Reevaluation of the toxicity of chlorinated water and the usefulness of MX as an index
Sadahiko Itoh, Atsushi Nakano and Toshiaki Araki

ABSTRACT
Changes in the toxicity in chlorinated water after chlorine addition were examined. For toxicity evaluation, the chromosomal aberration test and the transformation test were conducted as indexes of initiation activity and promotion activity, respectively, in the carcinogenesis process. Activity inducing chromosomal aberrations in chlorinated Lake Biwa water gradually decreased over time after chlorination. In contrast, activity inducing transformations determined by the two-stage assay gradually increased. Thus, toxicity that decreases or increases is present in chlorinated water. Furthermore, activity inducing transformations determined by the non-two-stage assay gradually decreased over time. This direction of change is opposite to that of activity inducing transformations determined by the two-stage assay and is consistent with that of activity inducing chromosomal aberrations. The drastic decrease in initiation activity detected as activity inducing chromosomal aberrations could be the main cause for the decrease in activity inducing transformations determined by the non-two-stage assay (an index of the sum of initiation and promotion activity). MX change was quantitatively consistent with those of activity inducing chromosomal aberrations and transformations determined by the non-two-stage assay. On the other hand, directions of changes in concentrations of typical by-products such as chloroform were consistent only with that of activity inducing transformations determined by the two-stage assay. Findings of this study suggest that MX is appropriate as an index for comparing the carcinogenicity of tap water near and far from a water purification plant.

Key words | chlorination, chromosomal aberration test, disinfection by-products, MX, transformation test

INTRODUCTION
In general, concentrations of trihalomethanes and haloacetic acids in chlorinated drinking water increase in water distribution systems. This strongly suggests that the toxicity of disinfected water is not stable and changeable. From this viewpoint, the change in the toxicity of chlorinated humic acid after chlorine injection has been examined by authors (Itoh et al. 2001). For the measurement of toxicity, in vitro bioassays as indexes in the carcinogenesis process were carried out. A chromosomal aberration test using Chinese hamster lung cells was carried out as an index to initiation activity, and a transformation test using mouse fibroblast cells was carried out as an index to promotion activity.

As a result, it was found that activity inducing chromosomal aberrations in chlorinated humic acid gradually decreased over time after chlorination. In contrast, activity inducing transformations gradually increased. Thus, toxicity that decreases or increases is present in chlorinated water. Since typical by-products such as trihalomethanes and haloacetic acids increase after chlorination, it is widely believed that the toxicity of drinking water also increases.
We have pointed out, however, that it might be early to conclude that the toxicity of drinking water increases in water distribution systems.

In this study, MX (3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone) known as a strong mutagen is focused on in addition to typical chlorination by-products. MX has received much attention because of the strong genotoxic activity. After Komulainen et al. (1997) reported the carcinogenic potency of MX in rat, the necessity of the water quality management of MX has been discussed (Melnick et al. 1997; Hirose et al. 1999).

In addition, it has been found that MX is unstable in water and reacts with residual chlorine (Meier et al. 1987; Kinae et al. 1992). This means that a concentration of MX might decrease after it is formed by chlorine. This direction of change is in reverse to those of concentrations of trihalomethanes and haloacetic acids. A problem is which of these carcinogenic by-products is appropriate as an index of the change of the toxicity of chlorinated water. In the previous study (Itoh et al. 2001), experiments were conducted using commercial humic acid as the first step. Natural water was used in this study in order to propose an appropriate index for comparing the toxicity of chlorinated drinking water in distribution systems.

MATERIALS AND METHODS

Chlorination and concentration of Lake Biwa water

Lake Biwa is the largest lake in Japan and also the major water source for 14 million people in Kansai area in Japan. The surface water of Lake Biwa was used in this study. Lake Biwa water filtered with a 1.0 μm membrane filter of which DOC (dissolved organic carbon) was 1.9 mg l⁻¹ was chlorinated at an initial concentration of 2.0 mg l⁻¹. Sodium hypochlorite stock solution (Wako Pure Chemical Industries, Ltd.) was used for chlorination. Available chlorine in the stock solution was analyzed by the DPD ferrous titrimetric method (APHA/AWWA/WEF 1998) just prior to use. The chlorination proceeded at 20°C in a dark room. The pH was not adjusted, however, it was maintained at 7.4 to 7.8 during the chlorination. Residual chlorine was detected during the chlorination, however, dechlorination was not carried out so as not to change the activity inducing chromosomal aberrations and transformations in chlorinated waters. It was confirmed that the trace residual chlorine had no influence on bioassays.

Organic matters in a sample water were concentrated by adsorption and desorption method using Sep-Pak Plus (Long) CSP800 (Nihon Waters K.K.) resin according to the procedure described by Urano et al. (1994). 20 l of a sample water adjusted at pH 2 was fed to CSP800 cartridges at a flow rate of 50 ml min⁻¹. The adsorbed substances were desorbed by DMSO (dimethylsulfoxide). The final volume of DMSO was 2 ml, and the concentration factor was 10000 times.

Chromosomal aberration test

Chinese hamster lung cell (CHL/1U) was obtained from Dainihon Pharmaceutical Co., Ltd. Cells were cultured with Eagle's MEM (Nissui Pharmaceutical Co., Ltd) supplemented with 10% fetal bovine serum (Gibco Oriental Co., Ltd). CHL culture was grown in 12 ml media, in a glass silicon-capped bottle. 0.06 ml of a concentrated solution in DMSO was added to a 1-day-old culture. As a result, a concentration of DMSO in the media was 0.5%. Bacteria in a sample were eliminated by a 0.22 μm filter. Only activity inducing chromosomal aberrations without activation was measured in this study. Chromosome preparation was made after 24 hours culturing of the addition (Sofuni 1999).

The previously developed image analysis was used to objectively evaluate results of chromosomal aberration test (Itoh et al. 1992). The shapes of chromosomes were analyzed by an image analyzer (Image-Pro Plus Ver4.0). Chromosomal aberrations can be divided into broken-type and exchanged-type. Exchanged-type aberrations are detected by the developed method. 50 metaphases in a specimen were analyzed. As a CHL cell has 25 chromosomes, 1250 of chromosomes were analyzed by each specimen. Image analysis of negative control gave a mean of 4.5 chromosomes/50 metaphases and a standard deviation of 2.6. Activity inducing chromosomal aberrations is expressed as a mean value of test results of triplicate specimens.

When activity inducing chromosomal aberrations of certain chemical has to be judged, and when a test result has
to be compared with data obtained by other laboratories, the standard method (Sofuni 1999) should be used and the method developed by ourselves cannot be used. The developed method is effective in order to compare the relative intensity of activity inducing chromosomal aberrations only in this study.

**Transformation test**

The in vitro cell transformation assay utilizing BALB/3T3 A31-1-1 cells (Japan Health Sciences Foundation) was carried out as an indicator of tumor-promotion activity. The standard method of transformation test using BALB/3T3 cells had been established by IARC/NCI/EPA Working Group (1985), however, it has been improved by Tsuchiya and Umeda (1995) to enhance transformation frequency and shorten the culture period. Culture conditions of the assay in this study followed the procedure described by Tsuchiya and Umeda (1995). 3-methylcholanthrene (3-MC) was used to induce DNA lesions in the cells as the initiation step. The cells were treated with 0.5 mg l\(^{-1}\) of 3-MC for two days. After the initiation, 0.015 ml of a concentrated solution was added to the cells cultured in 6 ml media. As a result, a concentration of DMSO in the media was 0.25%. This testing method is called the two-stage transformation assay, since a chemical or a sample is given to the cells in two steps. Only activity inducing transformations without activation was measured.

In contrast, in the case of the non-two-stage transformation test, a sample is added from the beginning without adding 3-MC. The assay is completed by the continuing addition of a sample. This testing method is useful for measuring the toxicity including genotoxicity of a sample.

The image analysis was also used for detecting transformed foci (Sumitomo et al. 1998). Since this method is not the standard method of transformation test, it is effective to compare the relative intensity of activity inducing transformations only in this study. More than 100 colonies were analyzed by each specimen. Image analysis of negative control cells initiated with 3-MC gave a mean of 3.2% transformation foci and a standard deviation of 0.5%. Image analysis of negative control cells without 3-MC in the non-two-stage transformation assay gave a mean of 1.1% transformation foci and a standard deviation of 0.2%.

Transformation efficiency is expressed as a mean value of test results of triplicate specimens.

**Assays of chemicals**

Chromosomal aberration tests and transformation tests of some chemicals including chlorination by-products were carried out. 14 chemicals that are suspected to have initiation and/or tumor-promotion activity were selected from review papers (Ishidate et al. 1988; Sakai et al. 1993; Budunova & Williams 1994; Sofuni 1999). When ethyl alcohol and DMSO were used to dissolve a chemical, the final concentrations of ethyl alcohol and DMSO are needed less than 1% and 0.5%, respectively, in the media. It has been confirmed that ethyl alcohol and DMSO do not influence the chromosomal aberration test up to concentrations of 1% and 0.5%, respectively (Sofuni 1999). No influence was confirmed in the transformation test either.

**Analytical procedures**

Chloroform was extracted with hexane, and the concentration was determined by a gas chromatograph with an electron capture detector (GC-ECD, Shimadzu GC-14B) using a 2 m × 2.6 mm i.d. column packed with silicone GE SE-30 on Chromosorb W AW-DMCS 80/100 mesh. The usual operating conditions were as follows. The injector temperature was 150°C. The column oven temperature was initially held at 70°C for 3 min, ramped to 145°C at 15°C min\(^{-1}\), and held at 145°C for 2 min. The detector temperature was 200°C. Haloacetic acids were methylated with diazomethane and analyzed by a GC-ECD. TOX (total organic halides) was measured by a TOX-10\(^{\text{TM}}\) analyzer (Mitsubishi Chemical Corporation). TOC (total organic carbon) was measured by a TOC-5000A analyzer (Shimadzu).

**Measurement of MX**

20 l of a sample water adjusted at pH 2 was fed to CSP800 cartridges at a flow rate of 30 ml min\(^{-1}\). The adsorbed substances were desorbed by 15 ml of ethyl acetate at a flow rate of 0.3 ml min\(^{-1}\). The eluate was concentrated to 200 µl after the addition of mucobromic acid (MBA) as the
internal standard. 100 μl of BSTFA + 1% TMCS (N,O-bis(Trimethylsilyl)trifluoroacetamide with 1% Trimethylchlorosilane, PIERCE) was added to derivatize MX in the residue. This solution was concentrated to 200 μl for the analysis by a gas chromatograph (Agilent, 6890plus) with a mass spectrometer (JOEL, JMS-AX505). 40 μl of the sample was injected by multiple injection (2 μl × 20) with the programmed temperature vaporizing (PTV) mode. The gas chromatography column was a DB-5 capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific). The injector temperature was 78°C (10.5 min) → 720°C min⁻¹ 200°C (21 min), and the column oven temperature was 80°C (10.5 min) → 6°C min⁻¹ → 200°C (0 min) → 25°C min⁻¹ → 250°C (2 min). The m/z = 135, 273, 275 ions were selected for TMS-MX and the m/z = 313, 315, 317 ions for TMS-MBA analyses.

RESULTS AND DISCUSSION

Result of bioassays of chlorinated Lake Biwa water

Figure 1 shows the result of bioassays of Lake Biwa water after the addition of chlorine. The residual chlorine after four days was 0.8 mg l⁻¹, and pH was maintained at 7.4 to 7.8 during the chlorination. Activity inducing chromosomal aberrations of Lake Biwa water was produced by chlorine, however, it was unstable and gradually decreased over time after chlorination. In contrast, activity inducing transformations measured by the two-stage assay gradually increased. Thus, the toxicity that decreases or increases is present in chlorinated water.

In addition to these two kinds of assays, the non-two-stage transformation test, which is useful for measuring the whole toxicity including genotoxicity of a sample, was carried out. Figure 1 shows activity inducing transformations measured by the non-two-stage assay gradually decreased. This direction of change was reverse to that of activity inducing transformations by the two-stage assay and consistent with that of activity inducing chromosomal aberrations. The non-two-stage transformation test can detect the toxicity including initiation and promotion step. It is important to note that the index of the sum of initiation and promotion activity gradually decreases. These results are qualitatively consistent with those in the case of commercial humic acid conducted by authors (Itoh et al. 2001).

Comparison with activity of some chemicals

Figure 2 shows activity inducing chromosomal aberrations and transformations measured by the two-stage assay of 15
tested chemicals including MX. The horizontal axis shows activity inducing chromosomal aberrations expressed as (chromosomes/50 cells)/(mg l\(^{-1}\)), and the vertical axis shows activity inducing transformations expressed as (mg l\(^{-1}\))\(^{-1}\). mg l\(^{-1}\) in these units means the weight per liter of the 15 tested chemicals in the culture media and TOC of Lake Biwa water before chlorination.

The change in activity inducing chromosomal aberrations and transformations by the two-stage assay of chlorinated Lake Biwa water shown in Figure 1 is plotted in Figure 2. The toxicity changed in the direction of the arrow. Firstly, it was found that activity inducing chromosomal aberrations of chlorinated water is relatively stronger than activity inducing transformations, when the plotting position of initial chlorinated water (the initial point of the arrow) is compared with those of other chemicals. Secondly, activity inducing chromosomal aberrations decreases sharply, while activity inducing transformations increases slightly.

Since the chromosomal aberration test and transformation test are carried out as indexes to initiation activity and promotion activity, respectively, it appears that initiation activity in chlorinated water is stronger than promotion activity. It also seems that initiation activity of chlorinated water decreases sharply and promotion activity increases slightly. It is assumed that the decrease of activity inducing transformations measured by the non-two-stage assay shown in Figure 1 can be attributed to this reason.

This phenomenon is illustrated in Figure 3. The increasing toxicity (promotion activity) is present in chlorinated water, however, initiation activity drastically decreases. Since the toxicity of water is measured by \textit{in vitro} assays in this study, it is not possible to get a conclusion on the change of toxicity on the human body. However, it should be noted that the whole toxicity associated with carcinogenic activity can be mainly attributed to initiation activity and presumably decreases over time after chlorination.

**Changes of typical by-products and MX**

Figure 4 shows changes in concentrations of chlorination by-products and TOX. All by-products and TOX increased after chlorine injection. These directions of changes of typical by-products are consistent only with that of activity inducing transformations by the two-stage assay and reverse to those of activity inducing chromosomal aberrations and transformations by the non-two-stage assay. By-products shown in Figure 4 are widely measured, however, they would not be appropriate as indexes to compare the toxicity of chlorinated drinking water in distribution systems.

In contrast, Figure 5 shows the change in concentration of MX. It was found that MX decreases over time after it is formed by chlorine. This decrease could be attributed to hydrolysis and the reaction of MX with residual chlorine (Meier \textit{et al.} 1987; Kinae \textit{et al.} 1992). Figures 1 and 5 show that the change of MX is qualitatively consistent with those of activity inducing chromosomal aberrations and transformations.

**Figure 3** | Proposed change of the toxicity of chlorinated water.

**Figure 4** | Concentrations of by-products in chlorinated Lake Biwa water.
transformations by the non-two-stage assay. This suggests that MX can be one of the indexes for the toxicity detected by these bioassays.

**Behavior of MX in water**

The stability and the toxicity change of MX in distilled water and chlorine aqueous solution were examined. The MX aqueous solution in a phosphate buffer of 67 mM at pH 7.0 was treated with chlorine. The chlorination proceeded at 20°C in a dark room. Figure 6 shows the change in concentration of MX. It shows MX decreases slightly even in water without chlorine, and the decreasing rate of MX increases with increasing chlorine added to the solution.

**Figure 6** | Change of concentration of MX in water and chlorinated water.

Figure 7 shows the change in activity inducing chromosomal aberrations of MX after chlorine was added to the MX aqueous solution. It shows that activity inducing chromosomal aberrations of MX decreases slightly even in water without chlorine, and it decreases gradually after chlorine is added to the solution. This change is almost correspondent to the change of MX concentration shown in Figure 6. It is reasonable to suppose that activity inducing chromosomal aberrations of MX decreases while MX is decomposed by reacting with residual chlorine.

**The usage of MX as an index**

To evaluate the usage of MX as an index, reaction rates of changes in the toxicity and the concentration of MX are determined. Decreasing rate constant $k$ for MX is given by assuming first-order reaction at the initial stage after reaching the maximum.

$$\frac{dC}{dt} = -kC \quad (1)$$

where $C$ is concentration of MX (ng l$^{-1}$), $t$ is reaction time (day), and $k$ is decreasing rate constant (day$^{-1}$). Decreasing rate of MX was taken as the slope of the decrease in Figure 5, and calculated constant $k$ was 0.19 day$^{-1}$. Subsequently, decreasing rate constants were calculated with Figure 1, by replacing $C$ in the equation (1) with results of bioassays. Obtained observed rate constants of activity

**Figure 7** | Change of activity inducing chromosomal aberrations of MX in water and chlorinated water.
inducing chromosomal aberrations and transformations by the non-two-stage assay were 0.14 day$^{-1}$ and 0.18 day$^{-1}$, respectively. It is apparent that these rate constants are similar to the constant for MX. Consequently, the change of MX was quantitatively consistent with those of activity inducing chromosomal aberrations and transformations by the non-two-stage assay.

Next, the effect of residual chlorine concentration on the reaction rate of MX and the toxicity change was examined in order to clarify an application range of MX as an index. As shown in Figure 6, MX decreases rapidly with a higher concentration of residual chlorine as a result of decomposition by the reaction with chlorine. On the other hand, Itoh et al. (2003) have confirmed that activity inducing chromosomal aberrations of chlorinated water decreases slowly with increasing concentration of residual chlorine. This means that activity inducing chromosomal aberrations decreases mainly by hydrolysis. Thus, mechanisms of changes would be different between MX and activity inducing chromosomal aberrations. Therefore, it is supposed that the change in MX concentration and results of bioassays could be correspondent within a limited range of residual chlorine.

In the following examination, only activity inducing chromosomal aberrations was measured, because on the basis of results obtained by authors (Itoh et al. 2001), it can be assumed that the change in activity inducing chromosomal aberrations would indicate the change in the toxicity of chlorinated water.

Sodium hypochlorite was added to Lake Biwa water filtered with a 1.0$\mu$m membrane filter. Added chlorine was 25 mg l$^{-1}$ and 100 mg l$^{-1}$ as initial concentrations, since this experiment is the addition to the one by which Figure 1 and Figure 5 were obtained. Other conditions in chlorination are described in MATERIALS AND METHODS. After the change in activity inducing chromosomal aberrations and MX concentration in chlorinated waters were measured, decreasing rate constants were calculated. All decreasing rate constants calculated in this study were plotted in Figure 8 as a function of concentration of residual chlorine.

The result of activity inducing chromosomal aberrations shows that the decreasing rate constant is small, that is, the activity is slow to decrease with increasing concentration of residual chlorine. In contrast, the result of MX concentration shows that the decreasing rate constant is large, that is, MX is rapid to decrease with increasing concentration of residual chlorine. This phenomenon suggests that MX is not appropriate as an index in drinking water with a higher concentration of residual chlorine, since the difference in rate constants between activity inducing chromosomal aberrations and MX concentration becomes large with increasing concentration of chlorine.

Under the condition of actual drinking water, however, decreasing rate constants of MX and activity inducing chromosomal aberrations were 0.19 day$^{-1}$ and 0.14 day$^{-1}$, respectively. It seems to be possible to use MX as an index for usual drinking water, since a concentration of residual chlorine is approximately 0.5 mgCl$_2$ l$^{-1}$ in actual tap water.

On the other hand, it has been revealed that pH affects the stability of MX in water. Kinae et al. (1992) and Meier et al. (1987) have shown that MX is unstable under alkaline condition compared to acidic condition. In addition, it was pointed out that there is a discontinuous region where MX is more stable at pH 8 than at pH 6. In contrast, Itoh et al. (1993) have shown that there is not a discontinuous region in the effect of pH on activity inducing chromosomal aberrations and it decreases faster under alkaline condition than under acidic condition. That is, the effect of pH on behaviors of MX and activity inducing chromosomal aberrations suggests the difference in mechanisms of their changes in water.

It follows from what has been described that pH and concentration of residual chlorine have to be limited for
utilizing MX as an index. In this study, it was pointed out that MX would be an appropriate index under the condition of neutral pH and chlorine dosage typically used in practice.

This study demonstrates that MX can be utilized for comparing the toxicity of tap water near and far from a water purification plant. When polluted raw water is chlorinated, however, higher concentrations of trihalomethanes and haloacetic acids are formed. In this sense, these typical by-products are still useful as indexes. The important point would be that indicator by-products have to be selected in view of the purpose of water quality management.

**CONCLUSIONS**

Activity inducing chromosomal aberrations in chlorinated Lake Biwa water gradually decreased over time after chlorination. In contrast, activity inducing transformations determined by the two-stage assay gradually increased. Thus, toxicity that decreases or increases is present in chlorinated water. Furthermore, activity inducing transformations determined by the non-two-stage assay gradually decreased over time. This direction of change is opposite to that of activity inducing transformations determined by the two-stage assay and is consistent with that of activity inducing chromosomal aberrations.

It was found that activity inducing chromosomal aberrations of chlorinated water is much larger than activity inducing transformations. The drastic decrease in initiation activity detected as activity inducing chromosomal aberrations could be the main cause for the decrease in activity inducing transformations determined by the non-two-stage assay (an index of the sum of initiation and promotion activity). An important finding is the toxicity presumably decreases over time after chlorination because of the drastic decrease in initiation activity and the slight increase in promotion activity.

MX change was quantitatively consistent with those of activity inducing chromosomal aberrations and transformations determined by the non-two-stage assay. On the other hand, directions of changes in concentrations of typical by-products such as chloroform were consistent only with that of activity inducing transformations determined by the two-stage assay. Findings of this study suggest that MX is appropriate as an index for comparing the carcinogenicity of tap water near and far from a water purification plant. It was also pointed out that indicator by-products have to be selected in view of the purpose of water quality management.

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**REFERENCES**


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