For submission to *Water Research*

**Toxicity assessment of chlorinated wastewater effluents by using
transcriptome-based bioassays and Fourier Transform Mass
Spectrometry (FT-MS) analysis.**

Toshikazu Fukushima^a, Hiroe Hara-Yamamura^a, Makoto Urai^b, Ikuro Kasuga^b, Futoshi
Kurusu^b, Taro Miyoshi^c, Katsuki Kimura^a, Yoshimasa Watanabe^c, Satoshi Okabe^{a*}

^a Division of Environmental Engineering, Faculty of Engineering, Hokkaido

University, North-13, West-8, Kita-ku, Sapporo Hokkaido 060–8628, Japan;

^b Research Center for Water Environment Technology, The University of Tokyo, 7-3-1

Hongo, Bunkyo-ku, Tokyo 113-8656, Japan;

^c Center for Environmental Nano and Bio Engineering, Hokkaido University, Sapporo

060-8628, Japan

* Corresponding author e-mail: sokabe@eng.hokudai.ac.jp (SATOSHI OKABE);

phone: +81-11-706-6266; fax: +81-11-707-6266,

19

20 **Contributors**

21 T.F., H.H-Y., and S.O. designed research; T.F., performed whole research and analyzed
22 data; M.U., I.K. and F.K. performed FT-MS analysis, T.M., K.K. and Y.W. operated
23 MBR, T.F. and S.O wrote the paper

24

25

26

27

28

Abstract

Effects of chlorination on the toxicity of wastewater effluents treated by activated sludge (AS) and submerged membrane bioreactor (S-MBR_B) systems to HepG2 human hepatoblastoma cells were investigated. In addition to the cytotoxicity and genotoxicity assays, the DNA microarray-based transcriptome analysis was performed to evaluate the change in types of biological impacts on HepG2 cells of the effluents by chlorination. Effluent organic matter (EfOM) and disinfection by-products (DBPs) were also characterized by using Fourier transform mass spectrometry (FT-MS). Although no significant induction of genotoxicity was observed by chlorination for both effluents, the chlorination elevated the cytotoxicity of AS effluent but reduced that of S-MBR_B effluent. The FT-MS analyses revealed that more DBPs including nitrogenated DBPs (N-DBPs) were formed in the AS effluent than in the S-MBR_B effluent by chlorination, supporting the increased cytotoxicity of AS effluent. The lower O/C ratio of S-MBR_B EfOM suggests that a large number of organic molecules were detoxified by chlorination, which consequently decreased the cytotoxicity of S-MBR_B effluent. Integration of all the results highlights that both cytotoxicity and biological impacts of chlorinated wastewater effluents were clearly dependent on the EfOM characteristics such as DBPs and O/C ratio, namely, on types of treatment systems.

47 **Keywords**

48 Chlorination, Cytotoxicity, DBPs, DNA microarray, Fourier transform mass
49 spectrometry, Wastewater effluents

50

51 **A list of abbreviations**

52 AS: activated sludge

53 DAVID: Database for Annotation, Visualized and Integrated Discovery

54 DBP: disinfection by-product

55 DCFH-DA: 2',7'-dichlorofluorescein diacetate

56 DPD: *N, N*-dimethyl-*p*-phenylenediamine

57 EfOM: effluent organic matter

58 FT-MS: Fourier transform mass spectrometry

59 NRU: neutral red uptake

60 MF: microfiltration

61 MOA: modes of toxic action

62 MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

63 PPCP: pharmaceuticals and personal-care product

64 ROS: reactive oxygen species

65 RT PCR: reverse transcription polymerase chain reaction

66 S-MBR_B: submerged membrane bioreactor

67 SMP: soluble microbial product

68 SPE: solid phase extraction

69 SRT: solid retention time

70 WWTP: wastewater treatment plant

71

72

1. Introduction

Globally, water resources in various regions and countries are expected to face unprecedented pressures in the near future (Asano et al., 2007). Wastewater reclamation can be considered as an alternative urban water source. Disinfection is an essential component of many wastewater reuses. Although the most common method of disinfection is chlorination, chlorination of wastewater effluent has been known to result in the formation of toxic disinfection by-products (DBPs) (Buschini et al., 2004; Marabini et al., 2006; Wang et al., 2007; Bayo et al., 2009; Maffei et al., 2009). More than 600 DBPs had been identified, however, only a small number of DBPs has been assessed their toxicity and regulated (Richardson et al., 2007). Watson *et al.* (2012) demonstrated that toxicity of wastewater DBPs cannot be predicted by chemical monitoring of the regulated individual DBPs. It has been generally accepted that hydrophobic dissolved organic matter represents the major source of DBP precursor sites (Leenheer and Croué, 2003). Fourier transform mass spectrometry (FT-MS) has recently been applied to understand the reactivity of natural organic matter toward chlorine and to identify DBPs (Zhang et al., 2012a; Zhang et al., 2012b; Lavonen et al., 2013). The characteristics of effluent organic matter (EfOM) were changed by advanced biological treatments, such as membrane bioreactor (MBR) (Krasner et al., 2009;

Mesfioui et al., 2012). Thus, it is assumed that treatment system influence on the DBPs production, which consequently determines the toxicity of wastewater effluent. The potential ecological impacts of DBPs in chlorinated wastewater effluents have been studied with various types of bioassays using ecotoxicology model organisms (Cao et al., 2009). The evaluation of potential human health risks becomes important when the reclaimed wastewater is used for any types of wastewater reuse practices with human body contacts. However, the potential human health risks of chlorinated wastewater effluents are not well understood.

The *in vitro* bioassays using human cell lines, such as genotoxicity and cytotoxicity assays, have been applied to assess the human health risks of DBPs in drinking water (Marabini et al., 2006; Maffei et al., 2009; Shi et al., 2009). However, since a variety of contaminants induce toxicity via different modes of toxic actions (MOAs), it is difficult to assess all MOAs by using those bioassays because each bioassay can assess only a single endpoint. On the other hand, the DNA microarray analysis enables us to examine global gene expressions in human, and has been used to evaluate biological impacts of hazardous chemicals (Kawata et al., 2007, 2009a, 2009b) and wastewater effluents (Hara-Yamamura et al., 2013) on human cell lines.

The aim of this study was to investigate the effects of chlorination on the toxicity of

wastewater effluent using HepG2 human hepatoblastoma cell lines. The wastewater effluents were collected from activated sludge (AS) and submerged MBR systems (S-MBR_B) treating the same domestic wastewater to compare the chlorine effect by different treatment systems. The cytotoxicity and genotoxicity assays and DNA microarray-based transcriptome analysis were performed to investigate changes in the cytotoxicity, genotoxicity and types of biological impacts on HepG2 cells by chlorination. Furthermore, the EfOM and DBPs were characterized by using FT-MS to explain the difference in toxicity developed by chlorination between two wastewater effluents.

2. Material and methods

2.1 Sample collection

Sampling was carried out from December 2011 to June 2012, at the full-scale municipal wastewater treatment plant (WWTP) in Sapporo, Japan. Wastewater effluent samples were collected from an activated sludge system (AS), which consists of aeration tank and secondary sedimentation basin, and a pilot-scale submerged membrane bioreactor (S-MBR_B) installed at the WWTP plant, which equipped with hollow-fiber polytetrafluoroethylene membranes with 0.3 µm nominal pore size. The

solid retention times (SRTs) of AS and S-MBR_B are 7 and 50 days, respectively. Other detailed operational conditions were described previously (Hara-Yamamura et al., 2013). Grab samples were taken around 10:00 am, from the effluents of the grid chamber (considered as raw wastewater; RAW), the secondary sedimentation basin of the AS, and the S-MBR_B. The effluent samples were transferred into glass bottles and stored in cold boxes with refrigerants during transportation.

2.2 Chlorination

Sodium hypochlorite solution (30 %) was added to effluent samples. Initial concentrations of chlorine were fixed at 0, 2 or 5 mg/L, respectively. Chlorination was carried out in the capped amber glass bottle at room temperature for 24 h with continuous mixing using a magnetic stirrer. The duration of chlorination simulates the disinfection process and following water storage and distribution processes. After 24 h, residual chlorine was not quenched because dechlorination agents, such as sodium thiosulfate, have been reported to reduce some chlorinated DBPs (Freuze et al., 2004). Residual chlorine concentration was measured by using *N*, *N*-dimethyl-*p*-phenylenediamine (DPD) method.

2.3 Solid phase extraction (SPE)

For RAW and other effluent samples, 0.1 and 1 L of samples were subjected to solid phase extraction (SPE), respectively. To prepare negative control samples for toxicity assays, DNA microarray analysis and FT-MS analysis, 1 L of pure water was also subjected to SPE. Samples were filtered with glass fiber membrane (GB-140, Advantec, Tokyo, Japan). After filtration, the SPE was conducted according to the protocol described previously (Macova et al., 2010, 2011). The detailed procedure of SPE is found in supplementary information.

2.4 Cytotoxicity assay

The culture condition of HepG2 is found in supplementary information. Cytotoxicity induced by each wastewater effluent sample was assessed by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and neutral red uptake (NRU) assays. The detailed procedures of MTT and NRU assays can be found elsewhere (Kawata et al., 2009; Hara-Yamamura et al., 2013) and in supplementary information.

2.5 Alkaline Single-Cell Gel Electrophoresis (Comet Assay)

Alkaline comet assay was carried out using CometAssay® kit (TREVIGEN,

Gaithersburg, MD) in accordance with manufacturer's specifications. The detailed procedure of the assay is found in supplementary information.

2.6 Reactive Oxygen Species (ROS) Measurement

The generation of intracellular reactive oxygen species (ROS) was measured by the 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay (Wang and Joseph, 1999). The detailed procedure of ROS measurement is found in supplementary information.

2.7 Human DNA microarray-based transcriptome analysis

The detailed procedure of DNA microarray analysis can be found elsewhere (Hara-Yamamura et al., 2013) and in supplementary information. Briefly, Human Genome Focus Array (Affymetrix, Santa Clara, CA), which contains probes for 8,795 verified human genes, was used for DNA microarray analysis. The fragmented cDNA (10 µg) was hybridized to the human genome focus array for 16 h at 45 °C. After hybridization, the microarrays were automatically washed and stained with streptavidin-phycoerythrin by using a fluidics station (Affymetrix, Santa Clara, CA). Finally, probe arrays were scanned with the Genechip System confocal scanner (Affymetrix). All the analyses were performed in duplicate. SPE fraction from pure water was used as negative control. The

raw data files [cell intensity (CEL) files] are available on Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.gov/geo>) of the National Center for Biotechnology Information (NCBI) (accession number: GSE48157). The obtained data was analyzed by using Avadis 4.3 prophetic software. Differentially expressed genes were defined by fold change (at least 2-fold) and using a paired Student's t-test ($P < 0.05$). Gene ontology (GO) assignments and clustering into functional groups were performed using the Database for Annotation, Visualized and Integrated Discovery (DAVID) (<http://david.abcc.ncifcrf.gov/>) (Dennis et al., 2003).

To validate the microarray data, real-time reverse transcription polymerase chain reaction (RT PCR) was performed for selected 9 genes, which showed higher fold changes in microarray analysis. The detailed procedure of real-time RT PCR can be found in supplementary information.

2.8 ESI-Orbitrap Fourier transform mass spectrometry (FT-MS) Analysis

To characterize EfOM and DBPs, FT-MS analysis of the SPE fractions were performed by an Exactive mass spectrometer (Thermo Scientific, Waltham, MA) with an electrospray ionization source. The detailed analytical condition is found in supplementary information. The raw data were processed and compared using Sieve

version 2.0 (Thermo Scientific). All the ions with a signal-to-noise ratio ≥ 3 and in the m/z range from 100 to 1000 were extracted, converted to neutral mass and exported.

To calculate the chemical formula, we used Xcalibur version 2.1 (Thermo Scientific). Mass tolerance was set at 2 ppm (Krauss et al., 2010; Remucal et al., 2012). Formulas containing only carbon, hydrogen, oxygen, nitrogen, sulfur, phosphorus and chlorine were considered. The nitrogen rule was applied and the relative intensity of ^{37}Cl containing isotope peak was used to validate the number of chlorine atoms included in the molecular formula. The following conservative ratios were also applied to assign molecular formulas: $\text{N/C} < 0.5$, $\text{S/C} < 0.2$, $\text{P/C} < 0.1$, $(\text{S}+\text{P})/\text{C} < 0.2$, H/C : 0.2-3.1, Cl/C : 0-0.8 and O/C : 0-1.2 (Kind and Fiehn, 2007; Sleighter and Hatcher, 2008). The mass error ranges of formula assignments in AS and S-MBR_B were -1.995 to 1.980 and -1.656 to 1.991 ppm, respectively.

When the chemical formula which meets the above criteria could not be found, those peaks were not used for further analysis. The chemical species whose ratios of peak intensity were increased by chlorination were defined as DBPs.

3. Results and discussion

3.1 Influence of chlorination on cytotoxicity of effluents

Toxicity recovery by SPE was confirmed by comparing the cytotoxicity of non-concentrated and SPE-concentrated RAW samples (**Fig. S1** in supplementary information). The cell viabilities exhibited no significant difference between non-concentrated and SPE concentrated samples, suggesting that the major parts of toxicants were recovered by SPE from the RAW samples. Although the free chlorine cannot be adsorbed with the Oasis HLB sorbent and was not detected from most of the chlorinated effluents (**Table S2** in supplementary information), the influence of the residual free chlorine on the cell viability was evaluated using chlorinated pure water samples (**Fig. S2** in supplementary information). After chlorination, all of chlorine was remained as free chlorine, which did not influence on the cytotoxicity.

The cytotoxicity was determined for AS and S-MBR_B effluents collected different dates (**Fig. 1 and 2**). The cytotoxicity of AS effluents was elevated with increasing chlorine concentration. The elevated toxicity of chlorinated wastewater effluents have been observed using different bioassays such as *Daphnia magna* acute toxicity test (Cao et al., 2009) and an *in vitro* bioluminescence assay (Watson et al., 2012). On the other hand, the cytotoxicity of S-MBR_B effluent was reduced by chlorination. Similar trends were observed for all the effluent samples taken on different sampling dates even though the time variations of quantity and quality of domestic wastewater have been

observed (Butler, 1993). The cell viabilities of non-chlorinated AS and S-MBR_B effluents were comparable, while those after chlorination were different. This implies that the major factors affecting the cytotoxicity after chlorination could be attributed to different wastewater treatment systems, that is, the water quality of each effluent.

Clear differences in operation of AS and S-MBR_B systems are the sludge retention time (SRT), MLSS concentration and strict removal of suspended solids by membrane. The forty water quality parameters of the National Effluent Standards were measured for both effluents previously (Hara-Yamamura et al., 2013), while the sampling date was different. Both effluents satisfied all the Japanese National Effluent Standards. The concentrations of BOD and total nitrogen in S-MBR_B effluent were slightly higher than those in AS effluent, but those cannot explain the different chlorine effect (**Fig. 1 and 2**). The removal of emerging contaminants and biodegradable organic carbon was improved with increasing SRT (Leu et al., 2012). On the other hand, the longer SRT would cause the higher production of soluble microbial products (SMPs) (Barker and Stuckey, 1999; Huang et al., 2011). It has been speculated that a certain type of EfOM produced only under the longer SRT (S-MBR_B) posed adverse effects on the tested cells (Hara-Yamamura et al., 2013).

3.2 Influence of chlorination on genotoxicity

Previous studies have reported that chlorine disinfection induced genotoxicity (Guzzella et al., 2004; Marabini et al., 2006; Shi et al., 2009). However, chlorine did not significantly induce genotoxicity in HepG2 cells for any effluents in this study (**Fig. S3** in supplementary information). The genotoxicity assay was performed on the 25 times concentrated effluent samples exhibiting a cell viability >70%, as recommended by the International Workshop on Genotoxicity Test Procedures (Tice et al., 2000). No significant induction of genotoxicity was observed even for the 50 times concentrated S-MBR_B effluent samples (data not shown). The overproduction of ROS results in oxidative stress, which leads to the induction of genotoxicity (Jiang et al., 2007; Cao et al., 2008; Zou et al., 2009). However, the intracellular ROS production was not increased by chlorination for both effluents (**Fig. S4** in supplementary information), which are consistent with the genotoxicity results. Watson *et al.* (Watson et al., 2012) reported only a marginal increase of genotoxicity, while the majority of previous studies reported the increases in general genotoxicity by chlorination. Taken together, the influence of chlorination on the genotoxicity largely depends on the effluent water quality such as the presence of DBP precursors.

3.3 DNA microarray-based transcriptome analysis

DNA microarray data were validated by comparing the fold changes of selected genes determined by DNA microarray and real-time RT PCR (Canales et al., 2006). As shown in supplementary information Fig. S5, a good correlation ($R^2=0.9369$, $p<0.05$) was obtained between the results of DNA microarray and real-time RT PCR.

The number of altered genes in HepG2 cells after 48 h exposure to 25-time concentrated AS effluents increased with increasing the chlorine concentrations (**Fig. 3**), indicating the increase in types of biological impacts on HepG2 cells by chlorination. In contrast, the number of altered genes decreased with increasing the chlorine concentrations for the S-MBR_B effluent, indicating the reduction of types of biological impacts by chlorination. The numbers of commonly altered gene are summarized in **Fig. 4**, showing the different genes were differentially expressed by chlorination especially for the S-MBR_B effluent.

To evaluate the change in types of biological impacts by chlorination, biological functional analysis was conducted by DAVID to link altered genes and related biological processes. Based on the p values (significance of alteration) of each biological function, top 25 altered functions from each sample were considered as major altered functions. To investigate how these function were changed by chlorination, the

major biological functions and their *p* values of the S-MBR_B effluent are summarized in **Fig. 5**. The biological functions identified for the S-MBR_B effluent treated with 0 mg/L chlorine were “Response to nutrients (Group 1)”, “Response to xenobiotics” (Group 2), “Response to hormone stimulus” (Group 3), “Acute inflammatory response” (Group 4) and “Biosynthesis” (Group 5). Among them, many of biological functions responsible for Groups 2, 3 and 4 were not identified in the chlorinated effluent with 5 mg/L, suggesting that chemical compounds responsible for those biological functions were detoxified and oxidized by chlorination.

The major biological functions and their *p* values of the AS effluent are summarized in **Fig. 6**. The alteration of the genes responsible for “Response to hormone stimulus” (Group 2), “Acute inflammatory response” (Group 3) and Group 5 were suppressed by chlorination. On the other hand, the gene alteration in Group 4 was elevated. Many of the functions in Group 4 are related to “Cellular metabolic process” while those functions are not grouped by DAVID. The mechanism is presently unclear, but those changes might cause the elevation of cytotoxicity. Therefore, the gene expression analysis suggested that types of biological impacts of both effluents were changed by chlorination, while the further study is required to confirm the actual damage to cells.

3.4 Fourier Transform Mass Spectrometry (FT-MS) analysis

This study applied ESI-Orbitrap FT-MS to characterize EfOM and DBPs. Although most of the studies applied FT ion cyclotron resonance MS to identify DBPs (Zhang et al., 2012a; Zhang et al., 2012b; Lavonen et al., 2013), previous study reported that accuracy of the Orbitrap MS is also sufficient for determination of unique molecular formulas (Remucal et al., 2012).

The majority of peaks appears at odd m/z values by negative ionization mode in all effluents indicate a predominance of zero or an even number of nitrogen in the molecules. The van Krevelen diagram of the effluents, plotting the molar H/C ratios on the y-axis and the molar O/C ratios on the x-axis, is constructed to elucidate differences between the mass spectra (**Fig. S6** in supplementary information) (van Krevelen, 1950; Kim et al., 2003). The AS effluents contained more diverse molecular compounds than the S-MBR_B effluents did. Similar difference owing to the different type of treatment system was also reported in the previous study (Mesfioui et al., 2012). The percentages for the number of nitrogen-encompassed formulas of AS and S-MBR_B effluents without chlorination were 39 and 29 %, and chlorine-encompassed formulas of those effluents were 18 and 16 %, respectively. The Cl-containing formulas detected before disinfection may be (i) DBPs in tap water, (ii) DBPs produced by residual chlorine in tap water or

household chemicals, (iii) Cl-containing pharmaceuticals and personal-care products (PPCPs), such as a perfumery and a germicide, and (iv) The Cl reaction products from Cl⁻ in the HCl used when acidifying the samples prior to SPE (Inaba et al., 1998; Lavonen et al., 2013).

The average O/C ratio of S-MBR_B effluent was lower than that of AS effluent (**Table1**). Previous study reported that lower O/C molecules are more reactive toward free chlorine than high O/C molecules (Zhang et al., 2012a; Zhang et al., 2012b). Namely, the lower O/C molecules are easily decreased by chlorination. This suggests that the number of molecules which were decreased by chlorination in S-MBR_B effluent was larger than that in AS effluent.

The formulas whose ratios of peak intensity were increased by chlorination were identified as DBPs. The number of DBPs in AS effluent was much larger than that in S-MBR_B effluent (**Fig. 7**). Only 6 DBPs were common in both chlorinated effluents, indicating that the composition of DBPs in both effluents were different. The numbers of nitrogenated DBPs (N-DBPs) in AS effluent was larger than S-MBR_B effluent. N-DBPs was considered higher carcinogenicity and toxicity than carbonated DBPs (C-DBPs) (Chang et al., 2013). Although it is difficult to discuss toxicity based on the numbers of toxicants, the larger numbers of DBPs, especially N-DBPs, in chlorinated

AS effluent might cause the elevation of cytotoxicity. The average O/C ratios of DBPs in both effluents were larger than that of total molecules (**Table 1**). The average H/C ratio of DBPs in AS effluent was similar to the ratio of total molecules, whereas that of DBPs in S-MBR_B effluent was much lower than the ratio of total molecules. It is assumed that the chemical characteristics of DBPs were different, which results in the different behaviors of cytotoxicity.

The weighted average mass was not significantly changed by chlorination (**Fig. S7** in supplementary information). This implied that the chlorination did not significantly break down the molecules. Only 2 or 0 molecules were completely disappeared by chlorination in AS and S-MBR_B effluents, respectively. Seven and 1 molecules were newly produced by chlorination in AS and S-MBR_B effluents, respectively. Thus, organic component proportion was not significantly changed by chlorination; however, those small changes might affect the toxicity of effluents.

3.5 Combination of toxicity assays and EfOM characterization to investigate the effects of chlorination on toxicity of wastewater effluents

In this study, the conventional cytotoxicity and DNA microarray-based transcriptome analysis were combined with FT-MS analysis to assess the toxicity of whole chlorinated

wastewater effluents treated by different treatment systems. The obtained results clearly demonstrated that this approach has great potential to correlate the observed cytotoxicity and biological impacts on HepG2 cell by chlorination and EfOM characteristics. It has been recognized that the toxicity of wastewater DBPs cannot be predicted by conventional chemical monitoring alone (Itoh et al., 2011; Watson et al., 2012) and thus comprehensive studies are required. To our best knowledge, no work has been conducted to discuss the toxicity of water resources by combining toxicity assays with FT-MS analysis.

The results of EfOM characterization by using FT-MS enabled us to formulate a hypothesis that the different chemical composition in AS and S-MBR_B effluents caused the different chlorination-driven toxicity.

The lower O/C ratio of S-MBR_B effluent suggests that larger number of molecules were detoxified, which consequently decreased the cytotoxicity of S-MBR_B effluent. In addition, the results of gene expression analysis showed that “Response to hormone stimulus” and “Acute inflammatory response” were suppressed in both effluents by chlorination (**Fig. 5 and 6**). Those results suggested that molecules which have lower O/C ratio and were decreased by chlorination might influence on “Response to hormone stimulus” and “Acute inflammatory response” because alteration of those functions was

379 suppressed by chlorination. On the other hand, the detoxification of AS effluent by
380 chlorination might not be sufficient because of higher O/C ratio. DBPs and other high
381 O/C molecules were increased by chlorination and influenced on “Cellular metabolic
382 process”. This might cause the elevation of cytotoxicity. These results suggested that the
383 treatment system and operational condition are important factors affecting toxicity
384 induced by chlorination. Further studies are definitely required to confirm this
385 speculation.

386 Although our approach has great potential, the following limitations must be noticed.
387 First, SPE cannot recover some contaminants such as inorganic and highly hydrophilic
388 matters in wastewater samples. Other concentration methods or direct exposure need to
389 be considered to assess whole effluent impacts. Second, since there is still a large gap
390 between gene expression responses and the actual toxicity, gene alternations are
391 required to relate to the actual cell damages. Third, the size of target molecules for the
392 FT-MS analysis in this study was within the mass range 100–1000 m/z , because most of
393 the ionized molecules were in the range of 150-500 m/z (**Fig. S7**). However, it cannot be
394 ignored the contribution of high molecular weight organic matter to the change in
395 cytotoxicity by chlorination, suggesting the necessity of further studies. Forth, it is not
396 clear whether same correlation between EfOM characterization and toxicity results

would be true in other wastewater effluents or with other human cell lines.

4. Conclusions

- Combination of the traditional bioassays, DNA microarray-based transcriptome analysis and FT-MS analysis is a powerful tool to investigate the toxicity of whole chlorinated effluent.

- The more DBPs were formed in the AS effluent than in the S-MBR_B effluent, resulting in the increase in cytotoxicity after chlorination.

- The organic matter of S-MBR_B effluent had the lower O/C ratio after chlorination, suggesting that a large number of organic molecules were detoxified by chlorination, which consequently decreased the cytotoxicity of S-MBR_B effluent.

Since the toxicity of chlorinated wastewater effluent was dependent on the characteristics of EfOM, namely wastewater treatment systems, the better design and operation of wastewater treatment systems need to be chosen for wastewater reuse practices. Furthermore, the potential human health risks of the reclaimed wastewater must be quantitatively evaluated in the future.

415

416 **Acknowledgements**

417 The authors would like to thank the Construction Bureau of the city of Sapporo
418 making the sampling site accessible, as well as Kenzo Kudo for his supports during the
419 sampling, Rie Nomachi for the maintenance of cell lines. This research was financially
420 supported by Core Research of Evolutional Science & Technology (CREST) for
421 “Innovative Technology and System for Sustainable Water Use” from the Japan Science
422 and Technology Agency (JST).

423

424 **References**

425 Asano, T., Burton, L. F., Leverenz, L. H., Ryujiro, T., Tchobanoglous, G., 2007. Water
426 Reuse: Issues, Technologies and Applications. Metcalf and Eddy, New York, USA,
427 Barker, D.J., Stuckey, D.C., 1999. A review of soluble microbial products (SMP) in
428 wastewater treatment systems. Water Research 33 (14), 3063–3082.
429 Bayo, J., Angosto, J.M., Gómez-López, M.D., 2009. Ecotoxicological screening of
430 reclaimed disinfected wastewater by *Vibrio fischeri* bioassay after a chlorination-
431 dechlorination process. Journal of Hazardous Materials 172 (1), 166–171.

432 Buschini, A., Carboni, P., Frigerio, S., Furlini, M., Marabini, L., Monarca, S., Poli, P.,
 433 Radice, S., Rossi, C., 2004. Genotoxicity and cytotoxicity assessment in lake
 434 drinking water produced in a treatment plant. *Mutagenesis* 19 (5), 341–347.

435 Butler, D., 1993. The influence of dwelling occupancy and day of the week on domestic
 436 appliance wastewater discharges. *Building and Environment* 28 (1), 73–79.

437 Canales, R.D., Luo, Y., Willey, J.C., Austermiller, B., Barbacioru, C.C., Boysen, C.,
 438 Hunkapiller, K., Jensen, R. V, Knight, C.R., Lee, K.Y., Ma, Y., Maqsodi, B.,
 439 Papallo, A., Peters, E.H., Poulter, K., Ruppel, P.L., Samaha, R.R., Shi, L., Yang,
 440 W., Zhang, L., Goodsaid, F.M., 2006. Evaluation of DNA microarray results with
 441 quantitative gene expression platforms. *Nature Biotechnology* 24 (9), 1115–1122.

442 Cao, J., Liu, Y., Jia, L., Jiang, L.-P., Geng, C.-Y., Yao, X.-F., Kong, Y., Jiang, B.-N.,
 443 Zhong, L.-F., 2008. Curcumin Attenuates Acrylamide-Induced Cytotoxicity and
 444 Genotoxicity in HepG2 Cells by ROS Scavenging. *Journal of Agricultural and*
 445 *Food Chemistry* 56, 12059–12063.

446 Cao, N., Yang, M., Zhang, Y., Hu, J., Ike, M., Hirotsuji, J., Matsui, H., Inoue, D., Sei,
 447 K., 2009. Evaluation of wastewater reclamation technologies based on in vitro and
 448 in vivo bioassays. *The Science of the Total Environment* 407 (5), 1588–1597.

449 Chang, H., Chen, C., Wang, G., 2013. Characteristics of C-, N-DBPs formation from
 450 nitrogen-enriched dissolved organic matter in raw water and treated wastewater
 451 effluent. *Water Research* 47 (8). 2729-2741.

452 Dennis, G., Sherman, B.T., Hosack, D. a, Yang, J., Gao, W., Lane, H.C., Lempicki, R. a,
 453 2003. DAVID: Database for Annotation, Visualization, and Integrated Discovery.
 454 *Genome Biology* 4 (5), P3.

455 Freuze, I., Brosillon, S., Herman, D., Laplanche, A., Démocrate, C., Cavard, J., 2004.
 456 Odorous products of the chlorination of phenylalanine in water: formation,
 457 evolution, and quantification. *Environmental Science & Technology* 38 (15),
 458 4134–4139.

459 Guzzella, L., Monarca, S., Zani, C., Feretti, D., Zerbini, I., Buschini, A., Poli, P., Rossi,
 460 C., Richardson, S.D., 2004. In vitro potential genotoxic effects of surface drinking
 461 water treated with chlorine and alternative disinfectants. *Mutation Research* 564
 462 (2), 179–193.

463 Hara-Yamamura, H., Nakashima, K., Hoque, A., Miyoshi, T., Kimura, K., Watanabe,
 464 Y., Okabe, S., 2013. Evaluation of Whole Wastewater Effluent Impacts on HepG2
 465 using DNA microarray-based Transcriptome Analysis. *Environmental Science &*
 466 *Technology* 47 (10), 5425-5432.

467 Huang, Z., Ong, S.L., Ng, H.Y., 2011. Submerged anaerobic membrane bioreactor for
 468 low-strength wastewater treatment: effect of HRT and SRT on treatment
 469 performance and membrane fouling. *Water Research* 45 (2), 705–713.

470 Inaba, K., Shiraishi, H., Soma, Y., 1998. Source and material balance of total organic
 471 halogen pollution in the Ayase River system, Japan. *Toxicological &*
 472 *Environmental Chemistry* 67 (3-4), 391–407.

473 Itoh, S., Gordon, B.A., Callan, P., Bartram, J., 2011. Regulations and perspectives on
 474 disinfection by-products: importance of estimating overall toxicity. *Journal of*
 475 *Water Supply* 60 (5), 261–274.

476 Jiang, L., Cao, J., An, Y., Geng, C., Qu, S., Jiang, L., Zhong, L., 2007. Genotoxicity of
 477 acrylamide in human hepatoma G2 (HepG2) cells. *Toxicology In Vitro* 21 (8),
 478 1486–1492.

479 Kawata, K., Osawa, M., Okabe, S., 2009a. In vitro toxicity of silver nanoparticles at
 480 noncytotoxic doses to HepG2 human hepatoma cells. *Environmental Science &*
 481 *Technology* 43 (15), 6046–6051.

482 Kawata, K., Shimazaki, R., Okabe, S., 2009b. Comparison of gene expression profiles
 483 in HepG2 cells exposed to arsenic, cadmium, nickel, and three model carcinogens

484 for investigating the mechanisms of metal carcinogenesis. *Environmental and*
 485 *Molecular Mutagenesis* 50 (1), 46–59.

486 Kawata, K., Yokoo, H., Shimazaki, R., Okabe, S., 2007. Classification of heavy-metal
 487 toxicity by human DNA microarray analysis. *Environmental Science &*
 488 *Technology* 41 (10), 3769–3774.

489 Kim, S., Kramer, R.W., Hatcher, P.G., 2003. Graphical Method for Analysis of
 490 Ultrahigh-Resolution Broadband Mass Spectra of Natural Organic Matter, the Van
 491 Krevelen Diagram. *Analytical Chemistry* 75 (20), 5336–5344.

492 Kind, T., Fiehn, O., 2007. Seven Golden Rules for heuristic filtering of molecular
 493 formulas obtained by accurate mass spectrometry. *BMC Bioinformatics* 8, 105.

494 Krasner, S.W., Westerhoff, P., Chen, B., Rittmann, B.E., Nam, S.-N., Amy, G., 2009.
 495 Impact of Wastewater Treatment Processes on Organic Carbon, Organic Nitrogen,
 496 and DBP Precursors in Effluent Organic Matter. *Environmental Science &*
 497 *Technology* 43 (8), 2911–2918.

498 Krauss, M., Singer, H., Hollender, J., 2010. LC-high resolution MS in environmental
 499 analysis: from target screening to the identification of unknowns. *Analytical and*
 500 *Bioanalytical Chemistry* 397 (3), 943–951.

501 Van Krevelen, D., 1950. Graphical-statistical method for the study of structure and
 502 reaction process of coal. *Fuel* 29, 269–284.

503 Lavonen, E.E., Gonsior, M., Tranvik, L.J., Schmitt-Kopplin, P., Köhler, S.J., 2013.
 504 Selective Chlorination of Natural Organic Matter: Identification of Previously
 505 Unknown Disinfection Byproducts. *Environmental Science & Technology* 47 (5),
 506 2264-2271.

507 Leenheer, J.A., Croué, J.-P., 2003. Peer Reviewed: Characterizing Aquatic Dissolved
 508 Organic Matter. *Environmental Science & Technology* 37 (1), 18–26.

509 Leu, S.-Y., Chan, L., Stenstrom, M.K., 2012. Toward Long Solids Retention Time of
 510 Activated Sludge Processes: Benefits in Energy Saving, Effluent Quality, and
 511 Stability. *Water Environment Research* 84 (1), 42–53.

512 Macova, M., Escher, B.I., Reungoat, J., Carswell, S., Chue, K.L., Keller, J., Mueller,
 513 J.F., 2010. Monitoring the biological activity of micropollutants during advanced
 514 wastewater treatment with ozonation and activated carbon filtration. *Water*
 515 *Research* 44 (2), 477–492.

516 Macova, M., Toze, S., Hodggers, L., Mueller, J.F., Bartkow, M., Escher, B.I., 2011.
 517 Bioanalytical tools for the evaluation of organic micropollutants during sewage

518 treatment, water recycling and drinking water generation. *Water Research* 45 (14),
519 4238–4247.

520 Maffei, F., Carbone, F., Forti, G.C., Buschini, a, Poli, P., Rossi, C., Marabini, L., Radice,
521 S., Chiesara, E., Hrelia, P., 2009. Drinking water quality: an in vitro approach for
522 the assessment of cytotoxic and genotoxic load in water sampled along distribution
523 system. *Environment International* 35 (7), 1053–1061.

524 Marabini, L., Frigerio, S., Chiesara, E., Radice, S., 2006. Toxicity evaluation of surface
525 water treated with different disinfectants in HepG2 cells. *Water Research* 40 (2),
526 267–272.

527 Mesfioui, R., Love, N.G., Bronk, D. a, Mulholland, M.R., Hatcher, P.G., 2012.
528 Reactivity and chemical characterization of effluent organic nitrogen from
529 wastewater treatment plants determined by Fourier transform ion cyclotron
530 resonance mass spectrometry. *Water Research* 46 (3), 622–634.

531 Remucal, C.K., Cory, R.M., Sander, M., McNeill, K., 2012. Low molecular weight
532 components in an aquatic humic substance as characterized by membrane dialysis
533 and orbitrap mass spectrometry. *Environmental Science & Technology* 46 (17),
534 9350–9359.

535 Richardson, S.D., Plewa, M.J., Wagner, E.D., Schoeny, R., Demarini, D.M., 2007.
 536 Occurrence, genotoxicity, and carcinogenicity of regulated and emerging
 537 disinfection by-products in drinking water: a review and roadmap for research.
 538 Mutation Research 636 (1-3), 178–242.

539 Shi, Y., Cao, X., Tang, F., Du, H., Wang, Y., Qiu, X., Yu, H., Lu, B., 2009. In vitro
 540 toxicity of surface water disinfected by different sequential treatments. Water
 541 Research 43 (1), 218–228.

542 Sleighter, R.L., Hatcher, P.G., 2008. Molecular characterization of dissolved organic
 543 matter (DOM) along a river to ocean transect of the lower Chesapeake Bay by
 544 ultrahigh resolution electrospray ionization Fourier transform ion cyclotron
 545 resonance mass spectrometry. Marine Chemistry 110 (3-4), 140–152.

546 Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, a, Kobayashi, H.,
 547 Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay:
 548 guidelines for in vitro and in vivo genetic toxicology testing. Environmental and
 549 Molecular Mutagenesis 35 (3), 206–221.

550 Wang, H., Joseph, J., 1999. Quantifying cellular oxidative stress by dichlorofluorescein
 551 assay using microplate reader¹. Free Radical Biology and Medicine 27 (99), 612–
 552 616.

- Wang, L.-S., Wei, D.-B., Wei, J., Hu, H.-Y., 2007. Screening and estimating of toxicity formation with photobacterium bioassay during chlorine disinfection of wastewater. *Journal of Hazardous Materials* 141 (1), 289–294.
- Watson, K., Shaw, G., Leusch, F.D.L., Knight, N.L., 2012. Chlorine disinfection by-products in wastewater effluent: Bioassay-based assessment of toxicological impact. *Water Research* 46 (18), 6069–6083.
- Zhang, H., Zhang, Y., Shi, Q., Hu, J., Chu, M., Yu, J., Yang, M., 2012a. Study on transformation of natural organic matter in source water during chlorination and its chlorinated products using ultrahigh resolution mass spectrometry. *Environmental Science & Technology* 46 (8), 4396–4402.
- Zhang, H., Zhang, Y., Shi, Q., Ren, S., Yu, J., Ji, F., Luo, W., Yang, M., 2012b. Characterization of low molecular weight dissolved natural organic matter along the treatment trait of a waterworks using Fourier transform ion cyclotron resonance mass spectrometry. *Water Research* 46 (16), 5197–5204.
- Zou, J., Chen, Q., Tang, S., Jin, X., Chen, K., Zhang, T., Xiao, X., 2009. Oloquinoxindox-induced genotoxicity and oxidative DNA damage in human hepatoma G2 (HepG2) cells. *Mutation Research* 676 (1-2), 27–33.

List of Figures

Fig. 1 - Cytotoxicity of AS effluents determined by MTT (A) and NRU (B) assays. The effluents were collected on 2012.05.15, 05.22 and 06.01, chlorinated by 0, 2 or 5 mg/L of sodium hypochlorite, and then concentrated by solid phase extraction (SPE). The SPE fraction from pure water was used as control. The cell viability of control was defined as 100%. Data represent mean $\pm$ SD of six wells of a cell culture plate. Asterisks (*) indicate a statistically significant difference compared to non-chlorinated effluent for each concentrated sample ($p < 0.05$).

Fig. 2 - Cytotoxicity of S-MBR_B effluents determined by MTT (A) and NRU (B) assays. The effluents were collected on 2012.02.15, 03.12 and 04.26, chlorinated by 0, 2 or 5 mg/L of sodium hypochlorite, and then concentrated by solid phase extraction (SPE). The SPE fraction from pure water was used as control. The cell viability of control was defined as 100%. Data represent mean $\pm$ SD of six wells of a cell culture plate. Asterisks (*) indicate a statistically significant difference compared to non-chlorinated effluent for each concentrated sample ($p < 0.05$).

Fig. 3 - Numbers of altered genes after exposure to AS and S-MBR_B effluents. AS and S-MBR_B effluents was taken on 2012.06.01 and 03.12, respectively. Effluents were chlorinated by 0, 2 or 5 mg/L of sodium hypochlorite, and then concentrated 25 times by solid phase extraction (SPE). The genes with $p < 0.05$ and ≥ 2.0 fold changes in either direction were identified as being significantly altered.

Fig. 4 - Numbers of commonly altered gene of each effluent which treated with different chlorine concentrations.

Fig. 5 - The major biological functions and their p values (significance of alteration) (S-MBR_B).

Fig. 6 - The major biological functions and their p values (significance of alteration) (AS).

Fig. 7 - Elemental composition of disinfection by-products (DBPs) formed in AS and S-MBR_B effluents chlorinated by 5 mg/L of sodium hypochlorite. The formulas whose ratios of peak intensity were increased by chlorination were identified as DBPs.

608

609

List of Tables

610

611 **Table 1** - Summary of FT-MS data: the numbers of molecular formula whose ratio of
612 peak intensities were significantly ($p < 0.05$) decreased, increased and not
613 changed by chlorination, and their averages of O/C and H/C ratios of AS and S-
614 MBR_B effluents. The formulas whose ratios of peak intensity were increased by
615 chlorination were identified as DBPs.

616

Table 1

1 **Table 1** - Summary of FT-MS data: the numbers of molecular formula whose ratio of peak intensities were significantly ($p < 0.05$)

2 decreased, increased and not changed by chlorination, and their averages of O/C and H/C ratios of AS and S-MBR_B effluents. The

3 formulas whose ratios of peak intensity were increased by chlorination were identified as DBPs.

	AS				S-MBR _B			
	Increased	Decreased	Not changed	Total	Increased	Decreased	Not changed	Total
Number of formulas	77(11.7%)	470(71.5%)	110 (16.7%)	657	30(5.6%)	443(83.1%)	60 (11.2%)	533
H/C	1.355	1.333	1.497	1.363	1.081	1.351	1.266	1.327
O/C	0.355	0.284	0.311	0.297	0.337	0.239	0.313	0.253

4

1

2

3

4

5

6

7

8

9

10

11

Fig. 1 - Cytotoxicity of AS effluents determined by MTT (A) and NRU (B) assays. The effluents were collected on 2012.05.15, 05.22 and 06.01, chlorinated by 0, 2 or 5 mg/L of sodium hypochlorite, and then concentrated by solid phase extraction (SPE). The SPE fraction from pure water was used as control. The cell viability of control was defined as 100%. Data represent mean $\pm$ SD of six wells of a cell culture plate. Asterisks (*) indicate a statistically significant difference compared to non-chlorinated effluent for each concentrated sample ($p < 0.05$).

1

2

3

4

5

6

7

8

9

10

11

Fig. 2 - Cytotoxicity of S-MBR_B effluents determined by MTT (A) and NRU (B) assays. The effluents were collected on 2012.02.15, 03.12 and 04.26, chlorinated by 0, 2 or 5 mg/L of sodium hypochlorite, and then concentrated by solid phase extraction (SPE). The SPE fraction from pure water was used as control. The cell viability of control was defined as 100%. Data represent mean $\pm$ SD of six wells of a cell culture plate. Asterisks (*) indicate a statistically significant difference compared to non-chlorinated effluent for each concentrated sample ($p < 0.05$).

1

2

3

4

5

6

7

8

9

Fig. 3 - Numbers of altered genes after exposure to AS and S-MBR_B effluents. AS and S-MBR_B effluents was taken on 2012.06.01 and 03.12, respectively. Effluents were chlorinated by 0, 2 or 5 mg/L of sodium hypochlorite, and then concentrated 25 times by solid phase extraction (SPE). The genes with $p < 0.05$ and ≥ 2.0 fold changes in either direction were identified as being significantly altered.

1
2
3
4
5
6
7
8

Fig. 4 - Numbers of commonly altered gene of each effluent which treated with different chlorine concentrations.

Figure 5

※1: response to wounding also relates to “Acute inflammatory response”.

Fig. 5 - The major biological functions and their p values (significance of alteration) (S-MBR_B).

Figure 6

Fig. 6 - The major biological functions and their p values (significance of alteration) (AS).

Figure 7

1

2 **Fig. 7** - Elemental composition of disinfection by-products (DBPs) formed in AS and S-
3 MBR_B effluents chlorinated by 5 mg/L of sodium hypochlorite. The formulas whose
4 ratios of peak intensity were increased by chlorination were identified as DBPs.

1 Graphical abstract

2