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# An *in vitro*/*in vivo* screening assay as a sensitive tool to assess endocrine disruptive activity in surface water

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**Abstract**

Adult male fathead minnow were exposed for 14 or 28-days under flow-through conditions to undiluted filtered water samples from the rivers Meuse and Rhine in the Netherlands. The experiment included two vessels per treatment each containing 10 fish and samples of five fish were taken after 14 and 28 days. Additional groups were exposed to 17α-ethinylestradiol (EE2) as a reference and untreated drinking water as a negative control. Major endpoints examined included induction of vitellogenin (VTG) synthesis, VTG mRNA activity, hepato- and gonadosomatic indices (HSI and GSI) and gonadal histology. No significant difference was recorded in body weight or mean GSI values between the various treatments. Only exposure to Meuse water resulted in significantly higher HSI means after 14 days. Histological examination showed no apparent effects on gonadal tissue except for eosinophilic blood plasma in fish exposed to Meuse water or EE2. After 14 and 28 days, elevated VTG and VTG mRNA levels were measured in most livers of the fish exposed to Meuse water, but not in the fish exposed to Rhine water. This was confirmed by measuring estrogenic responses in the *in vitro* ER CALUX® assay. Induction of VTG synthesis proved to be the most sensitive endpoint in the Non Spawning Male Fish Assay for *in vivo* detection of bio-available estrogenic activity supplementary to a sensitive *in vitro* assay. The other endpoints examined varied too much and required a higher number of fish or replicates to achieve sufficient power for statistical testing making them less animal friendly.
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## 1. Introduction

For already almost a decennium significant concern has grown worldwide regarding the risk of synthetic chemicals and environmental contaminants acting as endocrine disrupting chemicals (EDCs). This concern focused especially on the potential of chemicals to adversely affect the reproductive systems of both human and wild life populations around the world. In the Netherlands, a preliminary inventory was started by the Kiwa Water Research, the research institute of the Dutch Waterworks in corporation with the Association of River Waterworks (RIWA) in 1997 (Denneman et al., 1997), which resulted in a project plan to examine the possible presence of estrogenic active chemicals (EACs) in surface waters at locations where water is extracted for drinking water preparation. In 1999, the Dutch National Health Council published a report (Health Council of the Netherlands, 1999) stating that there were sufficient reasons for concern about the presence of EDCs in especially the aquatic environment in the Netherlands.

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The Council came up with 34 groups of compounds that were suspected to have the potential to disrupt the endocrine system of the organisms present and thus endangering the reproduction of certain species. In addition to natural and synthetic hormones, these compounds included alkylphenols, bisphenol-A, polybromobiphenyls, polybrominated diphenylethers and to a lesser extent phthalates. In an early study concerning analytical detection of estrogenic hormones in surface and wastewaters, most of the estrogens were below detection limits, however, at some locations levels up to 6 ng/l were found (Belfroid et al., 1999).

As in various other European countries, a nation-wide survey has been performed in the Netherlands (LOES Project) to make an inventory of the presence of estrogenic active substances in Dutch surface waters (Vethaak et al., 2005). This survey showed that at certain locations sewage treatment works (STW) effluents were estrogenic to fish but the Dutch STWs appeared to be very effective in removing natural and synthetic estrogens from the untreated wastewater. A limited sampling performed on wild populations of freshwater fish showed that intersex bream (*Abramis brama*) were found in the only small river included where a municipal STW discharged relatively large volumes of effluent. Follow-up sampling of fish from other small rivers with comparable discharge ratios (Rijs et al., 2004) revealed male bream or roach (*Rutilus rutilus*) with intersex and/or increased levels of the female protein vitellogenin (VTG). However, no intersex fish were found in main rivers like the Rhine and Meuse. The authors of the report recommended that authorities responsible for monitoring the surface water quality should further examine and list locations with a potential for a risk based on endocrine disruption. In the slipstream of the LOES-Project, various research projects started for detection of estrogenic activity in surface water serving as sources for drinking water. The research encompasses projects concerning the various stages of the fresh water cycle, varying from drinking water to waste water treatment and all related environmental aspects (e.g. endocrine disruption in fish). The main target of the present paper was to examine whether the Non Spawning Male Fish Vitellogenin *in vivo* Assay is sufficiently sensitive to confirm *in vitro* determined estrogenic activity by simultaneous *in vitro* and *in vivo* testing of the water phase of surface waters of two major rivers (Rhine and Meuse) sampled at locations where water is extracted for drinking water preparation.

The *in vitro* experiments were performed in the ER-mediated chemically activated luciferase gene expression (ER CALUX®) assay (Legler et al., 1999). In the ER CALUX® assay, reporter gene expression is a measure of the ER-mediated cascade of intra-cellular events resulting in activation of genes. The assay is performed with T47D human breast adenocarcinoma cells with endogenous estrogen receptors, which were stably transfected with an estrogen-responsive luciferase reporter gene containing three estrogen-responsive elements. In earlier studies, this assay has proven to be more sensitive than other assays (YES or CARP-HEP) and accurate in detection of estrogen activity in samples of EACs, effluents or sediments (Vethaak et al., 2005; Meerts et al., 2001; Murk et al., 2002).

The *in vivo* counterpart asked for a sensitive short-term bioassay in an aquatic organism, preferably fish. Induction of VTG in male fish has proven to be a sensitive endpoint for detection of estrogenic active EDCs as well as of effluents and surface waters (Kramer et al., 1998; Sheahan et al., 2002; Parrott et al., 2003; Jobling et al., 2004). VTG is an oestrogen-dependent yolk protein precursor synthesized in the liver and transported by the blood stream to the female gonads where it is incorporated in the developing oocytes. As male fish do not produce VTG except when they are exposed to estrogenic active substances it serves as a biomarker for exposure to estrogens in oviparous vertebrates. VTG levels can be determined in blood or homogenized liver tissue. The *in vivo* non-spawning male fish assay further addressed several other biological endpoints including secondary sexual characteristics, the gonado–somatic index (GSI), the hepato-somatic index (HSI), VTG mRNA levels in liver tissue, and histopathology of excised gonads. Female fish were not included to prevent elevation of the background VTG production in males in response to female excretion of compounds such as 17β-estradiol (Versonnen et al., 2003). The assay was designed to assess estrogen activity in the water samples taken from Rhine and Meuse at extraction sites for the preparation of drinking water, without identifying specific mechanisms of hormonal disruption (such as hormone receptor agonistic or antagonistic effects or disturbance of hormone synthesis or metabolism). An additional objective was to test whether the standard exposure period of 28 days could be shortened without loss of information, which significantly decreases the inconvenience for the test animals.

## 2. Materials and methods

### 2.1. Surface water samples

Between October 7th and November 4th 2003, surface water samples were taken from the river Rhine at sampling point Nieuwegein (Lek Channel) and from the river Meuse at sampling point Hedel, both in the Netherlands. Samples were taken with a bucket at 10 to 50 cm under the water surface. The water was transferred to coded 10 or 20 l mess tins and transported to NOTOX three times a week on Monday, Wednesday and Friday in the late afternoon and stored at 7 °C until further use (within 24 h). Incidentally, extra samples were delivered on other or additional days of the week. After emptying the mess tins, they were rinsed with tap water before being re-used for sampling. All mess tins were allocated to a specific location to prevent contamination with sampling water from the other location.

### 2.2. Chemicals and solvents

17α-Ethinylestradiol (EE2) was obtained from Sigma, The Netherlands (Product Number E4876) with a purity >98% (by HPLC). The solvent triethyleneglycol was supplied by Merck, Darmstadt, Germany and of synthesis quality with a purity of ≥99%.

### 2.3. Test organisms

Fathead minnows (*Pimephales promelas*, Teleostei, Cyprinidae) (Linnaeus, 1758) were bred from an in-house culture. Fish were held in tap water (hardness ca. 180 mg $CaCO_3$) at a temperature range of 21–26 °C in a 200-liter tank in a recirculating system with a bio-filter. Physical water parameters, i.e. pH, hardness, nitrate, nitrite and ammonia concentration were measured once a week and temperature every day. The stock was fed with frozen adult brine shrimp and

pellet food. The male fish selected for testing were acclimatized to the test conditions from seven days before the start of the test.

### *2.4. Preparation of surface water samples*

The river water was used without prior dilution, but filtered under high pressure through a glass filter and a 0.2 μm membrane filter (Schleicher and Schuell) to remove all suspended particles before dosing. Volumes of surface water to be filtered amounted to 50 l per sample per day and an additional 50 l or L for the weekly renewal of the whole vessels. When the complete samples could not be filtered on the same day of delivery, fractions of the samples were stored in a climate room at maximum 7 °C until they were filtered. Period of storage never exceeded 24 h.

### *2.5. Design of the in vivo assay*

The assay was performed with adult male fish from single non-spawning adult male populations. The assay was conducted using undiluted river water from the Rhine and Meuse Rivers, one EE2 exposure concentration (positive control) and untreated tap water (negative control), with two vessels per treatment each containing 10 males. The exposure was conducted for 28-days. Test vessels were 30 l vessels with an actual volume of ca. 25 l. The light period consisted of 16 h photo-period daily, between 10–20 $\mu Em^{-2}s^{-1}$ or 600–1000 lux.

As EE2 was not readily soluble in water, stock solutions of 50 μg/l were prepared in tri-ethyleneglycol (Trigol). The stock solutions with EE2 were dosed, via a computer-controlled system consisting of a dispenser (Gilson 402), into a mixing flask where dilution water (tap water) entered through a flow meter. In the mixing flasks the dosed volumes were mixed with the dilution water under continuous stirring providing a target concentration of 5 ng/l (in 0.01% Trigol). From the mixing vessels, the test solutions entered a distribution vessel for each replicate separately. From the distribution vessels, the test solution entered the replicates at an average flow rate of 1.1 l/h, i.e. sufficient to reach a one-volume exchange of test solutions per 24 h. The dilution water control vessels were dosed with tap water in a similar set-up as used for the EE2 treated solutions and defined as the blank or drinking water control. The resulting 0.2 μm-filtrates of the river water samples were dosed using tubing-pumps at a flow rate up to 1 l/h per replicate. The whole system was checked daily. Weekly samples of ca. 1 l were taken from the replicates of the drinking water control and the EE2 treated group. These samples were shipped to KIWA N.V., Nieuwegein where they were used for preparation and further testing in the ER CALUX® assay at BioDetection Systems, Amsterdam, The Netherlands.

The animals were weighed before they were randomly assigned to the test vessels and allowed to acclimatize for 7 days before the start of the test. During the test period, fish were fed with frozen adult brine shrimp and pellet food. Within 1 h after each feeding, any food residues were removed by siphoning. Fish were not fed at least 24 h prior to termination of the experiment. Fish were observed daily and dead fish were removed when observed. On day 0, day 14 and day 28, observations of physical appearance were made on among others body colour, coloration patterns, body shape, specialized secondary sex characteristics. After 14 and 28 days of exposure, 10 fish per treatment (five of each replicate) were sacrificed and the complete livers were collected and weighed for determination of the hepatic somatic index (HSI). Thereafter they were treated for determination of VTG levels and VTG mRNA. The gonads were removed and transferred to fixative in pre-weighed vials. The vials with gonads sampled on day 28 were weighed for calculation of the gonadal-somatic index (GSI) and processed for subsequent histological analyses.

### *2.6. Quantification of secondary sexual characteristics*

Characteristics of physical appearance in adult fathead minnows potentially important in endocrine disrupter testing include body color, coloration patterns (i.e., presence or absence of vertical bands), body shape (i.e., shape of head and pectoral region, distension of abdomen), and specialized secondary sex characteristics (i.e., number and size of nuptial tubercles, size of dorsal pad and ovipositor). Nuptial tubercles are located on the head (dorsal pad) of reproductively-active male fathead minnows, and are usually arranged in a bilaterally-symmetric pattern. Mapping, counting and grading of the nuptial tubercles was conducted using a binocular microscope and based on the work of Smith (1978) and Jensen et al. (2001), where the grading relates to the progressive prominence of the tubercles.

### *2.7. GSI and gonadal histology*

Gonadal histology was performed only on gonads collected after 28 days, and gonads were fixed in modified Davidson's solution (Creasy and Jonassen, 1999). After 24 h, the gonads were transferred to 70%-ethanol. Dehydration of the gonad samples occurred in a graded ethanol series, clearing in xylene, paraffin wax infiltration and embedding; sectioning at 4 μm on a rotary microtome and staining with Mayer's Haematoxylin and Eosin. The resulting slides were examined for histopathological alterations in male gonadal tissue applying the following scores:

a) inflammation: 0 = absent, 1 = present;
b) eosinophilic blood plasma: 1 = normal, 2 = increased; 3 = severe, with apparent resorption vacuoles;
c) necrosis: karyopycnosis in spermatogonia: 0 = absent, 1–2 = moderate-extensive;
d) apoptosis: single cell necrosis in spermatogonia, judging from eosinophilic cytoplasm and pycnotic/rhectic nucleus: 0–1 = absent-sporadic;
e) clumped spermatocytes: clusters of spermatocytes in tubular lumen, phagocytes by macrophages/ sloughing of sertoli cells unclear; 0 = absent, 3 = severe;
f) sertoli cells: 1 = normal, 2 = nuclear hypertrophy, due to low numbers of sertoli nuclei in sections, these were often based on not more than 2 to 3 nuclei.

Further observations included, if present, Leydig cell hypertrophy, calcified structures in damaged parts of the gonads or local vacuolisation of spermatogonia.

### *2.8. Viltellogenin (VTG) and VTG mRNA analysis*

The livers were excised, weighed, and then divided into two subsamples of which one was weighed and stored for VTG analysis, while the other one was stored for VTG mRNA determination. All samples were frozen in liquid nitrogen and stored at −80 °C. VTG concentrations in liver homogenates were measured using a commercially available Enzyme–Linked Immunosorbent Assay (ELISA) supplied by Biosense Laboratories AS, Norway, together with a protocol. The assay is based on a competition for the fathead minnow-Vtg antibody between VTG coated on the wells of the ELISA-plate and free VTG in solution (sample). The VTG mRNA levels were measured based on binding of isolated-mRNA to a specific probe, resulting in emission of light according to manufacturer's instructions (Molecular Light Technology, Wales, UK). The VTG mRNA levels were normalized relative to β-actin mRNA contents measured in the same samples. The β-actin mRNA contents are relative constant and not sex related. Further, to a certain extent, it is a measure of the amount of liver tissue analysed.

### *2.9. Water quality parameters*

At the start and once per week during the exposure, a water quality check was performed on fresh tap water and river water samples by measuring pH, alkalinity, $NO_2^-$, $NH_4^+$, $NH_3$, chloride, hardness, salinity and conductivity. When values measured deviated from optimal, the content of the whole vessel was renewed and the respective parameter was measured again to confirm it had improved.

Dissolved oxygen concentrations were determined (one per treatment) at the start and three times a week during the first week of the test period and daily thereafter. Temperature was measured continuously in one vessel of the negative and one of the positive control, three times a week in both vessels of the river water samples.

### *2.10. Design of the in-vitro assay*

Prior to the ER CALUX® *in-vitro* bioassay, organic compounds, possibly including EACs, were isolated from water samples by liquid–liquid extraction with ethylacetate. A water sample of 1 L was filtrated using 0.45 μm cellulose

Table 1
Mean values for fish body weights on days 14 and 28 in tap water (blank control), surface water of the rivers Rhine and Meuse and the positive control ethynylestradiol (EE2, 5 ng/l). At the start of the study, mean body weight of all fish was 4.32±1.39 g

| Group | Fish body weight in grams | |
|---|---|---|
| | Day 14 | Day 28 |
| | Mean±S.D. | Mean±S.D. |
| Blank A | 4.86±2.14 | 4.12±0.82 |
| Blank B | 4.37±1.01 | 4.16±0.89 |
| Mean Blank | 4.62±1.67 | 4.14±0.86 |
| Meuse A | 4.60±1.41 | 4.84±1.83 |
| Meuse B | 3.62±0.82 | 4.20±0.67 |
| Mean Meuse | 4.11±1.15 | 4.52±1.38 |
| Rhine A | 3.95±1.84 | 3.80±1.23 |
| Rhine B | 4.49±0.60 | 3.36±0.39 |
| Mean Rhine | 4.22±1.37 | 3.58±0.91 |
| EE2 A | 3.78±1.29 | 3.25±0.69 |
| EE2 B | 3.75±1.31 | 4.40±1.70 |
| Mean EE2 | 3.77±1.30 | 3.83±1.30 |

nitrate filters and subsequently extracted three times with successively 200, 50 and 50 ml ethylacetate. The combined extracts were concentrated to nearly dryness, i.e. less than 10 μl in pre-weighed glass point vials. After addition of 50 μl dimethylsulphoxide (DMSO), the extracts were concentrated by a gentle stream of nitrogen to remove the remaining ethylacetate. Finally, DMSO was added up to 50 μl by weight to achieve a final concentration factor of 20,000.

The T47D human breast adenocarcinoma cells were cultured as described previously (Murk et al., 2002) in Nunc 96-well plates (5000 cells/well) in assay medium (1:1 mixture of Dulbeccos's modified Eagle's medium/Ham's F12 medium) supplemented with nonessential amino acids and fetal calf serum (stripped from hormones). After incubation for 24 h, the assay medium was renewed followed by an additional 24 h of incubation. Before exposure, the assay medium was mixed with the extracts of the surface water samples or samples taken from the EE2 and blank solutions used in the *in vivo* test. The cells were exposed to these mixtures in triplicate for another 24 h. In addition, a 17β-estradiol (E2) calibration curve was tested in triplicate exposing cells to 0, 0.3, 0.6, 1, 3, 6, 10 en 30 pM. After exposure, the medium was removed and the cells were lysed in Triton lysis buffer. Luciferin substrate was added to each well and luciferase activity was measured with a luminometer (LUCYII, Anthos).

The estrogenic potency of the samples was expressed as estradiol equivalency (EEQ). The method used was comparable to that earlier described by Murk et al. (2002). The limit of detection (LOD) was 0.007 ng/l and the limit of quantification was 0.020 ng/l (lowest point of the calibration curve).

### 2.11. Data handling and statistics

All data on body and tissue weights, HSI and GSI were averaged per replicate and then compared with the combined means of the blank replicates. The values of the two blank replicates were compared using the Student's *t*-test for two sample analysis. They were only combined when there was no statistical difference. All data were tested for normality (Chi-square test) and homogeneity of variance (Bartlett test). The statistical analyses for pair-wise comparison with the combined control was performed with ANOVA–Dunnet or Bonferroni *t*-test, $\alpha=0.05$ two-sided. In addition, a multiple comparison was performed with the Tukey test ($\alpha=0.05$).

## 3. Results

### 3.1. Surface water samples: delivery and quality

The ultra-filtering of the river water samples was challenging due to the relatively large volumes of water that had to be filtered almost daily in order to maintain a constant flow during the experimental period. Although the flow rate of 1 l/h corresponded with one replacement of the actual water volume (ca. 25 l) in each vessel per day, it proved to be insufficient to maintain an optimal quality of the test solutions especially during the first week of exposure. Oxygen concentration generally was above 5 mg $O_2$/l (60% of saturation), except during the first week when incidentally oxygen dropped below this level in one or more vessels (3–4 mg $O_2$/l). The pH values measured generally ranged between 7.6 and 8.4, except on day 28 with three replicates where pH was >8.5 (8.6–8.8). The temperature remained largely between 22 and 24 °C. Difference in temperature between different exposure groups and replicates did not exceed 0.5 °C. Ammonium concentrations reached levels up to 5 mg/l and were comparable in all test solutions including the untreated controls, although the levels were mostly the highest in the vessels with Meuse or Rhine water. Nitrite concentrations ranged between 0.25 and 1 mg/l in Meuse water and between 0.1 and 1 mg/l in Rhine water. Although nitrite levels up to 1 mg/l were also

Table 2
Liver and hepatic somatic indices (HSI) measured after 14 days and 28 days of exposure; gonadal weights and somatic indices (GSI) measured after 28 days of exposure. Replicates contained 5 adult male fathead minnows each, except when otherwise indicated

| Group Replicates | Day 14 | | Day 28 | | | |
|---|---|---|---|---|---|---|
| | Liver weight (mg) | HSI | Liver weight (mg) | HSI | Gonadal weight (mg) | GSI |
| | Mean±S.D. | Mean±S.D. | Mean±S.D. | Mean±S.D. | Mean±S.D. | Mean±S.D. |
| Blank A | 64.0±42.1 | 1.33±0.57 | 53.8±26.5 | 1.29±0.47 | 56.2±15.6 | 1.43±0.61 |
| Blank B | 69.9±6.6 | 1.63±0.32 | 53.4±20.5 | 1.30±0.48 | 75.3±23.3 | 1.90±0.78 |
| Mean blank | 67.0±30.2 | 1.48±0.65 | 53.6±33.5 | 1.30±0.67 | 65.8±28.0 | 1.67±0.98 |
| Meuse A | 105.6±7.9 | 2.58±1.31* | 90.4±29.5 | 1.94±0.39 | 78.7±41.1 | 1.59±0.63 |
| Meuse B | 88.9±13.2 | 2.43±0.36* | 76.3±21.1 | 1.81±0.34 | 49.1±19.4 | 1.16±0.36 |
| Mean Meuse | 97.3±10.9 | 2.50±0.96 | 83.4±25.6 | 1.88±0.37 | 63.9±32.1 | 1.38±0.51 |
| Rhine A | 104.0±16.1 | 2.77±0.78* | 59.8±23.1 | 1.56±0.25 | 57.3±27.7 | 1.45±0.28 |
| Rhine B | 76.8±6.3 | 1.71±0.24 | 51.6±9.9 [a] | 1.54±0.38 [a] | 48.3±16.1 [a] | 1.45±0.56 [a] |
| Mean Rhine | 90.4±12.3 | 2.24±0.58 | 55.7±17.8 | 1.55±0.32 | 52.8±22.6 | 1.45±0.45 |
| EE2 A | 91.6±16.0 | 2.38±0.45* | 55.5±12.7 | 1.84±0.55 | 36.4±12.9 | 1.14±0.45 |
| EE2 B | 73.8±13.5 | 1.94±0.22 | 57.8±19.7 [b] | 1.61±0.46 | 42.1±20.2 | 0.88±0.41 |
| Mean EE2 | 82.7±14.8 | 2.16±0.36 | 56.7±32.6 | 1.72±0.51 | 39.2±16.9 | 1.01±0.43 |

* Value is significantly different from the combined blank control, $\alpha=0.05$ two sided, data Log base 10 transformed.

[a] Data of the one female fish are not included ($n=4$).

[b] Value excluding an outlying value of 149 mg, with this outlier the mean is 73.5±44.4.

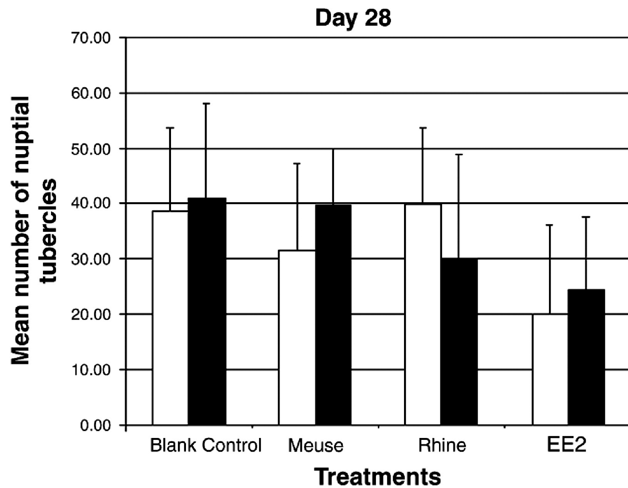


Fig. 1. Mean number of nuptial tubercles indicative of male sexual characteristic in fathead minnow after 28 days of exposure to surface water of the rivers Rhine and Meuse, the positive control ethynylestradiol (EE2, 5 ng/l) and tap water (blank control). White and black columns represent the two replicates of each treatment, the error bars represent the standard deviations.

incidentally detected in the untreated control solutions, the concentrations in the river water were always higher and detectable nitrite levels were already present in fresh samples before introduction into the test vessels. When the quality of the test solutions became below optimal, exposure water was completely renewed in addition to the daily exchange provided by the flow-through system.

### 3.2. Effects of surface water and EE2 exposure in fathead minnows

#### 3.2.1. Clinical effects and survival

Two fish died during the acclimatization period and were replaced by new male fish from the fish stock. One fish was found dead in a vessel of the EE2 treated solution on day 22. Otherwise, fish did not show any visible effects on behavior or appearance in any of the vessels throughout the test period.

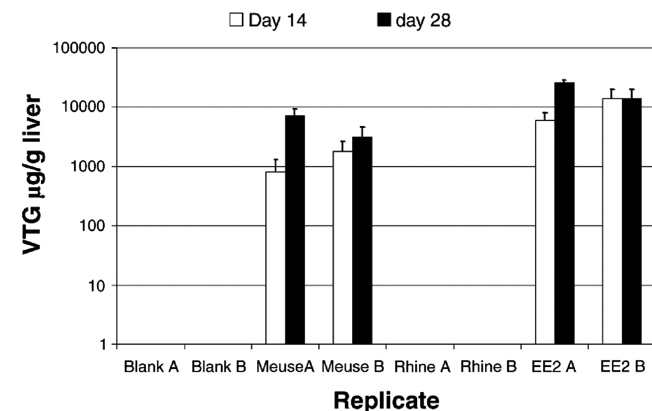


Fig. 2. Mean hepatic vitellogenin (VTG) concentrations in liver homogenates of male fathead minnows ($n=5$) exposed for 14 and 28 days in duplicate (vessels A and B) to tap water (blank), surface water of the rivers Rhine and Meuse, and the positive control ethynylestradiol (EE2, 5 ng/l). VTG concentrations in liver homogenates of male fathead minnows exposed to the blank and Rhine water were below the limit of detection (<1 µg/g liver). The error bars represent the standard deviations.

#### 3.2.2. Body and liver weights

At the start of the exposure, mean body weight of the fish was 4.32± 1.39 g. After 14 days of exposure the mean body weights of all replicates remained within that range (Table 1). Statistical analysis of body weights showed no significant difference between the various replicates ($\alpha=0.05$, two-sided). After 28 days of exposure, the mean body weights of all replicates remained within the initial range of 4.32± 1.39 g, although the means of replicates Rhine-B and EE2-A were near the lower limit of 2.93 g. Statistical analysis showed no significant difference between the various replicates ($\alpha=0.05$, two-sided).

Mean liver weights of fish exposed for 14 days to Meuse, Rhine and EE2 were 45, 35 and 23% higher than that of the control group, respectively (Table 2). The mean HSI values of both Meuse replicates were significantly higher than that of the combined blank control replicates, while this was true only for one Rhine and one EE2 replicate ($\alpha=0.05$).

After 28 days of exposure, no significant differences were observed both for mean liver weights and HSI values (Table 2). One of the fish in replicate EE2-B had a liver with an outlying weight of 149 mg. Fish exposed to Meuse water had the highest HSI values, but this was not statistically significant ($\alpha=0.05$).

#### 3.2.3. Nuptial tubercles

Exposure to EE2 for 28 days decreased the mean number of nuptial tubercles in both replicates (Fig. 1), but this was not statistically significant ($\alpha=0.05$). The mean numbers recorded in the other replicates were all in the same range.

#### 3.2.4. Gonadal weights, GSI and histopathology

Gonadal weights as well as GSI values varied considerably among the male fish with coefficients of variation (CVs) of up to 47% (see Table 2). There were no significant differences between the mean GSI values of the replicates exposed to river water or EE2 with the combined blank control replicates ($\alpha=0.05$, two-sided).

Gross histological examination of the gonads after 28 days of exposure revealed no apparent major effects on gonadal tissue in any of the slides originating from fish exposed to the negative control and Rhine water, except for one fish originating from the Rhine replicate B, which contained ovarian tissue with all stages of oocytes instead of testis. Apparently, this fish was female instead of male, which also explained the deviating value for gonadal weight. Of all histopathological parameters

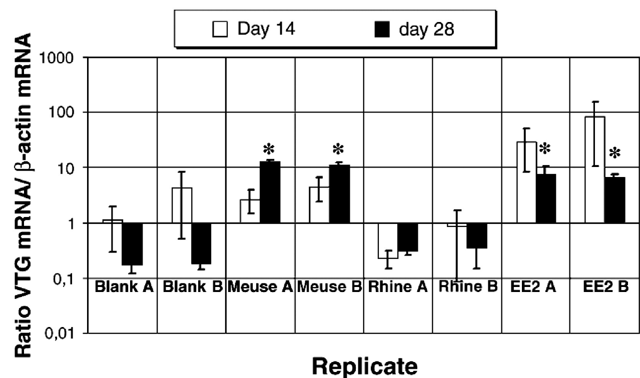


Fig. 3. Mean hepatic VTG mRNA normalized for β-actin mRNA induction ($y$-axis) in extractions of liver homogenates of male fathead minnow exposed for 14 and 28 days in duplicate (vessels A and B) to tap water (blank), surface water of the rivers Rhine and Meuse, and the positive control ethynylestradiol (EE2, 5 ng/l). The error bars represent the standard deviations. Asterices represent statistically significant differences compared to the combined controls.

analysed in fish exposed during 28 days, only significantly increased presence of eosinophilic blood plasma was observed in almost all slides originating from the fish exposed to EE2 and to a lesser extent in those originating from fish exposed to Meuse water (Kruskal–Wallis Test, $\alpha$=0.0001). More detailed examination showed peritubular granulomatous inflammation in some of the gonads originating from fish exposed to Meuse or Rhine water, where in incidental cases Leydig cell hyperplasia could not be excluded. Records of these tissues also included incidental occurrence of groups of epitheloid cells in tubuli, and/or sporadic intertubular macrophages. Further, only in one slide of the EE2 exposed group, very active spermatogonia were observed with few ripe spermatocytes. In the other slides of this group no differences in ripening or stages were recorded in comparison to those of the control group.

### 3.3. VTG and VTG mRNA measurements

No detectable VTG induction was recorded in any of the male fish of the blank control or Rhine water replicates after 14 or 28 days of testing (Fig. 2). Only in one liver sample of a fish from Rhine replicate B after 28 days of exposure VTG induction was measured, and this corresponded with the histological finding that this was a female fish instead of a male. In liver samples, elevated VTG levels were measured in 9 of the 10 fish sampled from the two Meuse replicates and from the two EE2 replicates after 14 days exposure. This was also observed in 8 samples from each of these groups after 28 days. The mean VTG levels induced by EE2 exposure were about a factor of 10 higher than those recorded in the Meuse samples.

The mean ratio's between VTG mRNA and β-actin mRNA were generally below 1 in the blank and river Rhine exposure groups indicating absence of VTG mRNA induction (Fig. 3). The higher responses in the Meuse and EE2 exposure replicates were not statistically significant when sampled after 14 days due to relatively high variations. After 28 days, however, the mean VTG mRNA values normalized for β-actin mRNA were significantly higher in all replicates of the Meuse and EE2 exposure groups compared to those of the blank control.

Table 3
The individual and mean estrogenic equivalencies (EEQs in ng/l) measured with the ER CALUX® assay for samples from surface water of the rivers Rhine and Meuse, the positive control ethynylestradiol (EE2, 5 ng/l) and tap water (blank control) tested in the *in vivo* experiment

| Date | Rhine | Meuse | EE2 | Blank |
|---|---|---|---|---|
| 3-Oct | 0.08 | 6.90 | nm | nm |
| 6-Oct | 0.20 | 5.00 | nm | nm |
| 8-Oct | 0.08 | 3.60 | 2.70 | 0.32 |
| 10-Oct | 0.25 | 5.30 | nm | nm |
| 13-Oct | 0.23 | 4.80 | nm | nm |
| 15-Oct | 0.24 | 3.80 | 6.90 | 0.47 |
| 17-Oct | 0.27 | 4.10 | nm | nm |
| 20-Oct | 0.30 | 7.10 | nm | nm |
| 21-Oct | – | 9.40 | nm | nm |
| 22-Oct | 0.27 | 6.80 | nm | nm |
| 23-Oct | – | – | 4.10 | 0.10 |
| 24-Oct | 0.36 | 3.90 | nm | nm |
| 27-Oct | 0.19 | 6.10 | nm | nm |
| 29-Oct | 0.25 | 7.70 | 4.00 | 0.09 |
| 31-Oct | 0.12 | – | nm | nm |
| 4-Nov | – | – | 3.30 | 0.06 |
| Mean | 0.22 | 5.73 | 4.20 | 0.21 |
| SD | 0.08 | 1.77 | 1.61 | 0.18 |

– = below the limit of quantification (0.02 ng/L).
nm = not measured (no sample available).

### 3.4. Estrogenic activity in surface waters with in vitro ER CALUX® assay

The estrogenicity was above the level of quantification (LOQ) of 0.020 ng/l in all 39 water extracts (Table 3). The mean value for the EEQ of Rhine water samples was in the same range as the mean value of the blank control (drinking water). Both mean values are one order of magnitude above the LOQ of 0.020 ng/l. In all Meuse samples, the EEQ was relatively high and ranged between 3.6 and 9.4 ng/l. The mean EEQ for the Meuse was slightly higher than the mean measured in the samples taken from the 5-ng/L EE2 positive control of the *in vivo* experiment. The EEQ's levels in the river water samples were relatively constant over time with a standard deviation of 18% (Meuse) to 36% (Rhine).

## 4. Discussion

Already within 14 days of exposure the *in vivo* non-spawning male fish assay detected significant estrogenic activity in surface water samples of the Meuse and EE2-spiked water but not in surface water samples of the Rhine. The ER CALUX® assay confirmed these findings. This is one of the first attempts to examine surface water samples simultaneously in *in vitro* and *in vivo* laboratory assays to assess estrogenic activity.

Filtration of the surface water samples excluded particulate and suspended matter as these were suspected to possibly contain adsorbed estrogenic compounds (Murk et al., 2002; Legler et al., 2003). The experiment was aimed to assess the possible estrogenic activity exclusively present in the water phase of the river water in the context of its application as source of drinking water.

The present assay included different endpoints of which especially measurement of VTG in liver homogenates, but also, to a lesser extent, VTG mRNA and gonadal histology were sensitive enough to assess significant changes in fish exposed to Meuse water.

Chemical analyses on EE2 concentrations in the positive control were performed but failed to provide reliable values (results not shown), because the target concentration of 5 ng/l was near the detection level of the method used. Still this concentration was applied as it approximated or was just one magnitude above the range of environmental concentrations detected in surface waters (Vethaak et al., 2005; Okkerman et al., 2001).

Water quality was not always optimal, especially during the first week of exposure. However, this had no dramatic effect on any of the fish exposed, supported by the high survival rate (only 1 dead fish), the absence of visible effects on behavior and appearance and the constant body weights during the 28-days of exposure.

Mean body weights in the control vessels A and B were in the same: 101 and 113% of the initial weight (4.32 g) on day 14 compared with 95 and 96% on day 28. Since fish were adults, they did not grow significantly during the test period.

The HSI is frequently included in experiments with fish, as the condition of the liver is thought to be closely related to estrogen exposure, e.g. to substantial VTG synthesis (Parrott

et al., 2003; Van den Heuvel and Ellis, 2002; Versonnen et al., 2003). However, increased HSI can also relate to exposure to other chemicals, e.g. P450 enzyme inducers also increase liver weight (McMaster et al., 1991). Although the HSI is a relative measure of liver and body weight, the sensitivity of this parameter is rather limited due to the relatively high variation between individual fish as seen in the present study after 14 and 28 days. The significantly higher means of HSI found in fish exposed to Meuse or Rhine water after 14 days of exposure did not correlate with the differences in VTG levels measured in the liver samples. Hence, the cause for higher HSI means in fish exposed to the river waters was probably not limited to estrogenic active compounds. Increased HSI values related to EE2 exposure were also reported in other studies (Parrott et al., 2003; Versonnen et al., 2003).

The GSI values in our study are in the range of the GSI values between 1 and 2% reported to be typical for adult male fathead minnows (Jensen et al., 2001). As for the HSI, the GSI varied considerably between fish with higher variations within vessels than between vessels. In our study, the average of the relative vessel-SEMs ($n=5$) was 17% (9–21%), while in a study of Parrott et al. (2003) with fathead minnow, this was 21% (13–36%) with $n$ varying between 9 to 35. Gimeno et al. (1997) reported even higher CVs than observed in our study, i.e. up to 107% for GSI in unexposed male carp ($n=7$ or 8). The high CVs for GSI makes this endpoint too insensitive to detect significant differences with sufficient power when not more than five fish are exposed per vessel. Although not statistically significant, exposure to EE2 reduced the average GSI by 31 and 47% in the two replicates, respectively. This is consistent with findings in other studies at higher concentrations of EE2 (Pawlowski et al., 2004). However, no apparent exposure related GSI differences were recorded for the river water treated groups, except a rather large difference between the vessel means of the Meuse group.

The major histopathological finding was eosinophilic blood plasma observed in slides originating from the fish exposed to EE2 or Meuse water. This feature has been reported earlier in fathead minnow (Palace et al., 2002), but also in carp (Gimeno et al., 1997), rainbow trout (Hermann and Kincaid, 1988), medaka (Metcalfe et al., 2001), and zebrafish (Weber et al., 2003) exposed to estrogens like E2 or EE2. In some cases eosinophilic fluids were reported also in cavities between as well as within organs (Gimeno et al., 1997; Metcalfe et al., 2001) and this was also observed in fathead minnows exposed in an extended fish early-life stage test to EE2 at 10 ng/l in our laboratory (Bogers et al., 2006). Some researchers attributed this response to estrogen related induction of VTG secretion, which accumulated in the blood and liver due to inadequate deposition in oocytes. Extensive appearance of eosinophilic fluids appears to be more related to chronic exposure of fish to estrogens (Bogers et al., 2006). Eosinophilic fluid of plasma was not present in fathead minnow after 14 days exposure to E2 (Miles-Richardson et al., 1999). However instead, these authors reported a dose dependent Sertoli cell proliferation and degeneration. No such finding was recorded in the tissues of the fatheads exposed to 5 ng EE2/l during 28 days in our study. The presence of eosinophilic plasma in the histological slides of fish exposed to Meuse or EE2 can be related to the relative high concentrations of VTG detected in their livers. Exposure to Meuse water induced elevated hepatic VTG levels in fish already within 14 days while livers of male fish exposed to Rhine water and the control fish completely lacked VTG. Induction of VTG synthesis in the liver or blood plasma of male fish is generally accepted as a sensitive indicator of estrogenic activity of industrial chemicals or pharmaceuticals (Ankley et al., 2001; Panter et al., 2002; Folmar et al., 2002) or effluents (Van den Heuvel and Ellis, 2002; Rodgers-Gray et al., 2000; Kirby et al., 2003, 2004).

The reference compound EE2 is a highly potent inducer of VTG synthesis in various fish species (Panter et al., 2002; Versonnen et al., 2003; Seki et al., 2002; Länge et al., 2001). In our study the EE2 concentration of nominal 5 ng/l (estimated actual value of $3.5 \pm 1.3$ ng/l, see below) induced significant induction of VTG in the male fatheads exposed already after 14 days of exposure. Länge et al. (2001) found a NOEC of $2.80 \pm 1.05$ ng/l (nominal 4 ng/l) for VTG induction in their full life cycle test with fathead minnow exposed to EE2 and Panter et al. (2002) found significant VTG induction in juvenile fathead minnow at levels down to $5 \pm 0.5$ ng/l. Since VTG levels in all males of the control group remained below the lowest limit of quantification, this parameter is very sensitive.

The VTG mRNA assay confirmed the VTG induction results, including the high response in the one female fish exposed to Rhine water (excluded from calculations). However, the responses recorded after 14 days were not statistically significant when compared to the negative controls. Interestingly the VTG mRNA levels had increased from day 14 to day 28 in the Meuse water fish indicating an increase of the internal dose of the compounds responsible for the VTG-induction. This higher internal dose may reflect possible bioconcentration of estrogenic compounds or fluctuations in the concentrations of compounds in the river water samples. Detection of hepatic VTG mRNA levels in fish has been used before as a biomarker of estrogenic contamination of effluents (Mellanen et al., 1999) or surface waters (Aravindakshan et al., 2004). Aravindakshan et al. (2004) found a relation between hepatic VTG mRNA levels and spermatogenic staging in immature shiners caught in St. Lawrence River near the island of Montreal (Canada). Folmar et al. (2000, 2001) found clear concentration-response and time-response relationships for VTG levels in plasma of fish exposed to various estrogenic compounds. However, the induction of VTG mRNA was quite variable for the estrogens E2 and diethylstilbestrol (DES) with only EE2 showing a clear concentration-response relationship. In addition, none of the estrogens showed a clear time-response relation for VTG mRNA. Retention of VTG in the plasma is much longer than that of the VTG mRNA in the liver (Bowman et al., 2000). Hemmer et al. (2002) found hepatic VTG mRNA rapidly diminishing after cessation of estrogenic exposure in sheepshead minnow, but plasma VTG clearance was at the level of days and appeared to be dependent on both concentration and exposure time. However, Brock and Shapiro (1983) showed that the half-life of vitellogenin-mRNA increased from 16 h to 3 weeks in the presence of estrogen.

The ER CALUX® assay could be applied to quantify the estrogenic potency of the different media the fish were exposed to. Assuming that 1 unit EE2 equals 1.2 estradiol equivalents (Legler et al., 2003) the average measured EEQ of 4.2 ± 1.6 ng/l (Table 3) corresponded with an average EE2 concentration of 3.5 ± 1.3 ng/l, i.e. 70% of the target concentration. Recoveries of EE2 below nominal have also been reported by others, i.e. down to 40% of nominal (Mellanen et al., 1999; Panter et al., 2002). The ER CALUX® results also showed a stable and substantial estrogen activity in the river Meuse water over the three week period. An attempt was made to identify possible estrogenic compounds in the samples by chemical analyses. Detectable concentrations of EE2 were found in two out of three samples taken from the EE2 treated medium and in one river Meuse sample (results not shown). An estrone (E1) response was found in another sample of the river Meuse. However, the results were only indicative and not considered as the actual concentrations present in the samples. In their extensive project Vethaak et al. (2005) found E1 more prominently present in the river Meuse (up to 4 ng/l) than in the river Rhine. Other steroids like E2 and EE2 were not detected except for E2 in the Meuse at Eijsden where the river enters the Netherlands. Concentrations of bisphenol-A were higher in the Rhine while more dimethyll-propylphtalate (DMPP) was found in the Meuse.

The ER CALUX® assay showed estrogenic activity in the samples from the drinking water control and the river Rhine above the LOQ, whereas no detectable estrogen related effects were recorded in the *in vivo* part of the studies. This response could be an indication of a false positive in the *in vitro* test or, a false negative in the *in vivo* test. Samples of normal drinking water originating from respective surface water sources did not induce significant responses above the detection limit (unpublished data provided by KIWA). The slightly positive ER CALUX® responses in the blank control could be caused by a possible contamination with xeno-estrogens from the experimental set-up including the flow-through system. However, the significant response in the river Rhine samples was in the same range as in the blank control and these samples did not originate from the flow-through system, but from separate samples from the sampling location. The lack of any response in the *in vivo* experiment for the blank control and Rhine samples might relate to the fact that the ER CALUX® extracts were prepared on 0.45 μM filtered samples, whereas the fish were exposed to river Rhine water filtered through 0.2 μM filters. This difference in type of filters used was discovered only after the *in vitro* and *in vivo* studies had been completed, due to the fact that they were performed at different laboratories. However, this difference was not relevant for the blank control samples (not filtered in the *in vivo* test) and had no effect on the corresponding responses between the *in vitro* and *in vivo* test for the Meuse samples. More likely, the significant responses in the blank control and Rhine samples could be a result of the extraction step applied in the *in vitro* test. The extraction of relatively large water samples in small volumes of organic solvents may concentrate traces of organic compounds present in these samples inducing a response in the *in vitro* test. The fact that the responses of the blank control and Rhine samples were in the same range in the *in vitro* test, indicate that these were not related to estrogenic compounds but were probably false positives.

The average EEQ-levels for the Meuse water and EE2 positive control determined by the ER CALUX® were comparable (Table 3) while the hepatic VTG-induction in the EE2-exposed fish was about 10-fold higher (Fig. 2). This is probably related to the fact that EE2 is several magnitudes more potent in fish compared to *in vitro* assays with mammalian cells and to other estrogens like E2 and E1 (Legler et al., 2003), which are believed to be the main estrogens present in the Meuse water (Vethaak et al., 2005). Further Legler et al. (2002) reported that relatively stable and accumulating compounds such as EE2 and o,p′-DDT were more estrogenic *in vivo* than *in vitro*, while on the other hand NP and BPA were more estrogenic *in vitro* than *in vivo*.

The positive result for estrogenic activity found in both the *in vitro* and *in vitro* test impelled KIWA to perform further research in the ER CALUX® assay on drinking water samples origination from the Meuse location. These results were not included in this publication as they fell outside the scope of the study, but no response was found in any of the samples tested. Hence, it could be safely excluded that there is any significant leaking of the estrogenic potential of Meuse water in the resulting drinking water.

The potential of the Meuse water samples to significantly induce VTG production in male fish reached levels comparable to an EE2 level of 5 ng/l within a relatively short exposure period of 28 days. Although this could indicate a possible risk for fish in the Meuse, the results of the present study did not show adverse effects on gonadal tissue after 28 days other than those related to increased amounts of extra-cellular VTG levels. Induction of elevated VTG levels in male fish, or even the induction of testis-ova does not automatically relate to a decrease in fertility or fecundity of the adult fish exposed (Seki et al., 2002).

## 5. Conclusion

Among the various endpoints examined, VTG-protein induction proves to be the most sensitive and robust parameter in a Non Spawning Male Fish Assay requiring no more than 14 days of exposure to detect significant estrogenic activity in surface water samples. The other endpoints, including nuptial tubercles, HSI and GSI were less sensitive even after 28 days. Further, a high control variability of HSI and GSI makes that these endpoints require a higher number of fish or replicates to achieve sufficient power for statistical testing. As a result, measurement of VTG-protein induction shortens the exposure period to 14 days without loss of information. This makes the Non Spawning Male Fish Vitellogenin Assay an important candidate as an *in vivo* short term (14 days) screening test for the detection or confirmation of estrogenic active substances or estrogenic potential of effluents and surface waters, by itself or in combination with *in vitro* assays like the ER CALUX® assay. Although the ER CALUX® assay has already proven its high sensitivity and ability to detect estrogen receptor related responses, a sensitive *in vivo* assay should be added in testing programs to avoid false positives or negatives based on the results of the *in vitro* test alone.

The Non Spawning Male Fish Vitellogenin Assay has also potential as a reliable assay to address effluents or surface water sources as low estrogenically active.

Finally, the ecological consequences of the current findings need to be studied further in chronic (full life cycle) tests with exposure to EEQ-levels in river water.

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