Genotoxicity testing of samples generated during UV/H_2O_2 treatment of surface water for the production of drinking water using the Ames test *in vitro* and the Comet assay and the SCE test *in vivo*

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ABSTRACT

UV/H_2O_2 treatment can be part of the process converting surface water to drinking water, but would pose a potential problem when resulting in genotoxicity. This study investigates the genotoxicity of samples collected from the water treatment plant Andijk, applying UV/H_2O_2 treatment with an electrical energy dose of 0.54 kWh/m^3 and a H_2O_2 dose of 6 mg/l. Genotoxicity was tested *in vitro* using the Ames and Comet assay. All samples showed negative results. Samples were also tested in *in vivo* genotoxicity tests in Eastern mudminnow fish (*Umbra pygmaea*) by a sister chromatid exchange (SCE) and a Comet assay. No significant increases in SCEs were observed, but gill cells isolated from fish exposed to water obtained immediately after UV/H_2O_2 treatment and to Lake IJsselmeer water showed significantly increased DNA damage in the Comet assay. All other samples tested negative in this Comet assay. This indicates that DNA damaging compounds may result from the UV/H_2O_2 treatment, but also that these can be efficiently eliminated upon granular activated carbon (GAC) treatment of the water. It is concluded that when combined with this subsequent GAC treatment, UV/H_2O_2 treatment for the production of drinking water from surface water is not of concern with respect to genotoxicity.

Key words | Comet assay, drinking water, sister chromatid exchange (SCE), surface water, *Umbra pygmaea*, UV/H_2O_2

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INTRODUCTION

PWN Water Supply Company North Holland (PWN) provides annually 105 million m^3 of drinking water to 1.5 million inhabitants in the province of North Holland in the Netherlands. The primary source of raw water for the production of drinking water is surface water that originates from Lake IJsselmeer which receives water from the River Rhine.

Generally in water treatment for the production of drinking water some type of advanced oxidation process (AOP) is applied as a general barrier for organic micro pollutants (i.e. pesticides, solvents, pharmaceuticals and endocrine disrupting compounds). These AOPs may include for example an ozone or UV/H_2O_2 based treatment. In view of formation of bromate in an ozone based AOP and the related possible adverse health effects (Kurokawa *et al.* 1990; Von Gunten 2003), PWN does not apply an ozone based AOP but has selected a UV/H_2O_2 based AOP in the water treatment plant at Andijk, since 2004. In this UV/H_2O_2 based treatment, PWN applies broad spectrum UV radiation with emission in the range of 200–300 nm (Kruithof *et al.* 2002; Martijn *et al.* 2009), with an electrical energy dose of 0.54 kWh/m^3 and a H_2O_2 dose of 6 mg/l. These process conditions of the UV/H_2O_2 treatment are set to meet 80% reduction in the concentration of atrazine

doi: 10.2166/aqua.2012.069

(as a model compound for organic pollutants). Besides degrading the organic micro pollutants present in the water, AOPs also oxidise fractions of the organic water matrix, produce easily assimilable organic carbon (AOC) and convert nitrate into nitrite. Biological processes in steps following the UV/H_2O_2 treatment, like granular activated carbon (GAC) filtration, reduce the levels of the produced AOC, the formed nitrite and other generated oxidation products.

In water treatment processes, such as AOPs, where compounds are transformed, the formation of genotoxic compounds potentially able to induce DNA damage may occur. The aim of this study is to determine and evaluate to what extent genotoxic compounds are formed during UV/H_2O_2 treatment and, if formed, to establish whether they are removed by the subsequent GAC filtration step. To this end concentrated water samples taken at specific locations in the water treatment plant Andijk during the process of treatment of surface water to generate drinking water were tested in *in vitro* genotoxicity assays with and without metabolic activation. As presented in most guidelines, in which new or existing chemicals are assessed for their genotoxicity hazards (Dearfield *et al.* 2011), an initial battery of *in vitro* genotoxicity assays is selected in this study, which provide information about small-scale genetic damage (e.g. point mutations as in the Ames test) and larger-scale genetic alterations (e.g. chromosomal damage as in the Comet assay). By concentrating the water samples 10,000 times, the initial battery of *in vitro* genotoxicity assays is sufficiently sensitive to detect substances/chemicals at environmentally relevant concentrations in water samples providing information on genotoxicity (Penders & Hoogenboezem 2003).

Additional *in vivo* genotoxicity testing is required to obtain further insight about the genotoxicity of compounds, when a positive result is obtained in the *in vitro* genotoxicity battery. In some guidelines, recommendations are made to use the *in vivo* genotoxicity assay next to *in vitro* genotoxicity in a standard test battery (Anonymous 1997, 2008) or to use *in vivo* genotoxicity also when negative *in vitro* results are obtained (Becks *et al.* 2006). Therefore, in addition to the use of *in vitro* genotoxicity tests, the water samples were also tested in an *in vivo* sister chromatid exchange (SCE) test and an *in vivo* Comet assay, both performed in gill cells obtained from Eastern mudminnow fish (*Umbra pygmaea*) exposed to the respective water samples for 11 days. These *in vivo* genotoxicity assays are sufficiently sensitive to detect substances/chemicals at environmentally relevant concentrations in water samples providing information on genotoxicity (Alink *et al.* 2007; Penders *et al.* 2012). This *in vivo* setup also has the advantage that it does not require a concentration step thus avoiding any potential loss of compounds present, because the fish are directly exposed to water samples *in vivo* and no concentration procedure is required (Alink *et al.* 1980, 2007).

MATERIAL AND METHODS

Site description of PWN drinking water production at Andijk

At the PWN's water treatment plant Andijk, located 60 km north-east from the city of Amsterdam, surface water from Lake IJsselmeer is treated to produce drinking water. The water treatment process consists of conventional pretreatment followed by a UV/H_2O_2 based AOP followed by a subsequent GAC filtration (Figure 1). For the study four samples at critical steps in the treatment process were taken as illustrated in Figure 1.

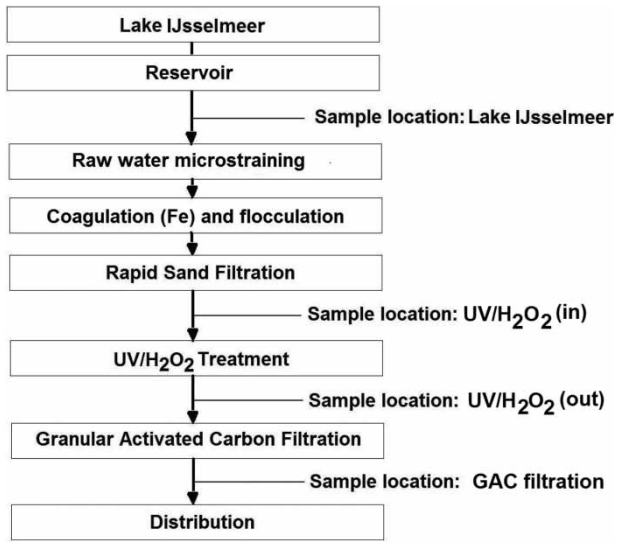

Figure 1 | Water treatment process scheme of water treatment plant Andijk and sample locations.

These four water samples included (1) raw water of lake IJsselmeer (sample Lake IJsselmeer), representing the surface water to be treated, (2) the influent for the UV/H_2O_2 treatment (sample UV/H_2O_2(in)), representing the water before the UV/H_2O_2 treatment but after the first treatment steps including microstraining, coagulation with ferric salts, flocculation and a rapid sand filtration step, (3) the effluent of the UV/H_2O_2 treatment (sample UV/H_2O_2(out)), representing the water immediately after the UV/H_2O_2 treatment and (4) the water of the effluent of the UV/H_2O_2 treatment after a subsequent GAC filtration (sample GAC filtration), also representing the water for distribution as drinking water. During the experiments and sampling activities, regular process conditions were applied. The conventional pretreatment: coagulation with ferric salts (up to 25 mg/l Fe), flocculation and settling in sludge blanket clarifiers followed by rapid sand filtration, removes turbidity and reduces total organic carbon to approximately 3 mg/l. The advanced UV/H_2O_2 based oxidation treatment is a non-selective barrier for organic micropollutants and provides primary disinfection. The UV/H_2O_2 installation is equipped with broad spectrum UV-lamps (emission between 200 and 300 nm). Nitrate absorbs UV in the lower wavelengths (<245 nm) and is converted into nitrite by these UV-lamps. Influent of the UV/H_2O_2 installation contains between 2 and 14 mg/l nitrate that is converted into approximately 200 μg/l nitrite by the UV/H_2O_2 treatment. A fraction of the organic water matrix is converted into easily AOC. Furthermore, after UV/H_2O_2 treatment an excess of H_2O_2 is still in the water (5 mg/l). During post treatment with GAC filtration, the bacterial flora present on the filters catalytically decomposes this surplus H_2O_2, converts nitrite into nitrate and decreases the AOC. The installed GAC empty bed contact time is 30 minutes at maximum capacity.

In vitro genotoxicity tests

For *in vitro* genotoxicity testing, the samples were concentrated using solid phase extraction with XAD-4 (Amberlite) as absorbent and diethyl ether as eluent. Reducing the volume of the collected diethyl ether eluent by evaporation with nitrogen gas at room temperature and adjusting the volume of the sample to 1.0 ml with MilliQ water, resulted in a 10,000 fold concentrated extract of the water sample which was the sample used for further *in vitro* genotoxicity testing. *In vitro* genotoxicity was tested using the plate incorporation Ames test with *Salmonella typhimurium* strain TA98 performed according to Maron & Ames (1983) with minor modifications. To this end the XAD concentrated water samples were mixed with a suspension of *Salmonella typhimurium*, strain TA98 (Johnson & Johnson Pharmaceutical Research and Development, Beerse, Belgium) in phosphate buffer solution or in a metabolic activation solution containing S9. The S9 was obtained from Molecular Toxicology Inc., USA, and is prepared from male Sprague Dawley rats induced with Aroclor 1254. Colonies were scored and the induction factor was calculated by dividing the number of colonies formed in incubations with the respective water extracts by the number of colonies formed in the incubations with the negative control (MilliQ water). When the induction factor of a sample was above 2, the sample was considered positive for genotoxicity. As positive controls, 2-aminoanthracene (Acros Organics) was used at a concentration of 2.5 μg per plate when S9 was present or 4-nitroquinoline oxide (Sigma) at a concentration of 0.2 μg per plate when S9 was not used.

The *in vitro* Comet assay was performed according to Singh *et al.* (1988) with minor modifications. The human lymphocytes of a donor in a phosphate-buffered saline (PBS) diluted blood sample were exposed for 2 hours (37 °C, 5% CO_2) in triplicate to the XAD concentrated water samples with or without the S9. After 5 minutes' centrifugation at 3,000 rpm and 4 °C, the cell pellet obtained was diluted in cold PBS. The cell suspension thus obtained was mixed with molten low melting point (LMP) agarose (0.8%) and added on the GelBond® Film. The GelBond® Films were placed in cold lysis solution overnight at 4 °C, were rinsed in electrophoresis buffer (pH >13) for 5 minutes at room temperature and were transferred to a cooled electrophoresis tank. After 40 minutes' incubation time, electrophoresis was performed. The GelBond® Films were rinsed carefully with cold PBS and MilliQ water and dried for 4 hours at room temperature. The DNA was stained with ethidium bromide solution. The Metafer Slide Scanning Platform (MetaSystems, Altlussheim, Germany) and software CometScan were used for scoring the comet tail lengths. Per sample location at least 425 comets were measured. As a positive control, benzo(a)pyrene was used

at a concentration of 25 μM when S9 was present and ethyl methanesulphonate at a concentration of 2.5 mM when S9 was not used.

In vivo genotoxicity tests

After permission from the Animal Welfare Committee of the Wageningen University and in collaboration with the Dutch Forest Service, 85 Eastern mudminnows (*Umbra pygmaea*) were collected in November 2008 from small ponds in the National Park 'De Groote Peel', a nature reserve near Ospel in the Netherlands. The size of the fish varied from 9 to 12 cm and the weight from 8 to 17 grams. Forty fish were transported to the intake station for the city of Amsterdam water works 'Waternet' at Nieuwegein. These fish were exposed to the different positive as well as negative controls. Forty-five fish were transported to PWN Water Supply Company at water treatment plant Andijk. Prior to the exposure to the different water samples, the fish were adapted slowly to the higher pH (from pH 5 to 8) and to the conditions of the Lake IJsselmeer water and control water in order to prevent stress. The mudminnows were fed daily with frozen red mosquito larvae (chironomids) until the end of the exposure. The mudminnows were exposed for 11 days to control water (negative control), and water from the four sample sites described above (Figure 1). The control water at Nieuwegein was natural groundwater of drinking water quality. It has been retained in deep aquifers for over 100 years. Before distribution to the community as drinking water, this water is aerated and rapidly filtered through sand without treatment with chlorine or any other disinfectants. This water is ideal as control water due to lack of influence from infiltrating river water. Sodium chloride (NaCl) was added at the exposure site to the control water to increase the conductivity to the same level as Lake IJsselmeer water (approximately 700 μS/cm). The pH of both waters was around 8. There were no other relations between the control water and the Lake IJsselmeer water, thus the control can be considered as a laboratory blank. Figure 2(a) presents the general experimental setup for exposure of the mudminnows. In addition to this general scheme, for some of the four test samples additional steps were included in the experimental design in order to avoid confounding factors. These included the following steps. For the removal of particles, the Lake IJsselmeer water (sample Lake IJsselmeer) was filtrated using a unit with four serially interconnected cotton candle filters (30, 10, 3 and 1 μm pore size respectively). Furthermore for the UV/H_2O_2 effluent (sample UV/H_2O_2(out)) an additional step was included to remove residual H_2O_2. This is essential since the UV/H_2O_2 effluent contains 147 μM H_2O_2, and H_2O_2 is known to induce toxicity in fish itself. A safety data sheet (Solvay Chemicals 2010) presented an LC_{50} (lethal concentration, 50%) value for H_2O_2 of 16.4 mg/l (482 μM) for the fathead minnow (*Pimephales promelas*) after 96 hours. By using the enzyme catalase from bovine liver (1,656,000 U/l), obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), the H_2O_2 level was reduced to 0.14 μM after 1 hour incubation time (level calculated from experimental data and first order kinetics). By providing the catalase solution via a pump into the water sample as presented in Figure 2(b), and by extending the incubation time for the catalase to 3 hours, a final concentration of 1.21×10^{-7} μM H_2O_2 was present in the water to which the fish were exposed. This residual concentration of H_2O_2 is not expected to induce toxicity in fish. The residual concentration is also not expected to induce genotoxicity in fish since literature data indicate that induction of genotoxicity by H_2O_2 *in vivo* in *Unio pictorum* requires concentrations >10 μM (Štambuk *et al.* 2008), or of H_2O_2 concentrations >5 μM when gill cells of *Mytilus edulis* are exposed *in vivo* (Wilson *et al.* 1998). Induction of genotoxicity by H_2O_2 *in vitro* in hepatocytes obtained from rainbow trout is observed with a Lowest Observed Effect Concentration (LOEC) of 3.35 μM (Devaux *et al.* 1997). In a second control, the fish used for the Comet assay were exposed to control water with the addition of the enzyme catalase (blank catalase).

Per assay and for each water sample to be tested, the mudminnows were kept in 50 l all-glass flow through tanks, with a flow rate of 1,200 l per day and continuous aeration. A temperature of approximately 12 °C was maintained by heating the incoming water samples. For the *in vivo* SCE assay, six fish were used in the negative control group, while eight fish were used for each group testing the water samples of Lake IJsselmeer, UV/H_2O_2(in), UV/H_2O_2(out), and GAC filtration respectively. For the *in vivo* Comet assay, four fish were used for all groups, according to the recommendation described by Hartmann *et al.* (2003). As a positive control, eight fish for the SCE test and four fish for

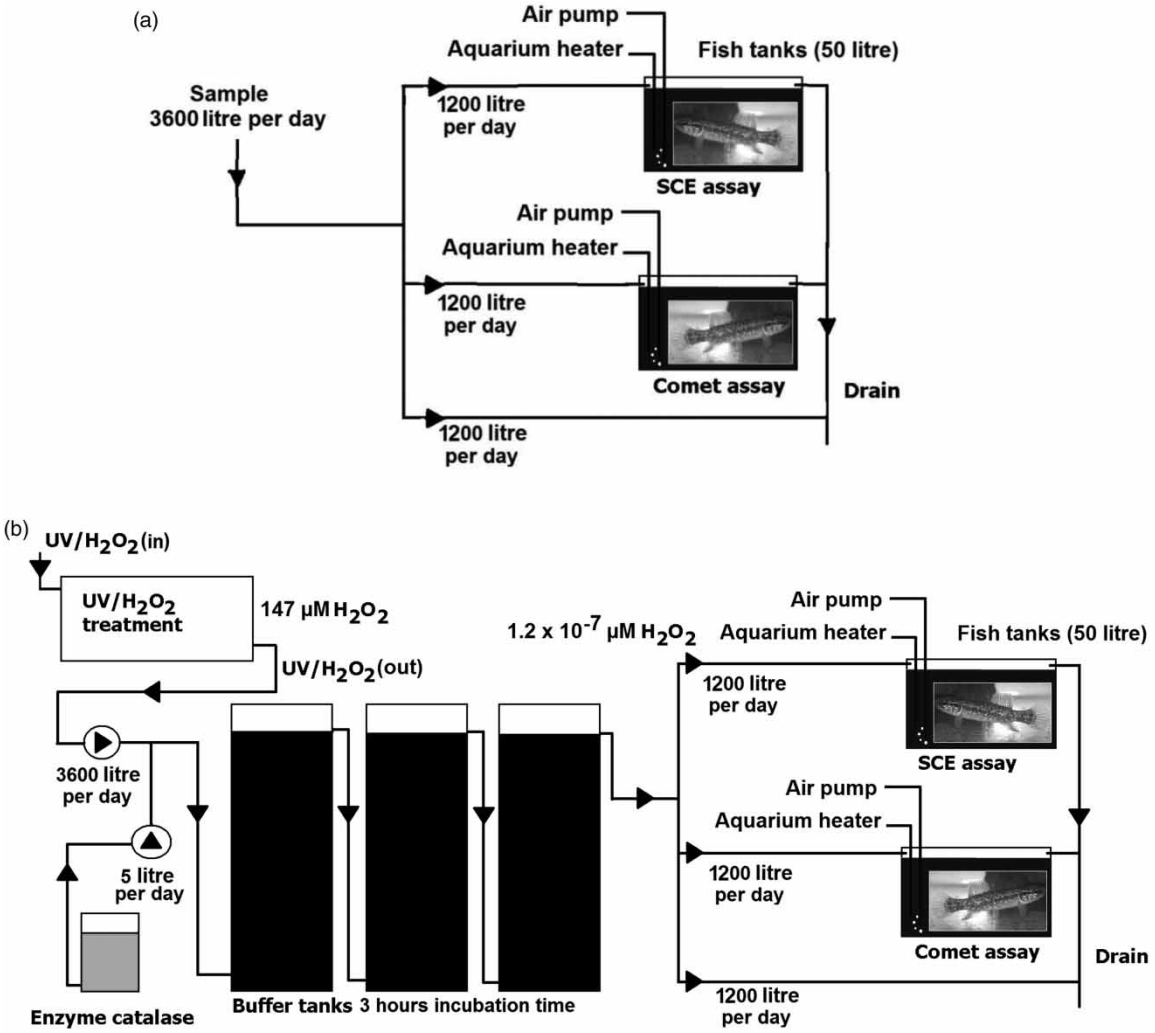

Figure 2 | The general experimental design for exposure of the fish (Eastern mudminnow) to water (a) and to UV/H_2O_2(out) water (b).

the Comet assay were exposed for 3 days in separate 5 l aquaria to 25 mg/l ethylmethanesulphonate (EMS), obtained from Fluka (Buchs, Switzerland). The gills of the exposed fish were used for the SCE test and the alkaline Comet assay, as described by Alink *et al.* (2007). The SCEs were scored in metaphases. With the Comet assay, the tail length of at least 750 comets per sample location was measured. The scoring was done in a double-blind fashion.

Statistics

Mean values and standard error of mean (SEM) of SCEs and tail lengths were determined. Each fish was considered as a test unit as described by others (Tice *et al.* 2000; Hartmann *et al.* 2003). Differences between groups were studied using the Student's *t*-test with significance level at $P < 0.05$.

RESULTS AND DISCUSSION

In vitro genotoxicity

In the Ames TA98 plate incorporation test, the induction factor of all the XAD concentrated water samples, was below the threshold of 2.0 for genotoxicity (Table 1), both in the absence and presence of metabolic activation (S9 mix). For the Comet assay, performed in the absence or presence of metabolic activation (S9 mix), no differences were

Table 1 | Response in the Ames (*Salmonella typhimurium* TA98) and Comet assay (*in vitro,* human lymphocytes) on XAD concentrated water samples obtained at PWN water treatment plant Andijk at different sampling dates and tested in the absence or presence of metabolic activation. Although samples were collected at different sampling dates (as indicated) they were all analysed on a single day for each test

Water sample	Sampling date dd-mm-yyyy	S9 mix	Induction factor Ames TA98 Plate incorporation	% DNA Comet assay Mean ± SEM ($n = 3$)
Negative control		−	1.00[a]	10.4 ± 0.6
Negative control		+	1.00[b]	8.8 ± 0.9
EMS positive control		−	NA	57.6 ± 3.8[d]
B(a)P positive control		+	NA	33.2 ± 5.4[d]
4-NQO positive control		−	5.92	NA
2-AA positive control		+	70.00	NA
Lake IJsselmeer	13-05-2008	+	1.23	14.4[c]
	18-11-2008	−	1.19	6.5 ± 0.4
	01-12-2008	+	1.22	8.2 ± 2.0
UV/$H_2O_{2(in)}$	13-05-2008	+	1.10	8.1 ± 1.3
	18-11-2008	−	0.96	5.7 ± 0.9
	01-12-2008	+	1.22	8.2 ± 0.8
UV/$H_2O_{2(out)}$	13-05-2008	+	1.31	6.8 ± 0.5
	18-11-2008	−	1.31	7.5 ± 0.7
	01-12-2008	+	1.25	8.8 ± 1.9
GAC filtration	13-05-2008	+	1.38	7.9 ± 2.9
	18-11-2008	−	0.81	9.6 ± 3.0
	01-12-2008	+	1.07	7.8 ± 1.3

NA = Not available because not tested.
[a]24 revertants.
[b]29 revertants.
[c]$n = 1$.
[d]$P < 0.05$.

observed in tail length between lymphocytes exposed to XAD concentrated water samples obtained at PWN water treatment plant Andijk at the different sampling sites and hepatocytes exposed to the negative control (Table 1).

From the results presented in Table 1, it can be concluded that the XAD concentrated water samples obtained from the sample location Lake IJsselmeer, do not reveal *in vitro* genotoxicity in the Ames test with strain TA98 and also do not result in DNA damage in the *in vitro* Comet assay in lymphocytes.

In vivo genotoxicity

In subsequent studies, the water samples were also tested in an *in vivo* SCE test and an *in vivo* Comet assay in Eastern mudminnow fish (*Umbra pygmaea*) exposed to the respective water samples for 11 days. This *in vivo* setup has the advantage that it does not require a concentration step thus avoiding any potential loss of compounds present since the fish are directly exposed to water samples *in vivo*. Figure 3 presents the results from the *in vivo* SCE test. For unknown reasons the fish in the group for the SCE test for the water sample from Lake IJsselmeer died before the end of the experiment. The fish in the other groups showed SCEs in gill cells per chromosome. The EMS group, representing the positive control, showed a statistically significant increase in the number of SCEs compared to the control group ($P = 0.024$). No significant increase in numbers of SCEs compared to the negative control were observed for the samples collected at the water treatment plant at Andijk (UV/H_2O_2(in), ($P = 0.70$), UV/H_2O_2(out), ($P = 0.91$) and GAC filtration ($P = 0.94$)). From these results

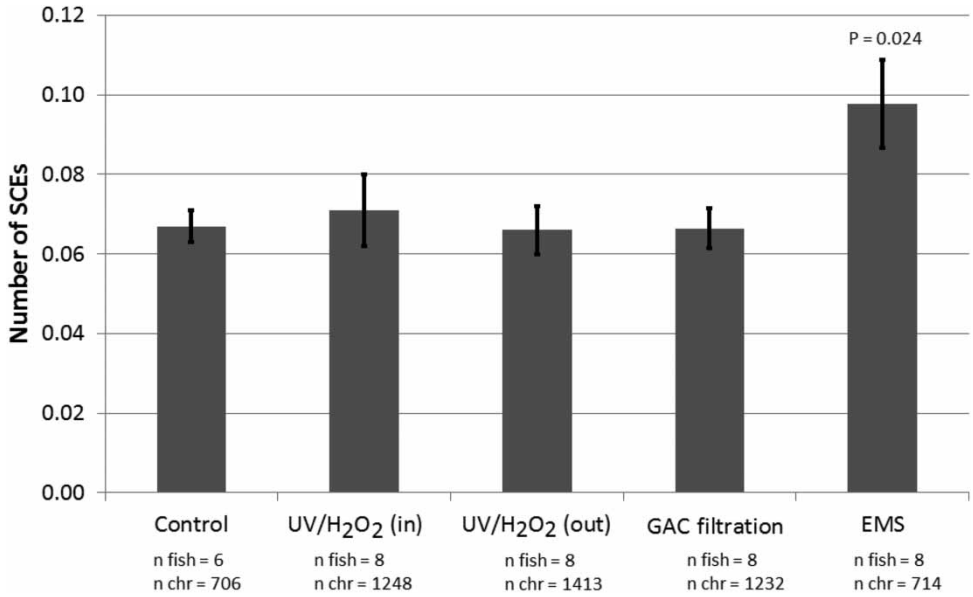

Figure 3 | Number of SCEs in gill cells per chromosome of the Eastern mudminnow after 11 days' exposure to control water, water containing the positive control EMS or water samples collected at the water treatment plant at Andijk including water samples UV/H_2O_2(in), UV/H_2O_2(out) and GAC filtration (Figure 1). Mean ± SEM, *n* fish = number of fish used, *n* chr = number of chromosomes scored.

it is concluded that compared to the negative control there is no significant increase in SCEs in the gill cells of fish exposed for 11 days to any of the water samples collected at Andijk including the water sample of the effluent of the UV/H_2O_2 treatment.

For the Comet assay, the viability of the gill cell suspension varied between 80 and 98% measured with the trypan blue assay. The prepared cell suspensions could thus be considered acceptable for use in the Comet assay. Figure 4 presents the results of the *in vivo* Comet assay. The results

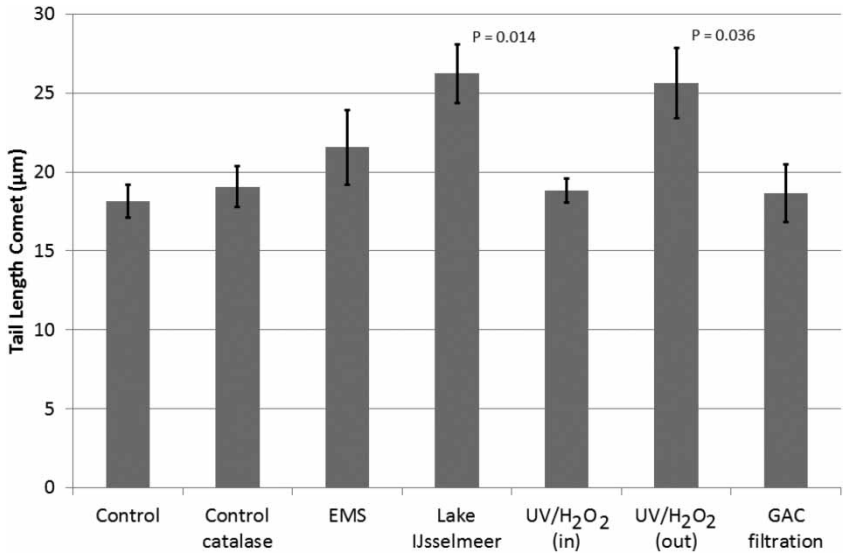

Figure 4 | Comet tail length in gill cells of the Eastern mudminnow after 11 days' exposure to control water (with or without catalase), water containing the positive control EMS, or water samples collected at the water treatment plant at Andijk including: water samples Lake IJsselmeer, UV/H_2O_2(in), UV/H_2O_2(out) and GAC filtration (Figure 1). Mean ± SEM, $n = 4$ fish.

obtained reveal that no significant differences in tail length were observed between the samples from the fish exposed to the ground water control and to the control catalase water sample ($P = 0.60$). There were also no significant differences in tail length between the samples from the fish exposed to the UV/H_2O_2(in) water sample and the control ($P = 0.61$), and the GAC filtration group and the control ($P = 0.82$). However, there was a significant difference in tail length between the fish exposed to Lake IJsselmeer water ($P = 0.014$) or to effluent UV/H_2O_2 ($P = 0.036$) and fish exposed to the groundwater control.

The samples from gill cells of fish exposed to EMS as the positive control showed a 1.2-fold increase in tail length which appeared non-significant ($P = 0.13$), but was in line with the 1.6-fold ($P = 0.039$) increase in the tail length obtained in a similar study performed previously (Table 2, Penders *et al*. 2012). A previous study (Alink *et al*. 2007), in which 120 mg EMS per litre was used as a positive control, showed a 2.1-fold increase in tail length ($P = 0.00039$).

In another study (Belpaeme *et al*. 1998), a statistically significant increase of DNA damage in gill cells was shown after *in vivo* exposure of marine flatfish to 50 mg EMS per litre.

The reason for the relatively lower induction by the EMS positive control than in previous studies remains unknown, but might be related to the relatively poor and thus difficult solubility of EMS in water.

The Lake IJsselmeer receives water from the River Rhine. The samples from gill cells of fish exposed to Lake IJsselmeer water showed a 1.45-fold increase in tail length compared to control, and is in line with the 1.32-fold increase in tail length obtained in the previous study (Alink *et al*. 2007) in which River Rhine water was tested.

It is of importance to note that, although there was a significant increase in tail length in gill cells of fish exposed to the UV/H_2O_2(out) sample, this effect was no longer observed for the GAC filtration sample due to a significant decrease in tail length in gill cells in fish exposed to GAC filtration water compared to the tail length in gill cells in fish exposed to UV/H_2O_2(out) water ($P = 0.054$). The DNA damage in gill cells of fish exposed to UV/H_2O_2(out) water is likely to be caused by reaction products formed during the UV/H_2O_2 treatment. These reaction products appear to be adsorbed or degraded during the GAC treatment, resulting in the tail length similar to the tail length of the control group.

Also important to note is that there is a significant increase in tail length in gill cells of fish exposed to Lake IJsselmeer water ($P = 0.014$) compared to fish exposed to control water, pointing to the presence of compounds able to cause DNA damage already in the surface water used for the drinking water production. The results for the fish exposed to the GAC filtration sample reveal however, that the treatment process as applied at the water treatment plant at Andijk effectively removes these compounds from the water.

To support the influence of the treatment process on the residual compounds present in the water, Table 3 presents results from an analysis of dissolved organic carbon (DOC), humic acids, UV254 absorption and suspended solids in the water samples collected at the water treatment plant at Andijk. Coagulation of organic matter and rapid sand filtration performed and turning water from Lake IJsselmeer into the sample called UV/H_2O_2(in) decreased the amount of DOC, humic acids, UV254 absorption and suspended solids. This process may contribute to the elimination of DNA damaging compounds present in the surface water used for preparation of the drinking water, and explain the decrease in effects on DNA tail length between the sample Lake IJsselmeer and UV/H_2O_2(in) as presented in Figure 4.

Table 2 | Comet tail length in gill cells of the Eastern mudminnow after exposure to control water and EMS performed in different studies

Study	Concentration EMS (mg/l)	Tail Length Control (μm) Mean ± SEM	Tail Length EMS (μm) Mean ± SEM	Fold Increase EMS/Control	*P*-value *t*-test
Pre-test EMS Alink *et al*. (2007)	120	23.36 ± 1.94	40.15 ± 2.39	1.7	0.0009
Alink *et al*. (2007)	120	17.71 ± 0.98	38.00 ± 1.70	2.1	0.0004
Penders *et al*. (2012)	25	33.92 ± 6.24	54.24 ± 3.33	1.6	0.0386

Table 3 | Indication ($n = 2$) of DOC, humic acids, UV 254 and suspended solids levels in the water samples collected at the water treatment plant at Andijk

Sampling location	DOC[a] µg/l Carbon	Humic acids[a] µg/l Carbon	UV 254 ext/m	Suspended solids mg/l
Lake IJsselmeer	6,400	2,940	10.5	23.96
UV/H_2O_2(in)	2,120	954	3.8	<0.2
UV/H_2O_2(out)	2,100	970	3.1	<0.2
GAC filtration	1,500	438	1.4	<0.2

[a]Based on Natural Organic Matter (NOM) data.

An increase in tail length in gill cells was observed in fish exposed to effluent UV/H_2O_2(out) water compared to fish exposed to the influent UV/H_2O_2(in) water sample ($P = 0.050$). Given that the UV/H_2O_2(out) water sample was treated with catalase before exposure of the fish, it can be concluded that this DNA damage is not due to a residual level of H_2O_2 in the water.

Thus, it is considered that the DNA damage in gill cells was caused by reaction products which were formed during the UV/H_2O_2 treatment. These reaction products are adsorbed or degraded during the GAC treatment. A decrease of DNA damage was observed in fish exposed to water collected after the GAC treatment compared to fish exposed to water obtained after the UV/H_2O_2 treatment ($P = 0.054$), resulting in tail length values for the fish exposed to the GAC filtration water sample that were similar to the tail length values of the control group.

Although the *in vivo* Comet assay is positive for the UV/H_2O_2(out) water sample, the *in vivo* SCE test was negative for this and also all other samples. This difference in the results of the *in vivo* Comet assay and the *in vivo* SCE assay may be caused by the fact that the UV/H_2O_2(out) water sample contains compounds which only induce single strand breaks. These single strand breaks do not lead to a positive response in the SCE assay (Bradley *et al.* 1979) but do result in DNA damage detected in the Comet assay (Singh *et al.* 1988). Another explanation may be related to the fact that in the Comet assay DNA damage is detected in cells at different stages of the cell cycle, whereas SCEs can only be detected in cells which are in C-metaphase and survived at least one mitotic cycle. In addition, repair of primary DNA damage during cell division or selective elimination of heavily damaged cells may contribute to the negative response in the SCE test (Kalweit *et al.* 1988; He *et al.* 2000).

The difference in result between the *in vitro* Comet assay and the *in vivo* Comet assay might be explained by the loss of compounds during the XAD solid phase extraction procedure. Such an explanation may also explain that negative results were obtained in the Ames test with strain TA98 for all water samples in the present study, whereas in another study positive results in the Ames II assay with strain TA98 for the UV/H_2O_2 treated water from the same location were reported (Heringa 2009). The water samples tested in the study reported by Heringa were pretreated with HCl to pH 2.3 and OASIS HLB resin was used for the absorption/desorption column. This method may result in extraction of other, more hydrophilic compounds, from the water. The XAD-4 resin used in the present study has a large capacity for uncharged lower molecular weight molecules (Malcolm & MacCarthy 1992). The difference between the present data and those reported by Heringa (2009) for the UV/H_2O_2 treated water may however also reflect different chemical compositions of the surface water used as starting material for the water treatment process, since these studies were performed at different moments in time.

In the study by Heringa (2009), no genotoxicity or DNA damage was observed for UV/H_2O_2(out) water extracts when tested by Ames II TAmix or in an *in vitro* Comet assay in which HepG2 cells were exposed. This illustrates that genotoxicity results obtained *in vitro* with concentrated water samples may turn out to give equivocal results depending on the experimental methods applied. This strengthens the importance of the use of *in vivo* tests for detecting genotoxicity in samples from water treatment plants, because this experimental set up does not require concentration of the water samples and allows long term exposure schedules, resulting in sufficient sensitivity to detect potential genotoxic or DNA damaging hazards in the water samples as such (Alink *et al.* 2007). To the best of our knowledge the present study is the first one using both the *in vivo* Comet assay and an *in vivo* SCE assay for the detection of genotoxic compounds in water samples collected from specific water plant processes for the treatment of surface water for the production of drinking water.

The increase in tail length in gill cells observed in fish exposed to effluent UV/H_2O_2 (out) might be the result of exposure to N-based disinfection by-products. These by-products are formed when the organic matrix reacts with nitrogen-containing intermediates formed by the photolysis of nitrate (Mack & Bolton 1999). Martijn & Kruithof (2012) suggested that the genotoxic response in UV/H_2O_2 treated water at PWN's water treatment plant Andijk is related to the UV-photolysis of nitrate. In their study, UV/H_2O_2 treatment of NOM-containing water in the absence of nitrate, via collimated beam experiments, showed no elevated response in the Ames II assay, relative to the blank and the negative control. However, in the presence of nitrate, a significant increase in the Ames II response was observed after UV/H_2O_2 treatment. Further research is required to identify the compounds which induce DNA damage and are formed during the UV/ H_2O_2 treatment, and further risk/benefit analyses of the usage of UV/H_2O_2 compared to the use of ozone or chlorine as disinfectant are needed.

CONCLUSIONS

The main conclusion of the present study is that after UV/H_2O_2 treatment of pretreated surface water, a significant increase in single strand breaks (Comet assay) is induced in gill cells of the Eastern mudminnow after 11 days' exposure *in vivo* to this UV/H_2O_2 treated effluent water. However, no increase in SCEs was observed during the same *in vivo* exposure. Also *in vitro* Ames TA98 and Comet assays performed on XAD concentrated samples of this UV/H_2O_2 treated effluent water, with or without metabolic activation, gave negative results. The negative *in vitro* results suggest that the compounds present in the effluent of the UV/H_2O_2 treatment which induce DNA damage have hydrophilic characteristics or that these assays were less sensitive. The nature of the substances formed during the UV/H_2O_2 treatment causing the *in vivo* DNA damage in the Comet assay remains to be elucidated.

As after GAC filtration, the level of DNA damage as detected by the Comet assay in gill cells of the Eastern mudminnow after 11 days' exposure *in vivo*, was similar to the level observed in the control samples, it is concluded that GAC filtration after UV/H_2O_2 treatment is suitable to adsorb or degrade the DNA damaging reaction products produced in the UV/H_2O_2 treatment finally resulting in the production of drinking water that is not of concern with respect to genotoxicity.

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First received 7 September 2011; accepted in revised form 22 October 2012