfection by-products (DBPs). This paper reviews evidence on the overall toxicity of disinfected water instead of focusing on the effects of individual DBPs. The possible health effects of ingesting DBPs include development of cancer and adverse reproductive/developmental outcomes. Only a few of the 600–700 chlorinated by-products are regulated, accounting for only a small portion of the overall toxicity of DBPs. This review showed that current water quality management, based on complying with standard values set for individual DBPs, is insufficient in responding to overall toxicity from DBP species. Because water suppliers typically focus their water quality management efforts on meeting the defined maximum concentration standards for individual regulated parameters, current water management practices may not adequately focus on effectively reducing overall DBP toxicity. Therefore, we recommend a progressive shift towards preventive and holistic DBP management based on a comprehensive health-based risk assessment that takes into account the overall toxicity and is supported by a validation of the control processes. We also present a prioritized research agenda that will help determine risk assessment and management and facilitate the development of regulations. This includes the development of an index for overall DBP toxicity.

Key words | carcinogenicity, disinfection by-products, drinking-water quality standards, reproductive/developmental toxicity

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WEAKNESSES IN THE CURRENT REGULATORY APPROACHES ON DBPS

Trihalomethanes (THMs) were originally recognized as a potential health concern in drinking water in the 1970s. Since then, there has been extensive effort by researchers internationally to detect and identify other disinfection by-products (DBPs) (Krasner *et al.* 1989; Stevens *et al.* 1990; Richardson 1998). Although THMs are the most commonly regulated DBP group, they only account for 20–30% of total organic halides (TOX) formed by chlorination. With advances in analytical technologies, 600–700 chlorinated by-products have now been identified. Despite these efforts, it is estimated that detectable by-products account for approximately 50% of TOX. Richardson *et al.* (2007) recently reviewed this issue focusing on carcinogenicity and genotoxicity of DBPs. In addition, the evidence to date has been considered adequate to set health-based values for less than 20 DBPs. As a matter of fact, a total of 18 DBPs currently have health-based values including provisional guideline values that have been derived by the World Health Organization (2006), the US (US Environmental Protection Agency 2006), European Union (1998), Canada (Health Canada 2007) and Japan (Council on Public Welfare Science 2003). No by-product has a health-based value that was determined to account for reproductive and developmental endpoints.

Internationally, current DBP-related norms and regulations (World Health Organization 2006; Karanfil *et al.* 2008) address only a relatively small fraction of the overall DBP toxicity. This proportion cannot be easily increased

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by monitoring increased numbers of DBP species, because regulation and monitoring of more DBPs has both scientific and financial constraints. These constraints are acknowledged in the *Guidelines for Drinking-water Quality* (World Health Organization 2006), which recommend a general shift in emphasis away from monitoring an increasing number of chemical parameters in favour of preventive risk management. Therefore, efforts have focused on the overall toxicity of drinking water.

Here we review the results of studies on the overall toxicity of disinfected water instead of focusing on individual DBPs. The toxicity described in this report includes not only carcinogenicity but also reproductive and developmental toxicity. First, this paper presents evidence that demonstrates the presence of toxicity in disinfected water that cannot be attributed to the currently regulated by-products. This confirms the importance of estimating the overall toxicity of drinking water. Next, we review attempts to evaluate the overall toxicity of disinfected water using *in vivo* bioassays. We discuss problems with these assays and describe ongoing related research by the US Environmental Protection Agency (US EPA). Finally, we highlight requirements of future drinking-water quality regulation and make recommendations.

IMPORTANCE OF ESTIMATING OVERALL TOXICITY OF DISINFECTED WATER

Contribution of individual by-products to the toxicity of chlorinated water

Some researchers have measured the concentrations of by-products and examined the toxicity of individual by-products by *in vitro* bioassays. Table 1 shows an example obtained by Itoh & Echigo (2008). The chromosomal aberration test using Chinese hamster lung cells and the transformation test using mouse fibroblast cells were performed as indices to estimate the initiation and promotion, respectively, in the carcinogenesis process. Three by-products: chloroform, dichloroacetic acid (DCA) and trichloroacetic acid (TCA), contributed 2.9% of the chromosomal aberration-inducing activity and 1.4% to the transformation efficiency. The contributions of MX (3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone) and bromate ion were almost negligible (less than 0.1%).

Previous research has also reported that individual DBPs make small contributions to overall mutagenicity, as reviewed by Donald *et al.* (1989). Meier *et al.* (1985) estimated that the summed mutagenicity of ten chlorinated by-products was only 7–8% on TA100 and less than 2% on TA98 by the Ames test. Research using the Ames test has shown that MX contributes from 0.2 to 60% of the mutagenicity of chlorinated water (Kronberg *et al.* 1988; Kinae *et al.* 2000). On the other hand, the contribution of MX to mutagenicity assessed by the Ames test and by a test using cultured mammalian cells differs. Plewa *et al.* (2002) measured the DNA-damaging activity of several DBPs and MX by alkaline single-cell gel electrophoresis (SCGE, comet assay) using Chinese hamster ovary (CHO) cells. This assay indicated that the genotoxicity of MX was very weak compared with that of bromoacetic acid. This is because MX has high affinity for protein and other nucleophiles to reduce its genotoxicity in mammalian cells. Thus, an estimate of MX based on the result of the Ames test may overestimate its cancer risk (McDonald & Komulainen 2005).

It is widely known that the individual by-products analysed in these studies account for a small proportion of the

Table 1 | Contribution of individual DBPs to the chromosomal aberration-inducing activity and the transformation-inducing activity in chlorinated water (Itoh & Echigo 2008)

DBPs	Chromosomal aberration-inducing activity	Transformation-inducing activity (by the two-stage assay)	Experimental conditions
Chloroform	0.5%	0.9%	
DCA	0.8%	0.25%	Humic acid solution chlorinated with Cl_2/TOC = 1.0
TCA	1.6%	0.25%	
MX	<0.1%	<0.1%	Chlorinated Lake Biwa water
Bromate	<0.1%	–	Humic acid solution treated with ozone/chlorine sequential treatment. Br^- in the humic acid solution; 37.5 mg/L

overall genotoxicity of chlorinated water and the toxicity of chlorinated water can be attributed predominantly to by-products other than those currently regulated. The overall toxicity measured by *in vitro* and *in vivo* bioassays is discussed below in the sections '*in vitro* mutagenicity testing' and '*in vivo* testing', respectively. There is no guarantee that the concentrations of the regulated DBPs track the concentrations of all DBPs of adverse health consequences. An implication is that current water quality management, based on standard values for individual by-products, is insufficient in responding to overall toxicity arising from all DBP species in drinking water.

Contributions of organobromine compounds and bromate ion

In general, the concentrations of brominated by-products formed by chlorination are lower than those of chlorinated by-products. However, brominated low molecular weight by-products such as brominated THMs and haloacetic acids (HAAs) are more toxic than chlorinated by-products (Plewa *et al.* 2002; Richardson *et al.* 2007). A complex mixture of by-products from humic acids formed by hypobromous acid has threefold greater mutagenicity than that formed by hypochlorous acid (Echigo *et al.* 2004).

A previous study assessed the contribution of organobromine by-products to the induction of chromosomal aberrations in chlorinated water (Echigo *et al.* 2004). Total organic chlorine (TOCl) and total organic bromine (TOBr) in TOX were measured separately. This study found that the contribution of TOBr ranged from 28 to 52% in the actual tap water conditions of $[Br^-]/TOC =$ 0.05–0.1 mg Br/mg C and $[HOCl]/TOC = 1.0–1.5$ mg Cl_2/mg C. In most chlorinated waters, the concentration of TOBr is far lower than that of TOCl; however, TOBr is more toxic. Thus, the contribution of TOBr can be unexpectedly large. In some parts of the world the concentrations of naturally occurring bromide ions in source waters are often over 100 μg/L. In these cases, the contribution of TOBr to overall toxicity may exceed that of TOCl.

The contribution of bromate ions to the toxicity of ozonated and chlorinated water is very small or negligible, as shown in Table 1.

Figure 1 summarizes these findings on ozonated/chlorinated water. The areas of ellipses approximately show the strength of mutagenicity based on the results of the chromosomal aberration test (Echigo *et al.* 2004). TOCl and TOBr are formed in chlorinated waters. Although the TOBr concentration is low, the contribution of TOBr to overall toxicity is significant. In waters that are both ozonated and chlorinated, oxidized by-products without halogen are formed, including bromate ions that contribute a very small proportion of the overall toxicity.

Figure 2 shows the induction of chromosomal aberrations by humic acid solutions containing bromide ion that had been chlorinated and ozonated/chlorinated (Echigo *et al.* 2004). When ozonation was followed by chlorination, the chromosomal aberration-inducing activity was less than that of water treated only with chlorine. Bromate ion up to 1.1 mg/L was formed by ozonation. Water containing

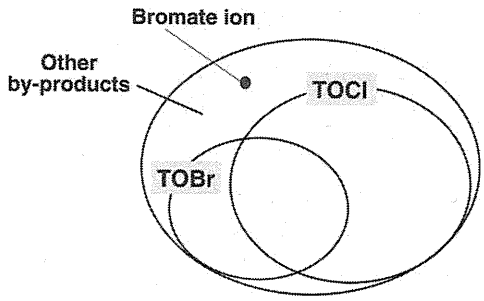

Figure 1 | Contributions of DBPs to the mutagenicity of ozonated/chlorinated water.

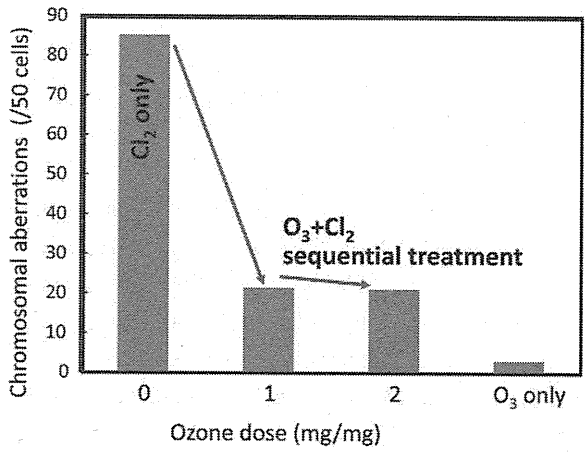

Figure 2 | Effect of ozonation on the chromosomal aberration-inducing activity in chlorinated water with Br^- (Echigo *et al.* 2004); conditions: humic acid concentration, 750 mg C/L; Br^-, 37.5 mg/L; reaction time, 1 day; temperature, 20 °C; chlorine dose, 1,500 mg Cl_2/L; pH, 7.0.

bromated ions that has been treated only with ozone has a weak chromosomal aberration-inducing activity. Because ozonation changes the chemical structures of natural organic matter (NOM), different by-products will be formed and induction of chromosomal aberration is less in chlorinated water if it has been ozonated. Thus, ozonation can produce safer (chlorinated) drinking water even with the formation of bromate ion.

Some water supply utilities reduce bromide ion in raw water by chlorination to decrease the concentration of bromate ions that is formed by subsequent ozonation (Buffle *et al.* 2004). Chlorination before ozonation can result in formation of organobromine by-products (TOBr). However, the contribution of bromate ion to the toxicity of chlorinated water as a final product is negligible, and organobromine by-products have a far greater contribution as shown in Figure 1. Therefore, this procedure may increase overall toxicity of drinking water and a careful safety evaluation should be performed before this is implemented.

Meeting water quality standards for individual DBPs (bromate ion in this case) may result in other potentially significant problems being overlooked, leading to potentially inappropriate and counterproductive treatment measures. Water quality standards for DBPs should be considered as a reference for water quality management. The relative evaluation of the toxicity of brominated organic by-products and bromate ion (Figure 1) and the result indicating the significance of ozonation (Figure 2) present examples of the necessity of measuring the overall toxicity of drinking water.

Change of the toxicity of chlorinated water and its index

As concentrations of THMs and HAAs in chlorinated drinking water increase in water distribution systems (Tanaka *et al.* 1991; Sasaki & Ueda 1992; Summers *et al.* 1996; Arora *et al.* 1997), it is widely believed by water supply utilities that the toxicity of drinking water also increases.

On the other hand, it has been found that mutagenicity of chlorinated water and some chlorinated by-products is not stable. Meier *et al.* (1983) have examined the effect of pH on the stability of mutagenicity of chlorinated water. Mutagenicity of chlorinated humic acids decreases with increasing pH. Nazar & Rapson (1982) have shown that mutagenicity of the known organochlorine mutagens decreases by cleavage of organically bound chlorine. As cleavage of chlorine proceeds by hydroxide ion, mutagenicity decreases faster at higher pH. These findings have shown that the structure of some organochlorine compounds produced by chlorination can be changed by hydrolysis.

Itoh *et al.* (2006) investigated changes in the toxicity in chlorinated water after chlorine addition. Figure 3 illustrates the results. The chromosomal aberration test and transformation test were carried out as indices to initiation activity and promotion activity, respectively. Firstly, it shows that initiation activity just after chlorination is stronger than promotion activity. This was found by a comparison between chlorinated water and various chemicals. Secondly, initiation activity is produced by chlorine; however, it is unstable and decreases sharply over time after chlorination even in the presence of residual chlorine. In contrast, promotion activity produced by chlorine increases slightly over time after chlorination.

Thus, toxicity that decreases or increases is present in chlorinated water. The increasing toxicity (promotion activity) is present in chlorinated water; however, initiation activity dramatically decreases. As the toxicity of water is measured by *in vitro* assays in this study, it is not possible to reach a conclusion on the change of toxicity on the human body. However, it should be noted that the overall

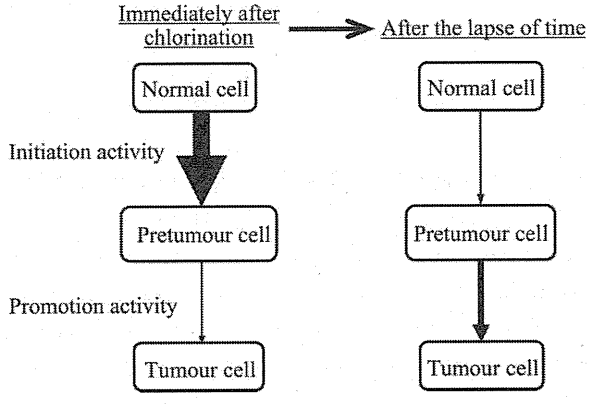

Figure 3 | Proposed change of the toxicity of chlorinated water (Itoh *et al.* 2006). The thickness of arrows shows the strength of initiation activity and promotion activity; changes of the thickness of arrows after the lapse of time indicate changes of initiation activity and promotion activity in the presence of residual chlorine.

toxicity associated with carcinogenic activity can be mainly attributed to initiation activity and presumably decreases over time after chlorination. This was also suggested by the non-two-stage transformation test that is an index of the sum of initiation and promotion activity.

It is well known that concentrations of typical by-products such as THMs and HAAs increase after chlorine injection based on studies on characteristics of DBP formation by chlorination and factors affecting the DBP yield (Rockhow *et al.* 1990; Zhuo *et al.* 2001; Liang & Singer 2003). Since many investigations have been carried out on the mutagenicity in chlorinated drinking water, some characteristics of the mutagenicity have been clarified. One of the representative characteristics is that the mutagenicity easily changes and decreases over time after disinfection depending upon pH and temperature of water (Rapson *et al.* 1980; Meier *et al.* 1983; Kinae *et al.* 1992; Ueda 1996; Itoh *et al.* 2001). These findings suggest that the direction of change in the mutagenicity is inconsistent with those of THMs and HAAs. In addition to these previous views, Figure 3 obtained by *in vitro* tests as indices of initiation activity and promotion activity shows that the toxicity of chlorinated water is not consistent with concentrations of THMs and HAAs. These by-products are widely measured; however, they would not be appropriate as indices to compare the toxicity of chlorinated drinking water in distribution systems.

The stability of some DBPs after production by chlorine has been examined and discussed (Glezer *et al.* 1999; Nikolaou *et al.* 2001; Lekkas & Nikolaou 2004; Xie 2004). MX, a strong mutagen and carcinogen (McDonald & Komulainen 2005), is also produced by chlorination; however, it has been found that it decreases over time after it is formed by chlorine (Meier *et al.* 1987; Kinae *et al.* 1992). This decrease could be attributed to hydrolysis and the reaction of MX with residual chlorine. This direction of change is the reverse of those of THMs and HAAs. In addition, the change in concentration of MX was quantitatively consistent with the change of the toxicity (Itoh *et al.* 2006). Consequently, MX is appropriate as an index for comparing the carcinogenicity of tap water near and far from a water purification plant.

This example suggests that we have to focus on the overall toxicity of chlorinated water and indicator by-products have to be selected in view of the purpose of water quality management.

Toxicity and characteristics of chlorine dioxide-treated water

The use of so-called 'alternative' (meaning non-chlorine) disinfectants can markedly reduce the levels of halogenated organic compounds, including THMs, in drinking water (Fielding & Farrimond 1999; Singer 1999; Barrett *et al.* 2000). DBPs formed by chlorine dioxide including inorganic by-products such as chlorite and chlorate ions have also been examined (Chang *et al.* 2000a, b; Dabrowska *et al.* 2003; Veschetti *et al.* 2005). Chlorine dioxide is generally thought to be suitable for practical disinfection processes with reducing levels of halogenated DBPs (Gates 1998). However, the use of alternative disinfectants has had unexpected consequences, including the production of a different set of toxic DBPs (Sedlak & Von Gunten 2011). For this reason, we have to consider the overall level of toxicity of water that is formed by these disinfectants, in addition to typical halogenated DBPs.

From this point of view, *in vitro* short-term genotoxicity tests are useful, because they can evaluate the combined action of DBPs present in drinking water as complex mixtures. Actually, there have been some studies on mutagenicity formation by chlorine dioxide and comparison between waters treated with chlorine dioxide and chlorine (Donald *et al.* 1989; Anderson *et al.* 1990; Itoh *et al.* 2001; Guzzella *et al.* 2004; Onarca *et al.* 2004). As described above in the section 'Change of the toxicity of chlorinated water and its index', the mutagenicity in chlorinated water changes over time after chlorination. A few studies show the change or persistence of DBPs formed by chlorine dioxide in distribution systems (Korn *et al.* 2002; Hoehn *et al.* 2003); however, no studies have been conducted on the change in the mutagenicity formed by chlorine dioxide over time after the water treatment. We have to consider that there are some differences in the mutagenicity level and the change rate of the mutagenicity over time after disinfection between chlorination and chlorine dioxidation.

In one study the toxicity of chlorine dioxide-treated water and associated changes were examined and compared with that of chlorinated water (Itoh *et al.* 2007). The

chromosomal aberration-inducing activity is produced by chlorination and chlorine dioxidation; however, this activity is unstable and gradually decreases over time after the treatments. Moreover, this activity decreases even under conditions where residual chlorine and chlorine dioxide can be detected. Changes in the chromosomal aberration-inducing activity were estimated to compare the safety of drinking water treated with chlorine and chlorine dioxide in distribution systems. The time to reach the maximum chromosomal aberration-inducing activity observed in chlorinated water or chlorine dioxide-treated water was set at 24 hours or 10 hours, respectively, based on the data obtained. Decreasing rate constants for the chromosomal aberration-inducing activity were calculated as a function of the concentration of residual disinfectants. It has been found that the decreasing rate constant is smaller, as the residual disinfectant concentration is higher. Residual concentrations in distribution systems were set at 0.1 and 0.4 mg/L. Figure 4 shows an estimated result based on typical drinking water in Japan. The 1.0 on the vertical axis indicates the maximum chromosomal aberration-inducing activity in chlorinated water.

The levels of chloroform and TOX formed by chlorine dioxidation were approximately 1% and 5–7%, respectively, of those formed by chlorination (Itoh *et al.* 2007).

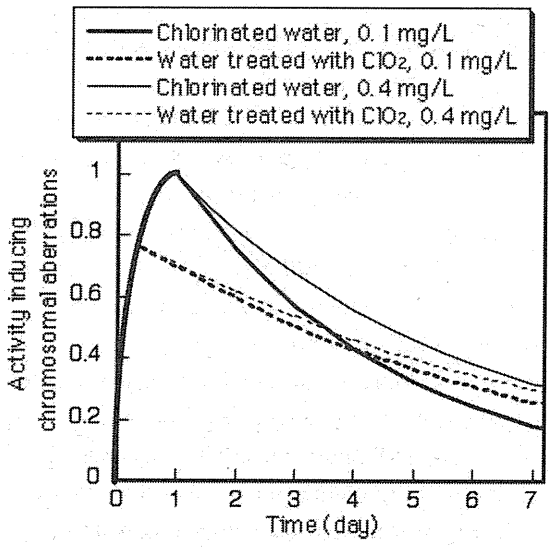

Figure 4 | Estimated changes in the chromosomal aberration-inducing activity in drinking water (Itoh *et al.* 2007). DOC of raw water, 2.0 mg/L; DOC after rapid sand filtration, 1.1 mg/L; added disinfectant, 1.1 mg/L (disinfectant/DOC = 1); assumed residual disinfectant concentrations, 0.1 and 0.4 mg/L.

A major advantage of chlorine dioxide over chlorine is that it produces significantly lower levels of halogenated organic compounds. Figure 4 shows, however, that the chromosomal aberration-inducing activity produced by chlorine dioxidation is stronger than would be expected based on the quantity of the formed by-products. Therefore, it is important to note that the use of chlorine dioxide instead of chlorine as an alternative disinfectant does not dramatically reduce the mutagenicity of the treated water.

Figure 4 shows that the activity in chlorine dioxide-treated water that induces chromosomal aberrations decreases more slowly, indicating that the mutagenicity of chlorine dioxide-treated water is more stable. The chromosomal aberration-inducing activity in chlorine dioxide-treated water is weaker than that in chlorinated water just after treatments; however, the difference in the two activities decreases over time after treatment. In particular, when the residual disinfectants are 0.1 mg/L, the activity in chlorine dioxide-treated water that induces chromosomal aberrations becomes equal to that in chlorinated water at approximately four days. After that, the relationship is reversed. When the residual disinfectants are 0.4 mg/L, the difference in the two activities does not rapidly decrease.

Assuming that the drinking water is retained in distribution systems typically for less than two days, Figure 4 also suggests that the mutagenicity of chlorine dioxide-treated water would be 70–80% of that of chlorinated water – a potential advantage of chlorine dioxide treatment. In addition, although chlorine dioxide-treated water is less mutagenic than chlorinated water, the difference is small when the drinking water remains in the distribution system for a long period of time.

Thus, while at face value chlorine dioxide treatment can 'solve' the THMs problem, it should be noted that it is similar to chlorine in terms of the mutagenicity of drinking water.

Chlorate ion and chlorite ion are formed as inorganic by-products by chlorine dioxide and standard values have been set for these by-products that prevent its widespread use because they are not easy to achieve. The finding presented here is an additional limitation in using chlorine dioxide.

Contribution of DBPs to the estrogenic effects of drinking water

The potential health risks of endocrine disrupting chemicals (EDCs) were of great public interest in the mid-to-late 1990s. Many epidemiological studies have been conducted to examine the relationship between adverse reproductive and developmental outcomes and exposure to chlorinated drinking water. Some reviews of these studies (Zavaleta *et al.* 1999; International Programme on Chemical Safety 2000; Nieuwenhuijsen *et al.* 2000; US Environmental Protection Agency 2006) have suggested that adverse outcomes, such as spontaneous abortion, stillbirth, low birth weight, neurotoxicity and birth defects, can be associated with THMs and chlorinated by-products. These associations were not reported in other studies and further research would be needed to confirm any association.

Hundreds of compounds have been listed as suspected EDCs (Endocrine Disruptor Screening and Testing Advisory Committee 1998), and most research on EDCs focuses on these individual micropollutants. In contrast, the relationship between the consumption of chlorinated water and reproductive and developmental toxicity has been explored in epidemiological studies as mentioned above. Therefore, chlorinated by-products formed from NOMs should be of interest in addition to typical EDCs.

We consider it is important to measure the estrogenic effects of raw water containing both micropollutants and NOMs, and of chlorinated by-products in addition to suspected EDCs. The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) (1998) established by the US EPA also recommended that a mixture of DBPs be evaluated for their potential to cause endocrine disruption.

Figure 5(a) illustrates the components of water that induce estrogenic effects and how they are changed by chlorination (Itoh *et al.* 2009). First, NOMs have a weak estrogenic effect that increases after chlorination. Itoh *et al.* (2000a) found that commercial humic acid exhibits the estrogenic effect, which increases upon chlorination. In addition, this study demonstrated that the estrogenic effect of concentrated Lake Biwa water using the XAD7HP resin increases up to 2.3 times upon chlorination. The reasons that chlorination increases the estrogenic effect could be: (1) chlorine produces by-products such as organochlorine substances, which are estrogenic; (2) a low molecular weight fraction, which may bind to the estrogen receptor in a cell, increases as a result of the oxidation and hydrolysis caused by chlorination; and (3) chlorine releases estrogenic substances, which interact with humic substances in the aqueous environment. Itoh *et al.* (2000b) revealed that the main factor affecting the increase in the estrogenic effect is the effect of chlorination by-products. However, it has not been successful in detecting specific by-products contributing to the increase in the estrogenic effect.

In addition, coagulation and activated carbon treatment decreased the estrogenic effect of the source water, but chlorination increased the estrogenic effect of the source water and treated waters (Itoh *et al.* 2009). These results suggest that the estrogenic effect is formed by the reaction of chlorine with organic matter that remains after water

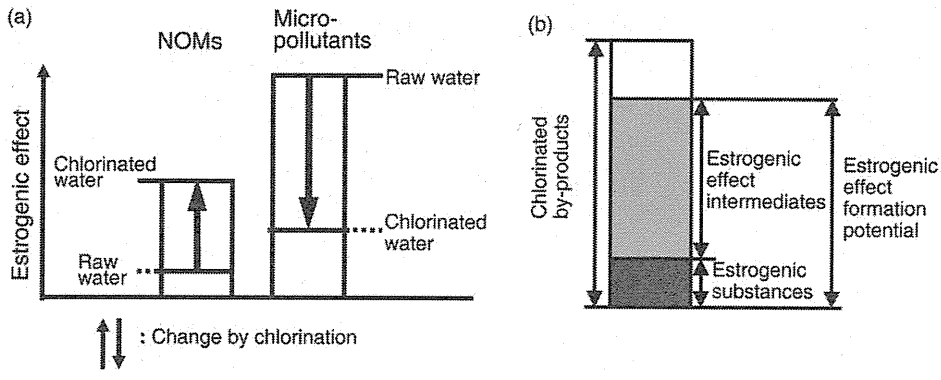

Figure 5 | Components of the estrogenic effects in chlorinated drinking water (Itoh *et al.* 2009).