Development of aquatic biomonitoring models for surface waters used for drinking water supply

Eric Jacobus Maria Penders

Thesis committee

Thesis supervisor
Prof. dr. I.M.C.M. Rietjens
Professor of Toxicology
Wageningen University

Thesis co-supervisors
Dr. G.M. Alink
Associate Professor Section Toxicology, Wageningen University
Dr. W. Hoogenboezem
Senior Consultant Biology, The Water Laboratory, Haarlem

Other members
Prof. dr. A.A. Koelmans, IMARES, Wageningen University
Prof. dr. S.E. Wendelaar Bonga, Radboud University Nijmegen
Dr. P.G. Stoks, Association of Rhine water works, Nieuwegein
Prof. dr. agr. habil. E. Dopp, University of Duisburg-Essen, Germany

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Thesis

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Eric Jacobus Maria Penders
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Contents

Chapter 1

General introduction

Drinking water from surface water

Formerly people obtained their drinking water directly from surface water, but due to increased populations, surface water quality decreased considerably. In 1852, the first Dutch drinking water company (Amsterdam) was founded and produced drinking water from water extracted from the dune area. As the population increased, the extracted amount of water exceeded the natural infiltration volume by rain water. The threat of emerging deep salt water made it necessary to switch over to artificial infiltration. From the 1940-ies, untreated river water was infiltrated in several dune areas. However, given the poor quality of the available surface waters, pre-treatment purification before dune infiltration and development of water treatment plants were required (Kosman, 1988; Vinke, 2002). Plans were developed to infiltrate pre-treated surface water from the river into the dunes. It took until 1957 before pre-treated surface water from the river Rhine was transported to the dune area "Vogelenzang" and "Noord Hollands Duinreservaat". The abstraction and pre-treatment of surface water from the river Rhine occurred at a plant in Nieuwegein. In its first operating year, 42 million m^3 of surface water was transported to the dune area (Beemsterboer, 2002). Delivery of drinking water to the citizens in and around Amsterdam was assured.
At this moment, 40 % of the drinking water in the Netherlands is produced from surface water, with an annual production of 490 million m^3 (Geudens, 2010). The major sources of the abstracted surface water are the river Rhine (41% of total volume) and the river Meuse (57% of total volume).

The threats of using surface water for drinking water

Water companies were aware of the fact that the quality of the river water was poor compared to the waters naturally present in the dunes. This is due to the facts that the rivers Rhine and Meuse collect domestic and industrial sewage water upstream, and that they may also contain residues of pesticides used in agriculture and in urban areas for killing weeds and vermin. The river Rhine was a symbol of water pollution in Western Europe. For the first time in June 1969, the water companies had to interrupt their abstraction of surface water from the river Rhine, due to pollution of the water with endosulfan resulting in massive fish kills.
A new law was set in 1970, which aims at the prevention of water pollution and contributes to the protection of the quality of the water. Located at the border of the Netherlands, monitoring stations at Lobith (river Rhine) and Eijsden (river Meuse) were in operation to monitor the quality of the rivers. However due to the Sandoz fire in 1986, in which many life forms in the river Rhine were killed, and due to the bentazon affair in 1987, in which the herbicide bentazon was found in drinking water exceeding the 0.1 µg/L level, the citizens of Amsterdam were highly concerned. The population

claimed poison-free drinking water and social pressure was put on the government to improve the water quality of the river Rhine. The International Commission for the Protection of the Rhine (ICPR) started the Rhine action program. This work was performed with three important results: 1) the drinking water companies installed reliable barriers in their treatment which eliminate micro-organisms efficiently and reduce the number and amount of chemicals present in surface water, in order to produce safe drinking water 2) the river managers faced more and more strict demands for directing discharges into the river, so that all cities and industries were forced to build waste water treatment plants, and 3) the government increased regulations and restrictions with respect to diffuse environmental pollutants and the use of pesticides in agriculture.

The quality of surface water of the river Rhine has improved since 1987. However, in spite of the decrease of industrial discharges into the river (Figure 1.1), other threats are still present. Water companies are now focused more on, for example, diffuse discharges of agricultural pesticides in river water.

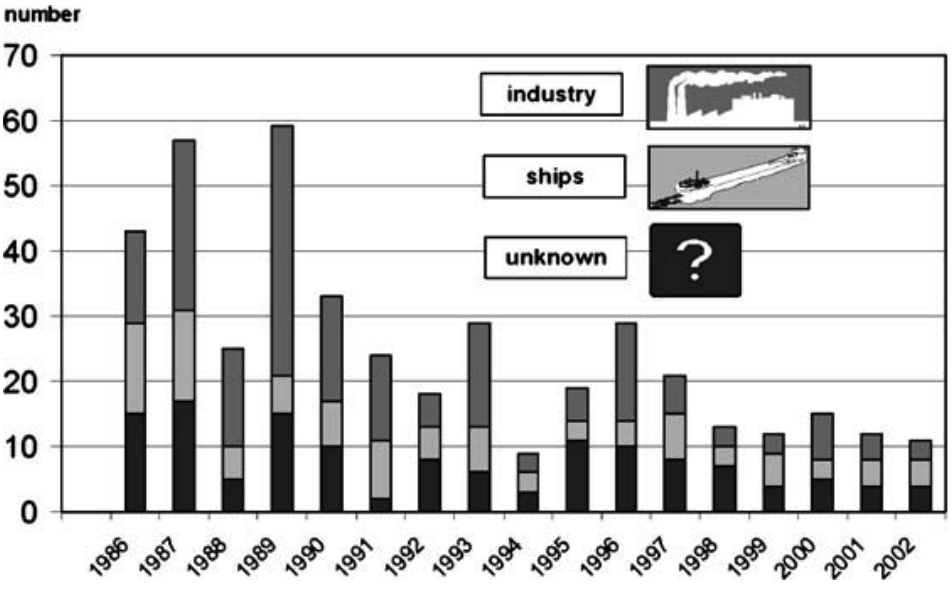

Figure 1.1: The number of discharges/reports according to the International Warning and Alarm Plan Rhine (ref: Diehl *et al.*, 2006). With kind permission from Springer Science+Business Media.

The present generation of pesticides is more hydrophilic and can present problems to the water companies. Also a growing number of emerging substances like pharmaceuticals, endocrine active substances, personal care products, complexion agents, nanoparticles, perfluorinated compounds, flame retardants, X-ray contrast media and fuel additives are discovered in surface water (Houtman, 2010). In addition, not only chemicals discharged in surface water may cause problems, but also their metabolites and/or degradation products formed in (a)biotic processes in the surface water and/or upon treatment to produce drinking water. During the advanced oxidation processes with ozone or UV/peroxide for example, required as disinfection treatment in the drinking water production, other metabolites or by-products may be formed. The presence of the different (unknown) chemicals in surface water in rivers is of concern for the drinking water companies.

Water quality control and source water protection approach

The organisation of the public drinking water supply and the supervision of the drinking water companies is controlled by the Dutch Water Supply act, active since 1957 (Anonymous, 1957). The purpose of this act was to protect the public health against risks at delivery and to ascertain the availability of drinking water. Drinking water companies were enforced to deliver reliable drinking water by performing analyses and controls frequently. Since 1960, the act was active and defines limits for a number of chemical and microbiological parameters. At the moment, for pesticides a maximum level of 0.1 µg/L is given for single compounds, and a maximum of 0.5 µg/L as summation of all detected pesticides, including their metabolites and reaction products which are toxicologically of concern (Anonymous, 2001). To protect the soil, flora and fauna in the dune area, the water companies may only infiltrate the surface water if the parameters used to monitor the quality of surface water are not exceeding limits stated in the act “infiltratiebesluit” (Anonymous, 1993). For 12 organochlorine pesticides, the limits are set to 0.05 µg/L (the sum of these pesticides must not exceed the 0.1 µg/L). For five organophosphate pesticides, three triazines, three chlorophenoxy herbicides, four urea herbicides and six chlorophenols, the limits are set at 0.1 µg/L. The summated concentration of pesticides must not exceed the 0.5 µg/L level. In both acts, no limits are at the moment given for newly emerging chemicals such as for example pharmaceuticals, endocrine active substances, personal care products, complexion agents, nanoparticles, perfluorinated compounds, flame retardants, fuel additives and X-ray contrast media (Houtman, 2010).

The drinking water companies develop new and expensive advanced treatment processes, for maximum purification of surface water in the production of drinking

water. However, the removal of chemicals from water depends on the physico-chemical properties of these compounds. Therefore, drinking water companies prefer the approach that emission of contaminants in the environment should be prevented.
Publication of the Danube, Meuse and Rhine Memorandum 2008 (Anonymous, 2008) formulates the most important principles of sustainable use of surface waters necessary for drinking water production. The goal, stated in this memorandum, is to achieve a water quality that allows drinking water companies to produce drinking water using only natural treatment methods.
To achieve this goal for the river Rhine for instance, the Association of Rhine Water Works (RIWA Rhine) together with the International Association of Water Works in the Rhine Basin (IAWR), interacts with politicians, governments and policy makers in water management and industry to safeguard a clean river. Information on water quality of the river Rhine is of importance. This quality control includes both analytical measurements of chemicals in water, using advanced and state-of-the-art detection techniques, but also *in vitro* and *in vivo* bioassays to obtain information on the toxicological characteristics of the water. The advantage of such bioassays over analytical methods is that the bioassays present effect based information of also non-detectable and unknown compounds present in the water, thereby adding an extra dimension to the water quality control.

Bioassays in water quality control

Aquatic toxicology is defined according Rand *et al*. (1995) as "the study of the effects of manufactured chemicals and other anthropogenic and natural materials and activities (collectively termed toxic agents or substances) on aquatic organisms at various levels of organization, from subcellular through individual organisms to communities and ecosystems". Such aquatic toxicity can be detected by biological tests, also called bioassays. Bioassays detect and quantify the adverse effects of contaminants on cells or organisms. In biological tests, organisms or parts of organisms (e.g. bacteria, algae, animals, cell cultures or tissues) are exposed to the water to be investigated or a concentrate of this, after which reactions by the test organisms (e.g. reduction in activity, reduction in reproduction or growth, or increased incidence of death) are recorded, which are then compared with the same endpoints in control samples. The advantages of the use of biological tests over the use of analytical chemical tests are that bioassays directly reveal any adverse effects on organisms or parts of organisms, and that they can detect compounds or mixtures containing compounds that cannot be chemically analysed or identified.
A whole series of bioassays for water quality control have become available and Table 1.1 presents an overview of the laboratory toxicity tests available. From this it appears

that the OECD (Organisation for Economic Co-Operation and Development) has a number of guidelines in which different freshwater toxicity test are defined (Table 1.1). Also some ISO (International Organization for Standardization) protocols are available.

Table 1.1: OECD guidelines and ISO protocols available for performing aquatic toxicity tests for water quality control in the laboratory.

Test code	Description
OECD 201	Algae, Growth Inhibition Test
OECD 202	*Daphnia* sp. Acute Immobilisation Test and Reproduction Test
OECD 203	Fish, Acute Toxicity Test
OECD 204	Fish, Prolonged Toxicity Test: 14-Day Study
OECD 210	Fish, Early-Life Stage Toxicity Test
OECD 211	*Daphnia magna* Reproduction Test
OECD 212	Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages
OECD 215	Fish, Juvenile Growth Test
ISO 6341:1996	Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) -- Acute toxicity test
ISO 7346-1:1996	Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)] -- Part 1: Static method
ISO 7346-2:1996	Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)] -- Part 2: Semi-static method
ISO 7346-3:1996	Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)] -- Part 3: Flow-through method
ISO 8692:2004	Freshwater algal growth inhibition test with unicellular green algae
ISO 10229:1994	Determination of the prolonged toxicity of substances to freshwater fish -- Method for evaluating the effects of substances on the growth rate of rainbow trout (*Oncorhynchus mykiss* Walbaum (Teleostei, Salmonidae))
ISO 10706:2000	Determination of long term toxicity of substances to *Daphnia magna* Straus (Cladocera, Crustacea)
ISO 11348-1:2007	Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) -- Part 1: Method using freshly prepared bacteria
ISO 11348-2:2007	Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) -- Part 2: Method using liquid-dried bacteria
ISO 11348-3:2007	Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) -- Part 3: Method using freeze-dried bacteria
ISO 12890:1999	Determination of toxicity to embryos and larvae of freshwater fish -- Semi-static method
ISO 20665:2008	Determination of chronic toxicity to *Ceriodaphnia dubia*
ISO 20666:2008	Determination of the chronic toxicity to *Brachionus calyciflorus* in 48 h

In addition to these laboratory toxicity tests, river water can also be monitored using biological early warning systems (BEWS). Table 1.2 presents an overview of biological early warning systems available for water quality control. A biological early warning system can be a system in which organisms are exposed in a continuous flow of water, such as occurs for example in the Kerren fish monitor (Table 1.2). Alternatively there are systems in which organisms are added to a water sample and the effect of exposure to this fixed water sample is measured after a certain exposure time, such as in the bbe (biological biophysical engineering) Algaetoximeter (Table 1.2).

Table 1.2: Overview of Biological Early Warning Systems (BEWS) used at monitoring locations along the Rhine or Meuse (with data from Diehl *et al*., 2006).

Biological early warning system	**Type of instrument**	**Organisms**	**Principle of measurement**	**Time Data output (minutes)**	**Producer**
Kerren fish monitor	Flow through system	*Leuciscus idus*	Avoidance behaviour of fish detected by touching sensors	12	Kerren, Viersen, Germany
bbe Fish Toximeter	Flow through system	*Leuciscus idus* or other fish species	Changes in behaviour of fish detected by video image processing	1	bbe Moldaenke, Kiel, Germany
Dreissena-Monitor	Flow through system	*Dreissena polymorpha*	Monitoring open/close position of mussels by reed contacts	5	Envicontrol, Frechen, Germany
Mosselmonitor®	Flow through system	*Dreissena polymorpha*	Monitoring the shell movements by electromagnetic fields	1-10	Aquadect, Brouwershaven, The Netherlands
bbe Daphnia toximeter	Flow through system	*Daphnia magna*	Changes in behaviour of daphnia detected by video image processing	1	bbe Moldaenke, Kiel, Germany
Dynamic Daphnia test	Flow through system	*Daphnia magna*	Activity of daphnia by passing IR light sensors	10	Electron GmbH, Krefeld, Germany
DF algae test	Organisms exposed to a fixed water sample	*Chlorella vulgaris*	Measurement of active algae by delayed flourescense detection	30-45	Regensburger University, Regensburg, Germany
bbe Algae toximeter	Organisms exposed to a fixed water sample	*Chlorella vulgaris*	Measurement of active algae according to Genty measurements	30-45	bbe Moldaenke, Kiel, Germany
TOXcontrol	Organisms exposed to a fixed water sample	*Vibrio fischeri*	Measurement of the decrease in bioluminescence	30-45	Microlan BV, Waalwijk, The Netherlands
Regensburger Luminescent Bacteria test	Organisms exposed to a fixed water sample	*Vibrio fischeri*	Measurement of the decrease in bioluminescence	30-45	Regensburger University, Regensburg, Germany

Both types of fully automated systems provide data about the acute toxicity of the monitored water, with a frequency varying from 1 data point every minute to 1 data point every 45 minutes. When a data point is exceeding a given threshold limit, an alarm can be sent to the duty manager of the drinking water treatment plant and follow up actions can be started (like for instance interrupting the abstraction of the surface water).

For a BEWS, the requirements can be the following (adapted from Brosnan,1999):

- It provides an alarm early enough for taking actions
- It covers all potential threats
- It is sensitive to quality changes of the river (at regulatory levels)
- It gives minimal false-positive or false-negative alarms

Apart from the sensitivity at regulatory levels (which in the Netherlands is defined as below < 0.5 µg/L for the total concentration of pesticides), the BEWS are fulfilling the stated requirements.

The mentioned assays (Table 1.1 and 1.2) detect adverse effects of water samples containing toxic substances on organisms or cells. The adverse effects detected in the various bioassays may represent overall toxicity or specific types of toxicity such as neurotoxicity or genotoxicity. Genotoxicity reflects an interaction of the chemical or its metabolite or degradation product with the DNA or chromosomes of the cells. This interaction can lead to changes in the DNA (mutations) or damage to chromosomes resulting in biological effects in the organism itself or in their offspring in the next generation. To monitor these kinds of adverse effects caused by chemicals present in the surface water, several genotoxicity tests are available. Since genotoxicity of surface water was one of the important endpoints studied in the present thesis, Table 1.3 presents an overview of the various OECD guidelines and ISO protocols available for performing aquatic genotoxicity tests in the laboratory. Figure 1.2 presents the principles of some of these assays in some more detail.

Table 1.3: OECD guidelines and ISO protocols available for performing aquatic genotoxicity tests in the laboratory

Test code	Description
OECD 471	Bacterial Reverse Mutation Test (Ames)
OECD 473	*In vitro* Mammalian Chromosome Aberration Test
OECD 474	Mammalian Erythrocyte Micronucleus Test
OECD 476	*In vitro* Mammalian Cell Gene Mutation Test
OECD 479	*In vitro* Sister Chromatid Exchange Assay in Mammalian Cells
OECD 480	*Saccharomyces cerevisiae*, Gene Mutation Assay
OECD 481	*Saccharomyces cerevisiae*, Miotic Recombination Assay
OECD 482	DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells *in vitro*
ISO 16240:2005	Determination of the genotoxicity of water and waste water -- *Salmonella*/microsome test (Ames test)
ISO 13829:2000	Determination of the genotoxicity of water and waste water using the umu-test
ISO 21427-1:2006	Evaluation of genotoxicity by measurement of the induction of micronuclei -- Part 1: Evaluation of genotoxicity using amphibian larvae
ISO 21427-2:2006	Evaluation of genotoxicity by measurement of the induction of micronuclei -- Part 2: Mixed population method using the cell line V79

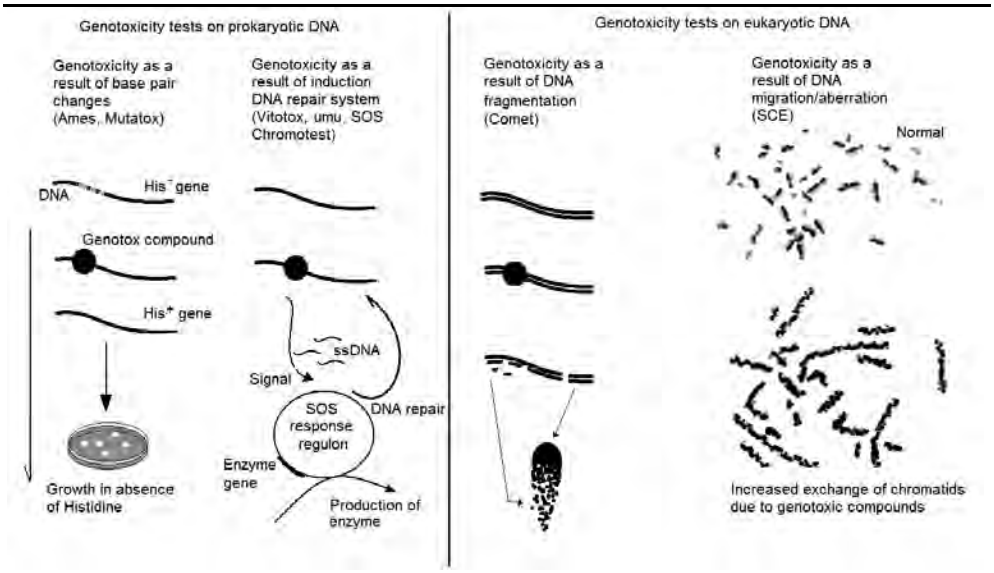

Figure 1.2: Principles of some important genotoxicity test, including tests for genotoxicity in prokaryotic DNA (Ames test, Mutatox, Vitotox, umu and SOS Chromotest ; left Panel) and tests for genotoxicity in eukaryotic cells (Comet and Sister Chromatid Exchange (SCE) test; right Panel). (Adapted from Hoogenboezem and Penders, 2003).

Results of quality control of River Rhine surface water

The first study using bioassays to monitor the toxicity of surface waters in the Netherlands took place in the late seventies. In this study the toxicity of Rhine water was determined by studying its effects on rainbow trout (*Salmo gairdneri*, R) after 18 months of exposure (Poels *et al*., 1978). The decrease in growth of rainbow trout in river Rhine water compared to growth of the control group and the significant increase in liver weight in the exposed versus the control group clearly indicated that river Rhine water had adverse chronic effects on the rainbow trout. In 1981 and 1982, organic concentrates of rivers Rhine and Meuse were tested for toxicity on guppies (*Poecilia reticulata*). Lethal effects among the fish were observed at concentration factors higher than 10 (Slooff *et al*., 1983). Fish were also the first organisms used as biological sensor in BEWS. At a Rhine water abstraction point in Nieuwegein (Water transport company Rijn Kennemerland), the rainbow trout was used in 1982, followed by the golden ide (*Leuciscus idus*) in 1986. Due to the demand for systems, which were more sensitive to selective group of compounds (herbicides) or present the acute toxicity to other trophic levels, from 1994 onwards, BEWS with other organisms, like *Daphnia magna*, *Dreissena polymorpha*, *Chlorella vulgaris* and *Vibrio fischeri* were also in operation (Vinke, 2002) at that location. Other intake stations along the river Meuse are equipped with several BEWS as well.

In the first study in which the genotoxic potential of surface water of the river Rhine was determined, fish of species *Umbra pygmaea* were exposed during 3, 7 and 11 days to Rhine water and an increase in chromosome aberrations in gill cells was observed (Prein *et al*., 1978). Also in this study, a doubling of mutants was detected in the Ames TA100 strain, when aromatic bases and aromatic hydrocarbons were extracted from water and tested in the presence of metabolic activating liver fraction.

In another study, the Ames genotoxicity test was performed on XAD extracts of waters, concentrated following the procedure described by Kool *et al*. (1981), and obtained during the production of drinking water from surface water (Kool *et al.*, 1982). In this study, it was concluded that “dune infiltration in combination with activated carbon (powder) and slow sand filtration were shown to be very effective in a waterwork in removing organic mutagens present in water”.

Thanks to the fact that XAD extracts of surface water of the rivers Rhine and Meuse were tested in the Ames test at regular time intervals by the RIWA, a time dependent decline in the genotoxicity of the river Rhine could be documented over the period 1980 till 2000. Figure 1.3 presents the data corroborating this conclusion.

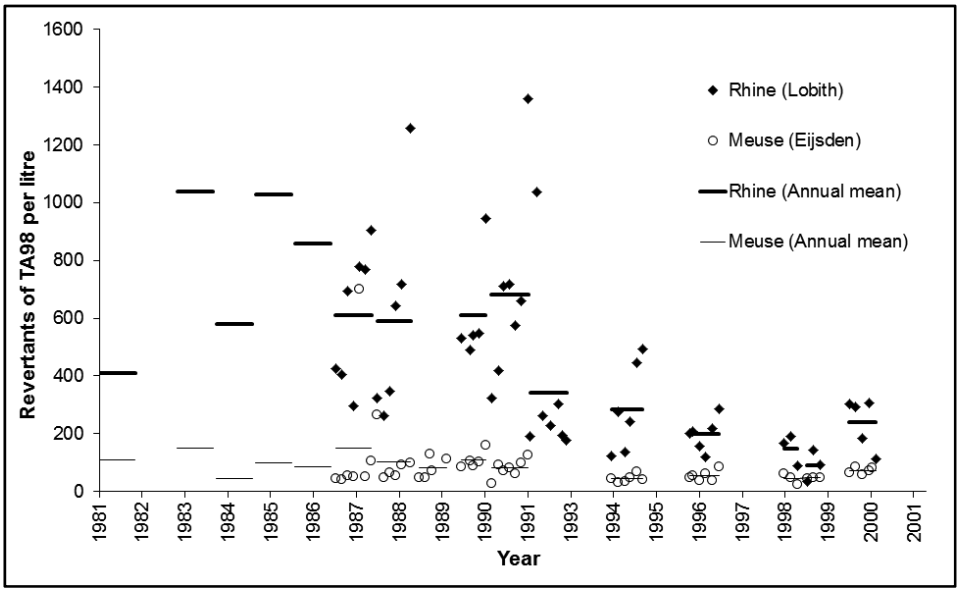

Figure 1.3: Decline of the genotoxicity of the River Rhine measured with the Ames TA98 assay using XAD based concentration of the water samples, as compared to the lower genotoxicity of the river Meuse (Adapted from Hoogenboezem and Penders, 2003).

However, in spite of this decline in the (geno)toxicity of surface water of the River Rhine over the years, there is at present still a need for continued quality control and bioassays with increased sensitivity because of i) the increasing number of chemicals present in surface water, and ii) the fact that concentrations of chemicals of concern may be toxicologically relevant but below the level of detection for toxicity in currently applied protocols for bioassays or biological early warning systems.

Aim and outline of the thesis

Given the need for continued quality control of surface waters used for the production of drinking water by state-of-the-art bioassays and biological early warning systems, the objective of the present thesis was to validate and improve some of the bioassays and biological early warning systems used for quality control of surface water. To achieve this overall objective the following studies were performed:

(i) determine which battery of (geno)toxicity tests and procedures is optimal to detect present trends in the water quality of surface waters (Chapter 2 of this thesis),

(ii) determine if an automated sample concentration procedure can be incorporated in currently applied biological early warning systems (BEWS), in order to increase their sensitivity, and still allow on-line detection of toxicity (Chapter 3).

(iii) evaluate results from *in vitro* genotoxicity testing of surface water extracts against results from *in vivo* genotoxicity tests under nearly field conditions with non-concentrated water samples, in order to detect if any genotoxicity detected *in vitro* will also be displayed *in vivo*, and if genotoxicity can be measured in water samples as such without the need for sample concentration (Chapter 4 and 5)

(vi) obtain information on the genotoxicity of formed products when surface water with its organics is treated with a newly developed advanced oxidation UV/peroxide process in the production of drinking water from surface water (Chapter 6)

The river Rhine is selected as a model river for our studies. The results obtained will enable further improvement and optimisation of procedures for effect based water quality control of surface water used for the production of drinking water.

Chapter 1 of this thesis presents an introduction to the topic and the aim of the thesis.

Chapter 2 of this thesis presents the data obtained in a variety of *in vitro* bioassays for several extracts of Rhine water and the evaluation of the different bioassays and genotoxicity tests in relation to sensitivity, selectivity and suitability for future routine monitoring programs. Ecological risk model evaluations based on either chemical analytical data or bioassay data are included to judge the ability of the *in vitro* bioassays to detect effects on the different organisms in the ecosystem.

Chapter 3 focuses on the development and validation of Solid Phase Extraction (SPE) as an online water concentration step for two BEWS, the bbe Algaetoximeter and the TOXcontrol system.

Chapter 4 describes the results from a study quantifying the *in vivo* genotoxicity of the river Rhine and the comparison of these data to those of similar studies performed 27 years ago with the same experimental design, in order to measure the effect of Rhine water on the induction of SCE in the Eastern mudminnow fish (*Umbra pygmaea*). Also results with a new test system, the single cell gel electrophoresis assay (Comet assay), were compared to the results of the SCE assay.

Chapter 5 reports whether prolonged exposure of the fish results in a further increase in *in vivo* genotoxicity, whether new data corroborate that *in vivo* genotoxicity of Rhine water is at present lower than in 1978, and whether the Comet assay is a suitable alternative to the SCE assay.

Chapter 6 presents the data of the genotoxicity of samples collected from the water treatment plant Andijk, applying UV/H_2O_2 treatment. Genotoxicity was tested *in vitro* using the Ames and Comet assay. Samples were also tested in *in vivo* genotoxicity tests in Eastern mudminnow fish (*Umbra pygmaea*). Genotoxicity was analysed by the Sister Chromatid Exchange (SCE) and the Comet assay performed with isolated gill cells.

Chapter 7 includes a summary, discussion and future perspectives of the research described in this thesis.

Al together the studies presented provide an overview of the trends in the quality of surface water from river Rhine over the last decades and indicate how bioassays and biological early warning systems used for quality control of surface water can be further improved and used for future quality control in the process of producing drinking water from surface water.

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Chapter 2

Evaluation of existing *in vitro* bioassays for toxicity testing of surface water

This chapter is based on: E.J.M. Penders, I.M.C.M. Rietjens, G. Alink, and W. Hoogenboezem, “Evaluation of existing *in vitro* bioassays for toxicity testing of surface water”, submitted.

Abstract

In vitro bioassays that monitor the toxicity of surface water used for the production of drinking water provide an important addition to existing chemical, physical and biological parameters. The *in vitro* toxicity bioassays applied may detect general toxicity or genotoxicity. The Association of River Water Works (RIWA) includes the Ames test in its measuring program already for over a decade and the objective of the present paper was to evaluate the performance of additional *in vitro* assays in monitoring the toxicity as part of the quality assessment of surface water used for the production of drinking water and to define the optimal *in vitro* bioassay test battery. To this end the paper presents the data obtained in a variety of *in vitro* bioassays for several extracts of Rhine water and the evaluation of the different bioassays and genotoxicity tests in relation to sensitivity, selectivity and suitability for future routine monitoring programs. Ecological risk model evaluation based on bioassay data is included to judge the ability of the *in vitro* bioassays to detect effects on the organisms in the ecosystem as a whole. It is concluded that an optimal bioassay battery of *in vitro* tests for water quality monitoring of the River Rhine includes the *Daphnia* IQ assay, the *Raphidocelis* sp. PAM test and the Microtox® test as general toxicity tests, together with the Ames TA98 test (with metabolic activation) to monitor genotoxicity. The results also reveal that the water quality of the River Rhine has improved over the years. The potentially affected fraction of organisms which is predicted to experience adverse effects of chemicals present in the surface water is lower than 5%. All together, it is concluded that the overall toxicity measured with bioassays provide essential information about the quality of surface water that is not obtained by chemical analysis.

2.1 Introduction

The water of the River Rhine is an important source for the production of drinking water in The Netherlands. Over 200 million m^3 surface water is yearly abstracted from the River Rhine by the drinking water companies Waternet and PWN in the Netherlands. After several treatment processes of this surface water, the drinking water is provided to 2.5 million consumers (Geudens, 2007). The Association of River Water Works (RIWA), a cooperative venture by the Dutch water supply companies which use surface water for the preparation of drinking water, strives for a quality level in the surface water of the Rhine catchment basin, so that simple purification processes are adequate to produce flawless drinking water (Anonymous, 2008). To achieve this objective RIWA-Rhine monitors the quality of water in the Rhine catchment basin. For a state-of-the-art monitoring system RIWA considers it essential to include biological tests in its measuring program to supplement the chemical, physical and biological parameters. In the previous decades RIWA undertook several toxicological studies on the quality of the River Rhine. An example is the study in which samples were taken from various sections of the Rhine basin and subjected to a number of different chemical, toxicological and biological analyses (Van Genderen *et al.*, 1997). RIWA has also carried out research into the genotoxicity of Rhine and Meuse water by means of the Ames test (Veenendaal & Van Genderen, 1997). In spite of the fact that the chemically detected contamination burden in the River Meuse is often higher than that in the Rhine, these tests have demonstrated higher genotoxicity of samples from Rhine water. This observation indicated the importance of *in vitro* bioassays that monitor the toxicity of surface water used for the production of drinking water as an important addition to chemical, physical and biological characterisation of water quality. The *in vitro* bioassays applied may detect general toxicity or genotoxicity. The objective of the present paper was to evaluate the performance of a wide range of *in vitro* bioassays used in the previous years by RIWA for monitoring the toxicity as part of the quality assessment of surface water used for the production of drinking water and to define the optimal *in vitro* bioassay test battery. To this end the paper presents the data obtained in a variety of *in vitro* bioassays for of several extracts of Rhine water and the evaluation of the different bioassays and genotoxicity tests in relation to sensitivity, selectivity and suitability for future routine monitoring programs. The bioassays should be straightforward and fully validated laboratory tests, thus directly suitable for implementation in routine water laboratories. Based on the results obtained the present study also presents an ecological risk model evaluation on bioassay data calculating the toxic potency pT value (De Zwart and Sterkenburg, 2002), to judge the ability of the *in vitro* bioassays to detect effects on the organisms in the ecosystem as a whole.

2.2. Materials and methods

Sampling locations

Samples of River Rhine surface water were obtained at location Lobith (Figure 2.1). This location was selected for the present study for two main reasons:

1. The availability of an extensive package of chemical-analytical data for samples from this location at different organisations Waterdienst and RIVM (National Institute for Public Health and the Environment) and laboratories of several waterworks.

2. The availability of data from previous (geno)toxicity measurements for samples from this location.

To extend the series of samples for the genotoxicity test, additional samples were collected at location Nieuwegein (Figure 2.1). Samples were taken over the whole year of 1998 and in 2000 (as indicated).

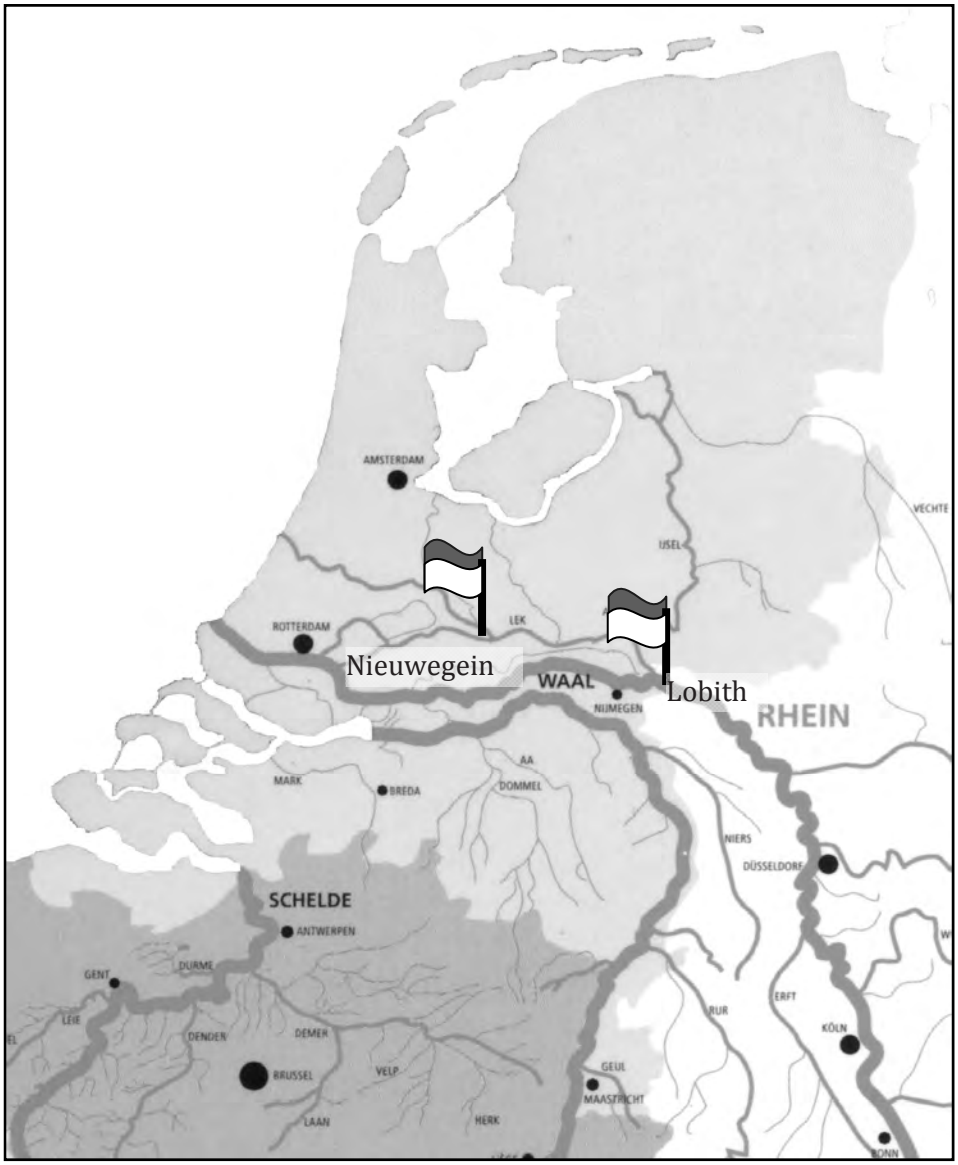

Figure 2.1: Sampling locations of the present study in the Netherlands.

For samples tested in *in vitro* bioassays for general toxicity, a volume of 100 litre of surface water, collected at Lobith, was transported to the RIVM (Bilthoven, The Netherlands), where samples were concentrated by extraction as described hereafter within 48 hours. Extracts were stored at -20 °C. Twenty-four hours before the extracts were sent to the laboratories VITO (Mol, Belgium), Waterdienst (Lelystad, The Netherlands) and RIVM carrying out the toxicity tests, they were diluted to the correct volume by means of the dilution medium required for each individual test.

For samples to be tested in the genotoxicity tests, 300 litre of surface water, collected at Lobith or Nieuwegein, was concentrated by extraction as described below, either at the sampling site or immediately after transport to KIWA Water Research (KWR) Nieuwegein. Extracts were dissolved in ethanol and sent to the testing laboratories VITO, KWR, Waterdienst and RWTH (Aachen, Germany) where they were stored at -20 °C until analysis.

Concentration procedures

Due to differences in sensitivities between the different bioassays, different concentration procedures were applied.

Concentration procedure bioassays for general toxicity
Water samples to be analyzed by the bioassays for general toxicity were concentrated following the procedure described by Roghair *et al.* (1997) based on solid phase extraction with XAD as absorbent. The XAD-4 (Rohm & Haas, Antwerp) and XAD-8 (DAX-8, Supleco) resins were purified thoroughly before they were used for concentrating the samples. By means of these resins, non-polar or mildly polar components will be extracted (Verweij *et al.*, 2010). Within 48 hours after sampling, the 100 litre non-filtered sample was divided to 10 litre borosilicate bottles, to which a resin mixture (with an XAD4/XAD8 ratio of 1:1) was added with a concentration of 2 ml resin mixture per litre water. Mixing took place by rolling the bottles at 20 °C in the dark for 24 hours, after which the resin granules were sieved from the bottles. The granules were dried using an air flow until their weight had stabilized to less than 6 grams granules per 20 ml. After drying, the ten different XAD batches of one sample were mixed and packed into an elution column. Elution took place using a bed volume of acetone. The acetone concentrate was transferred into a conical tube of a Kuderna-Dänisch distillation apparatus. Two ml of mineral water was added and the acetone was evaporated at 65 °C for approximately 30 minutes. Distillation was stopped when volume reduction and boiling symptoms ceased. The residue was purged for 20 minutes using a precisely tuned nitrogen stream in order to reduce the remaining acetone concentration to less than 0.1 % (v/v), which will not result in a positive result in the

toxicology test. After purging, EPA (Environmental Protection Agency, US) medium was added until a final volume of 10 ml was reached. The EPA medium is a moderately hard synthetic freshwater medium and consists of 96 mg $NaHCO_3$, 60 mg $CaSO_4.2H_2O$, 60 mg $MgSO_4$ and 4 mg KCl in one litre of deionized water, adjusted to pH 7.8. The water concentrate had a concentration factor of 1000 and was stored at -20 °C until further analysis.

Concentration procedure bioassays for genotoxicity

Water samples to be analyzed by the bioassays for genotoxicity were concentrated following the procedure developed by KWR (Noordsij *et al.,* 1983) in which XAD-4 (Amberlite) is used in a column with a bed height of 20 cm and a bed volume of 300 ml. Via a siphon system, the sample (300 litre) was conducted from a reservoir to the vertical resin column at a speed of 1 bed volume per minute. After the neutral sample (pH = 7) was pumped into the first column, the filtrate was brought in-line to pH = 2 by means of hydrochloric acid, and subsequently loaded on to a second XAD column. Both loaded XAD columns were rinsed with 10 bed volumes of ultra pure water (for the acid column, the water was first brought to pH = 2). After drying the columns with nitrogen gas, elution took place with five bed volumes of pure ethanol and five bed volumes of an azeotropic mixture with 30% (v/v) pure ethanol in cyclohexane. After this elution, the eluates were filtered with a 0.45 µm PTFE filter. Subsequently the eluates were concentrated by means of evaporation of eluens by heating and evaporation facilitated with a gentle flow of nitrogen gas, leading to the ultimate concentration factor of 25,000.

Test battery

An important aspect of a battery of tests is that it represents components of various parts of the ecosystem such as primary producers (algae), consumers (invertebrates) and decomposers, as well as tests determining effects on the genetic material (DNA).

In the various bioassays in which the concentrated water extracts were analyzed, the ultimate parameter used to quantify the toxicity is the LCf_{50} (Lethal Concentration factor 50%) value or the ECf_{50} (Effect Concentration factor 50%) value. This is the concentration factor resulting in the sample that results in 50% lethality or effect in the test population. The lower the LCf_{50} or ECf_{50} value, the more toxic the sample.

Bioassays for general toxicity

Bacterial assays

One bacterial test, the Microtox® test, was included in the test battery of the present study. In the Microtox® test, freeze-dried bacteria *Vibrio fisheri* are used, and after 1.5 hours of reconstitution, applied to a series of diluted extracts. Dilution of the extracts is performed with a solution of 20 g NaCl per litre. The bacteria are emitting light when their metabolism is normal. After 5 to 15 minutes of incubation at 15 °C, the luminescence level is determined with a luminosity meter (Bulich, 1979; Bulich and Isenberg, 1981). When the bacteria are in a toxic stress situation, a reduction in light emission can be observed and measured. On the basis of the numbers obtained the ECf_{50} value is determined, defining the concentration factor of the sample that results in 50% decrease in light output in comparison with the control.

Algae assays

The test battery of the present study contained three algae tests including the Micro Plate algae test performed with species *Raphidocelis subcapitata* and *Scenedesmus subspicatus* and the Pulse-Amplitude-Modulation (PAM) algae test with the species *Raphidocelis subcapitata.*

In the Micro Plate algae test, algae of the species *Raphidocelis subcapitata* and *Scenedesmus subspicatus* are added to a series of diluted extracts from the sample. Dilution of the extracts is performed with EPA medium. During the 72 hours of incubation, the population growth is measured by determining the number of cells by means of a cytofluorescent meter. On the basis of the numbers obtained the ECf_{50} value is determined, defining the concentration factor of the sample that resulted in 50% growth inhibition in comparison with the control.

In the PAM algae test, *Raphidocelis subcapitata* algae are added to a series of diluted extracts and incubated for a period of 4.5 hours under continuous lighting at 20 °C. Dilution of the extracts is performed with EPA medium. After this incubation period, the photochemical efficiency, or the photon yield, is determined with the Pulse-

Amplitude-Modulation fluorescent meter (Genty *et al.*, 1989; Hofstraat *et al.*, 1994). The ECf_{50} value is determined as the concentration factor that resulted in 50% reduction of the photochemical efficiency.

Invertebrate assays

Three invertebrate bioassays were included in the test battery of the present study, namely the ROTOX kit F, the Thamnotox kit F and the *Daphnia* IQ test.

In the Rotox kit F, rotifers (*Brachionus calyciflorus*) are used as test organisms. These rotifers are raised from cysts (Janssen *et al.*, 1993; Snell and Persoone, 1989; Snell *et al*., 1991) and placed in EPA medium with lighting for 16 to 18 hours at 25 ^{o}C. Within 2 hours, the rotifers are added to a dilution series of the extract in polystyrene multititre plates. Dilution of the extracts is performed with EPA medium. After a 24-hour incubation period in the dark, the survivors are counted by means of a stereo microscope. The ECf_{50} value is determined as the concentration factor that results in the sample that causes 50 % mortality.

In the Thamnotox kit F, the crustacean *Thamnocephalus platyurus* is used as test organism. These test organisms are raised from cysts from the test kit (Centeno *et al*., 1993) in medium under 24 hours light conditions at 25 ^{o}C. These organisms are acclimatized to the dilution medium for 4 hours, after which the extract is added in different dilutions. The dilution medium used consists of EPA medium. The tests are carried out in sealed off glass vials. After 24 hours of incubation in the dark, the number of surviving individuals is counted. The ECf_{50} value is determined as the concentration factor that results in the sample that causes 50 % mortality.

The *Daphnia* IQ test is an enzymatic inhibition test applied to non-fed young (less than 24 hours) *Daphnia magna*, which are exposed to a dilution series from the water extract. Dilution of the extracts is performed with EPA medium. After 1 hour of incubation a tracer compound is applied (4-methyl- umbelliferyl-ß-D-galactose) (*Daphnia* IQ, Aqua Survey Inc., 1993). After 15 minutes of incubation, the fluorescence of each *Daphnia* upon UV irradiation was scored by eye. The toxicity is determined on the basis of the inhibition of the enzymatic splicing of the galactose from the tracer. The lower the fluorescence production, the more toxic the sample analyzed. On the basis of the data obtained the ECf_{50} value is determined.

Data analysis

From the results of the bioassays, the ECf_{50} values and confidence limits are obtained via a dose effect curve from a logistics response model (Haanstra *et al.*, 1985). The LCf_{50} values and confidence limits are determined by means of the Spearmann-Kãrber method (Hamilton *et al.*, 1977, 1978).

Bioassays for genotoxicity

In this study several genotoxicity tests were applied in which damage in prokaryotic or eukaryotic DNA can be detected, and they included the Ames test, the umu test, the VITOTOX® test as well as the Comet assay performed in either human lymphocytes or *Daphnia*.

The Ames test was conducted according to the method described by Maron and Ames (1983) using *Salmonella typhimurium* strain TA98 for the detection of genotoxic substances causing frame shift mutations. In the umu genotoxicity test (Oda *et al*., 1985; Reifferscheid *et al*., 1991; Reifferscheid and Heil, 1996) a modified strain (TA1535/pSK1002) of the bacterium *Salmonella typhimurium* is used, detecting genotoxic compounds which cause DNA damage and the induction of the SOS-DNA recovery system. The same kind of genotoxic compounds are also detected using the VITOTOX® test (Van der Lely *et al*., 1997). In this assay a modified strain of *Salmonella typhimurium* namely strain TA104 *recN*2-4/TA104 *pr1* is used. Samples for the Ames, umu and VITOTOX® test were tested in duplicate, with and without a rat liver S9 metabolic activation system.

For the detection of DNA damage in eukaryotic DNA, the alkaline Comet assay was used (Tice, 1995). Human lymphocytes are exposed *in vitro* to the extract during 2 hours, after which the cells are lysed in a gel on a microscope slide. Also cells from *Daphnia* after 48 hours exposure to the extract are lysed. After gel-electrophoresis of the slides, the 'comets' are analyzed after staining with ethidium bromide by means of a fluorescence microscope. The content of DNA in the tail is the parameter for DNA damage in human lymphocytes and tail length for DNA damage in *Daphnia*.

With genotoxicity tests, the Lowest Observed Effect Concentration factor (LOECf) is determined, based on criteria defined for each test to judge when a sample is considered positive. The criterium for determining the LOECf of the Ames test is that a sample was judged positive when it displayed two times or more the number of revertants per litre compared to the negative control sample. The results for a sample tested in the umu test are considered positive when the induction factor is more or equal to 1.5 compared to the negative control sample. A positive result in the VITOTOX® is defined based on whether the signal from the test strain is 1.5 times higher than that of the primary strain.

For the Comet assay, the LOECf is determined by statistical analysis of the data. Statistical analysis is performed by the Kruskall-Wallis test for non-parametrical observations (*Daphnia* Comet assay) or Mann-Whitney U test (Lymphocytes Comet assay).

Correlations
Correlation between different bioassays is determined with the Pearson correlation on log-transformed data. A difference in response between the used genotoxicity tests, between presence and absence of metabolic activation and between acidity of the water sample is determined using the Mann-Whitney-U test.

Chemical analysis
The chemical analysis of water samples from the sampling location Lobith was performed at the institute Waterdienst, Lobith, The Netherlands. Using solid phase extraction, HPLC and GCMS, a large array of organics and other micro-pollutants were measured. Specific information about the performed analysis can be obtained from the first author.

Ecological risk model evaluation
Ecological risk model evaluation was carried out based on bioassay data in order to judge the ability of the *in vitro* bioassays to detect effects on the organisms in the ecosystem as a whole. For individual chemicals, the maximum permissible environmental concentration is generally derived from the No Observed Effect Concentration (NOEC), measured by performing toxicity tests with a single species using different concentrations of the compound. In its simplest form, the risk of the chemical in the ecosystem can be evaluated (Slooff *et al*., 1986) by regression analysis between the single species NOECs and data from field experiments.

A more sophisticated procedure is the use of a distribution in which the sensitivity of a set of species towards a compound is described. The ecotoxicological risk of one compound in water to a whole range of aquatic species can be estimated by fitting a generic species collection SSD (Species Sensitivity Distribution) log-logistically to no effect concentration (NOEC) values obtained in bioassays with different species.

According to De Zwart and Sterkenburg (2002), the ecotoxicological risk of a mixture of compounds in water for aquatic species, can be estimated by fitting a generic species collection SSD (Species Sensitivity Distribution) log-logistically to no effect concentration factor (NECf) values obtained in bioassays with different species.
De Zwart (2002) presented statistics indicating that for many compounds, the no effect level for toxicity averaged over species is a factor 10 lower than the average acute toxicity. Therefore, for estimating the NECf values, the ECf_{50} or LCf_{50} values measured by the different bioassays are divided by a factor 10.

The log-logistic SSD is characterized by only two values:

- The α (alpha), i.e. the average of the ^{10}log-transformed NECf values from the different bioassays used
- The ß (beta), i.e. the angle of inclination of the curve fitted. Beta is proportional to the standard deviation of the ^{10}log-transformed NECf values from the different bioassays used (ß = $\sqrt{3}/\pi$ * standard deviation of the ^{10}log-transformed NECf values)

With this fitted log-logistic SSD distribution (formula 1), the potentially affected fraction of species can calculated depending on the NECf values.

$$Potential\ affected\ \ fraction\ of\ \ species = \frac{1}{1 + e^{-\frac{^{10}\log NECf - \alpha}{\beta}}} \qquad (1)$$

The toxic potency (pT) of one sample, expressed in potentially affected fraction of aquatic species which are expected to display an adverse effect upon the exposure to the original non-concentrated water sample, can be calculated by substituting 1 for the NECf factor.

Next to the calculation of the toxic potency of surface water samples (pT sample), the toxic potency of blank water samples were also calculated (pT blank). With both values, the corrected toxic potency of the surface water is calculated (formula 2).

$$pT_{sample\ \ corrected} = \frac{pT_{sample} - pT_{blank}}{1 - pT_{blank}} \qquad (2)$$

The mean ecotoxicological risk, in which a fraction of aquatic species is affected by the compounds present in several non-concentrated water samples, is the geometric means of the individual pT values of the samples measured.

2.3. Results and discussion

Physico-chemical water quality and flow rate of the River Rhine

Differences in flow rate and physico-chemical water quality parameters of the River Rhine as obtained during the sampling years for the bioassays in the period 1998-1999 and documented in the International Commission for the Protection of the Rhine (ICPR) waterbase are presented in Table 2.1. The highest flow rate of 9413 m^3/s was observed in November 1998 and the lowest (1372 m^3/s) two months earlier (Table 2.1). With the high flow rate the amount of suspended solids (TSS), the dissolved organic carbon (DOC), total nitrogen (TN) and phosphorous (TP) in the Rhine water increased while there is a decrease in the chloride amount in the water and the conductivity. These results indicate more concentrated amounts of various substances in periods of low discharge and dilution during high discharge periods.

Table 2.1: Physico-chemical water quality and flow rate of the River Rhine 1998-1999 obtained from the ICPR database, Kolblenz, Germany.

Sample location and date	**Flow rate (m^3/s)**	**Chloride (mg/L)**	**TSS (mg/L)**	**DOC (mg/L)**	**Temperature (°C)**	**Conductivity (µS/cm)**	**TN (mg/L)**	**TP (mg/L)**
Lobith Jul 15 (98)	1636	134.6	19	3	22.0	840	3.74	0.17
Lobith Sept 9 (98)	1372	141.2	14	2	21.7	694	3.61	0.21
Lobith Nov 4 (98)	9413	44.4	110	6	8.7	572	4.76	0.45
Lobith Feb 24 (99)	6733	75.0	210	4	6.0	650	5.10	0.47
Lobith Apr 21 (99)	3528	76.0	28	3	11.5	631	4.08	0.11
Lobith Jul 16 (99)	2532	64.0	27	2	22.2	646	2.85	0.15

In addition to the measurement of the physico-chemical water quality and flow rate of the River Rhine, Waterdienst performed also analyses of several organic micro-pollutants on water samples at the same date as when the collection of water sample occurred for the bioassays. A set of organic micropollutants is selected and presented in Table 2.2, in which in at least one sample, a measurement was obtained above the detection limit. The concentration of the organic micro-pollutants in individual samples is presented (Table 2.2) next to the calculated toxic units. The toxic unit is the ratio between the concentration of the compound measured and the NOEC of the compound. Also in Table 2.2, the total toxic unit of the individual sample is presented.

Table 2.2: Set of organic compounds measured in different Rhine water samples with the individual Toxic Units and total Toxic Units.

Organic compound	Cas number	NOEC IUCLID[#] (µg/L)	Lobith Jul 15 (98) (µg/L)	T.U.	Lobith Sept 9 (98) (µg/L)	T.U.	Lobith Nov 4 (98) (µg/L)	T.U.	Lobith Feb 24 (99) (µg/L)	T.U.	Lobith Apr 21 (99) (µg/L)	T.U.	Lobith Jul 16 (99) (µg/L)	T.U.
Diazinon	333415	0.17	0.01	5.9E-02	<0.01		<0.01		<0.01		<0.01		<0.01	
Diuron	330541	**2.2**	<0.05		<0.05		<0.05		<0.05		<0.05		0.06	2.7E-02
Lindane	58899	2.9	0.002	6.9E-04	0.002	6.9E-04	0.002	6.9E-04	0.002	6.9E-04	0.003	1.0E-03	n.a.	
Isoproturon	34123596	**8**	<0.05		<0.05		0.36	4.5E-02	<0.05		0.17	2.1E-02	<0.05	
Atrazin	1912249	10	<0.05		<0.05		<0.05		0.04	4.0E-03	0.03	3.0E-03	0.09	9.0E-03
Chlorotoluron	15545489	24	<0.05		<0.05		0.11	4.6E-03	<0.05		<0.05		<0.05	
Simazin	122349	**29**	0.01	3.4E-04	0.02	6.9E-04	<0.01		0.02	6.9E-04	0.02	6.9E-04	0.02	6.9E-04
Glyphosate	1071836	**130**	0.08	6.2E-04	0.07	5.4E-04	<0.05		<0.05		0.05	3.8E-04	0.06	4.6E-04
Trichloroethene	79016	150	n.a.		n.a.		n.a.		0.02	1.3E-04	0.01	6.7E-05	0.01	6.7E-05
Tetrachloroethene	127184	1000	n.a.		n.a.		n.a.		0.04	4.0E-05	0.03	3.0E-05	0.02	2.0E-05
Benzene	71432	3100	n.a.		n.a.		n.a.		0.01	3.2E-06	0.01	3.2E-06	<0.01	
Trichloromethane	67663	3100	n.a.		n.a.		n.a.		0.02	6.5E-06	0.02	6.5E-06	0.01	3.2E-06
1,2-Dichloroethane	107062	11000	n.a.		<0.5		<0.5		0.01	9.1E-07	0.06	5.5E-06	0.01	9.1E-07
2-(2,4-dichlorophenoxy)propionic acid, 2,4-DP	120365	46400	<0.05		0.16	3.4E-06	0.13	2.8E-06	0.17	3.7E-06	<0.05		<0.05	
2-Methyl-4-chlorophenoxy propionic acid, MCPP	7085190	n.a.	<0.05		0.22		<0.05		n.a.		<0.05		n.a.	
Bentazon	25057890	n.a.	<0.05		<0.05		0.07		0.07		0.09		0.07	
∑ Toxic Units over above compounds				6.0E-02		1.9E-03		5.0E-02		5.6E-03		2.6E-02		3.8E-02

T.U. = Toxic Units; n.a.= not available; # = lowest NOEC from ecotoxicology data in the International Uniform Chemical Information Database (IUCLID, 2000). Bold number is an estimation of the NOEC by dividing the lowest reported EC_{50} value by 10.

Based on the concentration of organic compounds, the total Toxic Units range from 0.0019 to 0.06. The values are lower than the value of 1, which is a threshold value for toxicity. However, given that chemical analyses is directed at selected compounds, other substances of potential toxicity and concern may not be detected. This is why bioassays detecting overall toxicity may complement physico-chemical analysis of surface water quality.

Bioassays for overall toxicity

Table 2.3 presents the results of the testing of XAD4-concentrated water samples of the River Rhine in the various bioassays for overall toxicity. Considering the results obtained the most toxic sample was that collected in September 1998, since for all bioassays presented this sample showed the lowest LCf_{50} or ECf_{50} value, indicating that it required the lowest concentration factor to result in 50% lethality or effect. The sample of July 1999 was the least toxic for the bioassays based on organisms representing the trophic consumer level (*Daphnia* IQ, Thamnotox kit, Rotox kit), the sample February 1999 for the trophic producer level (*Raphidocelis* sp.Growth inhibition, *Raphidocelis* sp. PAM test, *Scenedesmus* sp. Growth inhibition) and the sample of April 1999 for the trophic decomposer level (Microtox ®). All together it can be concluded that with the current extraction steps the *in vitro* bioassays can all detect the presence of toxic compounds in surface water and that at different sampling times the nature of the toxic compounds and thus the bioassay and type of organism being most sensitive may vary. This indicates that in an optimal test battery for monitoring surface water quality an *in vitro* test for each trophic level needs to be included.

Table 2.3: The results of different bioassays on XAD4 concentrated water samples of the River Rhine and the corresponding blanks with 95% confidence limits.

Sample and date	*Daphnia* IQ		Thamnotox		Rotox kit		*Raphidocelis* sp. Growth inhibition		*Raphidocelis* sp. PAM test		*Scenedesmus* sp. Growth inhibition		Microtox®	
	ECf_{50}	95%	LCf_{50}	95%	LCf_{50}	95%	ECf_{50}	95%	ECf_{50}	95%	ECf_{50}	95%	ECf_{50}	95%
Lobith Jul 15 (98)	211.9	176-254	178.6	152-209	467.9	416-526	57.4	39.6-82.2	49.7	47-53	67.9	52.8-88.3	103.5#	94-113
Lobith Sept 9 (98)	86	72-104	73	64-83	128	114-145	32.9	22.0-48.0	34.8	31-39	56.6	16.0-194	47	41-54
Lobith Nov 4 (98)	139	105-184	79	66-93	216	192-244	60.7	43.6-83.2	51.7	48-55	60.6	40.5-90.3	47.7	40-58
Lobith Feb 24 (99)	87	69-109	98.2	79-124	288.4	261-318	97.4	∞	224.7	205-246	121.5	78.9-186	59.7#	51-71
Lobith Apr 21 (99)	115	94-142	108	92-130	259.3	233-289	75.1	∞	69.6	65-76	84.6	∞	445	317-625
Lobith Jul 16 (99)	300.3	n.d.	208.4	169-256	601.2	n.d.	54	45.1-63.1	75.1	69-81	87.1	∞	146.1#	122-175
Median value	126		103		274		59		60		76		47.7	
Blank Jul15 (98)	>250	n.d.	>500	n.d.	>500	n.d.	661	327-1339	2368	∞	>900	∞	100	80-124
Blank Sept 9 (98)	>250	n.d.	444	397-497	267	240-297	278	178-432	251	226-279	659	246-1760	327	235-456
Blank Nov 4 (98)	>250	n.d.	>500	n.d.	247	219-280	464	257-838	131	1-16000	>900	∞	98	86-116
Blank Feb 24 (99)	210	135-326	>500	n.d.	>500	n.d.	653	458-932	395	380-411	>900	∞	61	41-90
Blank Apr 21 (99)	>250	n.d.	>500	n.d.	>500	n.d.	317	∞	463	∞	811	719-914	118	104-135
Blank Jul 16 (99)	>250	n.d.	>500	n.d.	>500	n.d.	315	∞	509	∞	482	369-631	159	134-190

n.d. = not determined; ∞= confidence limit from 0 to infinity; # =excluded for evaluation due to comparable blank readings

Pearson correlation analysis of ^{10}log transformed ECf_{50} values of the bioassays (Table 2.4) reveal good correlations of the bioassays within the different trophic levels. For example, the ^{10}log transformed ECf_{50} values of the various algae tests show high correlation coefficients and this also holds for the ^{10}log transformed ECf_{50} values obtained in the three tests using invertebrates. The single decomposer Microtox® test results do not correlate with any of the other results. Moreover, no correlation was observed between algae tests and invertebrate tests. These results corroborate the importance of tests representing different trophic levels within an optimal test battery.

For the trophic consumer level tests, the order of sensitivity based on the median of all measurements (Table 2.3), is Thamnotox kit > *Daphnia* IQ > Rotox kit. Among the test at the trophic producer level, the order of sensitivity is *Raphidocelis* sp. growth inhibition > *Raphidocelis* sp. PAM test > *Scenedesmus* sp. growth inhibition. As concluded above, due to the correlation of the bioassays within the same trophic level, the selection of only one bioassay per trophic level is possible. Based on the results of the present study it can be concluded that for adequate and efficient water quality control based on *in vitro* bioassays with best performances, low costs, and providing results after a couple of hours incubation, the optimal selection of bioassays would be the *Daphnia* IQ, *Raphidocelis* sp. PAM test and the Microtox® test.

In 1994, several bioassays were performed at the same location (Van Genderen *et al.*, 1997). Also results from a measuring campaign of 2007 from the same location and procedure were available (Dirven *et al*., 2008). The results of five bioassays in samples from Lobith and measured in 1994, 1997 and 2007 are given in boxplots (Figure 2.2). The bioassays *Raphidocelis* sp. PAM test and Thamnotox kit were not selected or available for the bioassay set in 1994. It is apparent that the LCf_{50} values of 1994 were lower than in 1997 and that the values of 1997 were lower than in 2007. From this it can be concluded that the water quality of the River Rhine has improved considerably in the years following 1994. Most of the bioassays performed in 1997 and 2007 present LCf_{50} values above 100, indicating that for non-concentrated water chronic toxicity to aquatic organisms in River Rhine water is not expected (De Zwart and Slooff, 1993).

Table 2.4: Pearson correlation analyses of ^{10}log-transformed ECf_{50} and LCf_{50} values of Table 2.3.

	trophic consumer level			**trophic producer level**			**trophic decomposer level**
Variables	***Daphnia* IQ**	**Thamno tox**	**Rotox kit**	***Raphidocelis* sp. Growth inhibition**	***Scenedesmus* sp. Growth inhibition**	***Raphidocelis* sp. PAM test**	**Microtox®**
***Daphnia* IQ**	**1**						
Thamnotox	**0.88**	**1**					
Rotox kit	**0.851**	**0.954**	**1**				
***Raphidocelis* sp. Growth inhibition**	-0.088	0.123	0.361	**1**			
***Scenedesmus* sp. Growth inhibition**	-0.059	0.279	0.433	**0.812**	**1**		
***Raphidocelis* sp. PAM test**	-0.186	0.099	0.305	**0.847**	**0.959**	**1**	
Microtox®	0.039	0.3	0.295	0.525	0.475	0.525	**1**

Values in bold are significant correlations

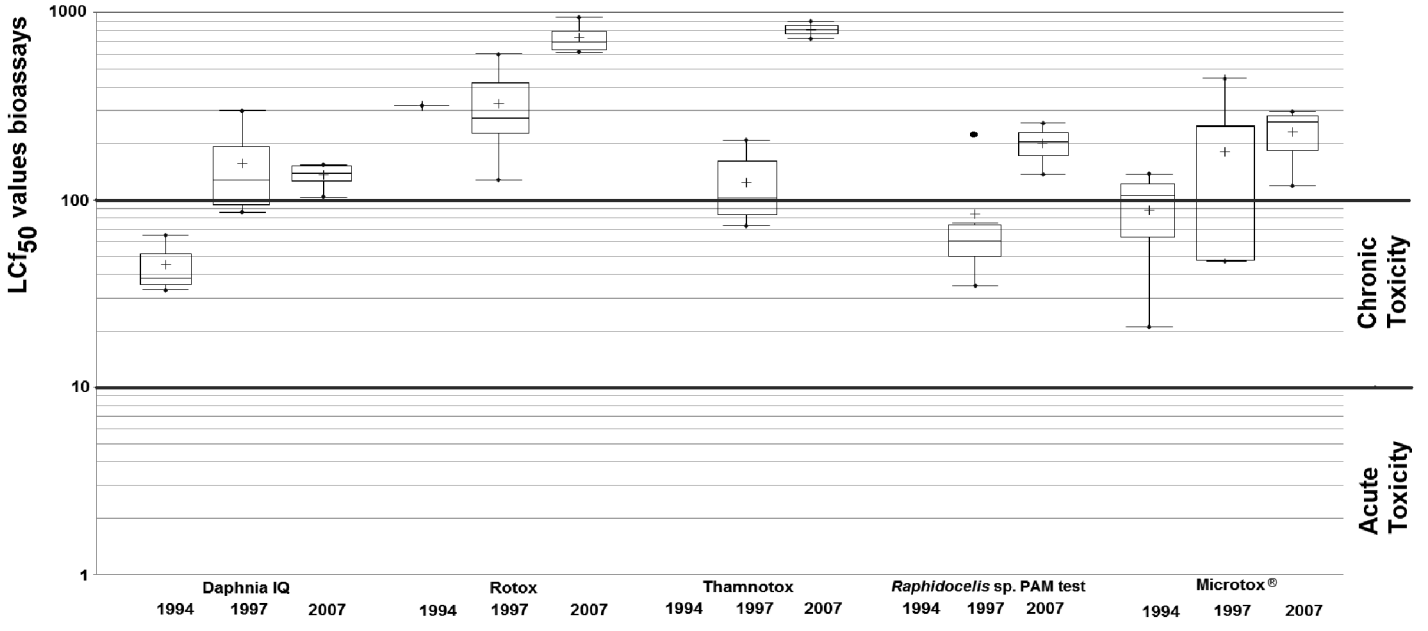

Figure 2.2: Boxplots of bioassays performed on XAD water extracts of the River Rhine at Lobith in 1994, 1997 and 2007 (data 1994 from Van Genderen *et al.* (1997) ; data 2007 from Dirven *et al.* (2008)). The minimum, 1st quartile, median, mean and 3rd quartile are displayed together with both limits (the ends of the "whiskers") beyond which values are considered anomalous and indicated as individual dots. The mean is displayed with a +, and a black line corresponds to the median.

Bioassays for genotoxicity

Table 2.5 and 2.6 present the results from the genotoxicity testing of the water samples collected at Lobith and at Nieuwegein. For the period 1998 and 1999, the sample taken at Lobith on July 15th in 1998 was the most genotoxic sample considering the low concentration factors needed to obtain positive outcomes in the Comet *Daphnia magna* and Ames test. In the year 2000, in which an additional number of genotoxicity tests was performed on samples from two sampling locations (Nieuwegein and Lobith), the most genotoxic sample was the sample taken at May 17th in Lobith. Based on the results obtained it is concluded that XAD concentrated water samples from the River Rhine may contain genotoxic compounds and that therefore an optimal battery of *in vitro* bioassays for monitoring surface water quality should also contain a test for genotoxicity.

Table 2.5: Lowest Observed Effect Concentration factor (LOECf) in different genotoxicity assays of XAD concentrated samples from Lobith and Nieuwegein. Concentrated samples were prepared at pH =7.

Sample and date	**Ames TA98**		**Umu**		**VITOTOX®**		**Comet Human lymphocytes**		**Comet *Daphnia magna***
pH level = 7	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	
Lobith Jul 15 (98)	275	138	< 520 >260	n.d.	250	>250	>780	n.d.	7.8
Lobith Sept 9 (98)	275	138	< 886 >443	n.d.	250	>250	>780	n.d.	31.2
Lobith Nov 4 (98)	410	138	260	260	>250	>250	>780	n.d.	>62.5
Lobith Feb 24 (99)	>545	410	<130 >65	260	250	250	>780	>780	>62.5
Lobith Apr 21 (99)	275	138	33	260	250	125	>780	780	>62.5
Lobith Jul 16 (99)	275	275	<260 >130	260	250	250	>780	>780	>62.5
Lobith Mar 22 (00)	*275*	*138*	750	750	n.d.	n.d.	780	>780	n.d.
Lobith May 17 (00)	*275*	*138*	750	750	n.d.	n.d.	390	195	n.d.
Lobith Jul 12 (00)	410	275	750	>750	n.d.	n.d.	>780	780	n.d.
Lobith Sept 6 (00)	410	138	750	750	n.d.	n.d.	780	195	n.d.
Lobith Nov 1 (00)	>545	275	750	>750	n.d.	n.d.	780	390	n.d.
Nieuwegein Apr 19 (00)	*275*	*138*	750	750	n.d.	n.d.	780	780	n.d.
Nieuwegein Jun 14 (00)	410	275	>750	>750	n.d.	n.d.	195	195	n.d.
Nieuwegein Aug 6 (00)	410	275	750	>750	n.d.	n.d.	195	195	n.d.
Nieuwegein Oct 5 (00)	>545	275	750	>750	n.d.	n.d.	780	780	n.d.
Nieuwegein Nov 29 (00)	545	275	750	>750	n.d.	n.d.	780	780	n.d.

-S9 = without addition of metabolic activation system; +S9 with addition of metabolic activation system; n.d. = not determined

Table 2.6: Lowest Observed Effect Concentration factor (LOECf) in different genotoxicity assays of XAD concentrated samples from Lobith. Concentrated samples were prepared at pH =2.

Sample and date	**Ames TA98**		**Umu**		**VITOTOX®**		**Comet Human lymphocytes**		**Comet *Daphnia magna***
pH level = 2	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	
Lobith Jul 15 (98)	410	410	65	n.d.	>250	>250	>780	n.d.	31.2
Lobith Sept 9 (98)	410	275	55	<886 >443	250	>250	>780	n.d.	62.5
Lobith Nov 4 (98)	275	138	<260 >130	521	125	>250	780	n.d.	>62.5
Lobith Feb 24 (99)	545	>545	130	260	125	>250	>780	>780	>62.5
Lobith Apr 21 (99)	410	275	260	260	>250	>250	>780	390	31.2
Lobith Jul 16 (99)	410	275	n.d.	n.d.	250	>250	390	780	>62.5

-S9 = without addition of metabolic activation system; +S9 with addition of metabolic activation system; n.d. = not determined

Evaluating the data obtained with the Mann Whitney U test, it appears that significant differences were found between results obtained in the presence and absence of a metabolic activation system in the Ames TA98 test ($p = 0.029$), in which the samples were more genotoxic when tested with S9 mix compared to testing without S9 mix. In contrast, the VITOTOX® and umu tests resulted in significantly higher genotoxicity for samples tested without the S9 mix compared to samples tested in the presence of S9 ($p = 0.036$ and $p = 0.035$ respectively).

Comparing the Lobith data of 1998 and 1999 with data of 2000, in 2000 significantly higher LOECf values (lower genotoxicity) were seen in the umu test ($p < 0.0001$) and a significant decrease of LOECf values (higher genotoxicity) in the Comet assay with human lymphocytes ($p<0.0001$). No differences were found in LOECf values of the Ames test.

It is concluded that although detection of DNA damage by the different genotoxicity tests is based on different mechanisms of genotoxicity, the use of the Ames TA98 assay only would provide adequate and robust data for water quality control of the River Rhine surface water. The data also reveal that the Ames TA98 assay can be best performed using XAD extraction at pH 7 and with the use of metabolic activation.

Ecological risk evaluation

An ecological risk model based on outcomes of the bioassays was applied to allow judgment of the ability of the *in vitro* bioassays to detect effects on the organisms in the ecosystem as a whole. The acute LCf_{50} or ECf_{50} values presented in Table 2.3 were divided by a factor 10 and presented as the no effect concentration factor (NECf) of the given bioassay. The cumulative distribution function of the species sensitivity was fitted by Equation 1 to the NECf of the different bioassays (an example is given in Figure 2.3).

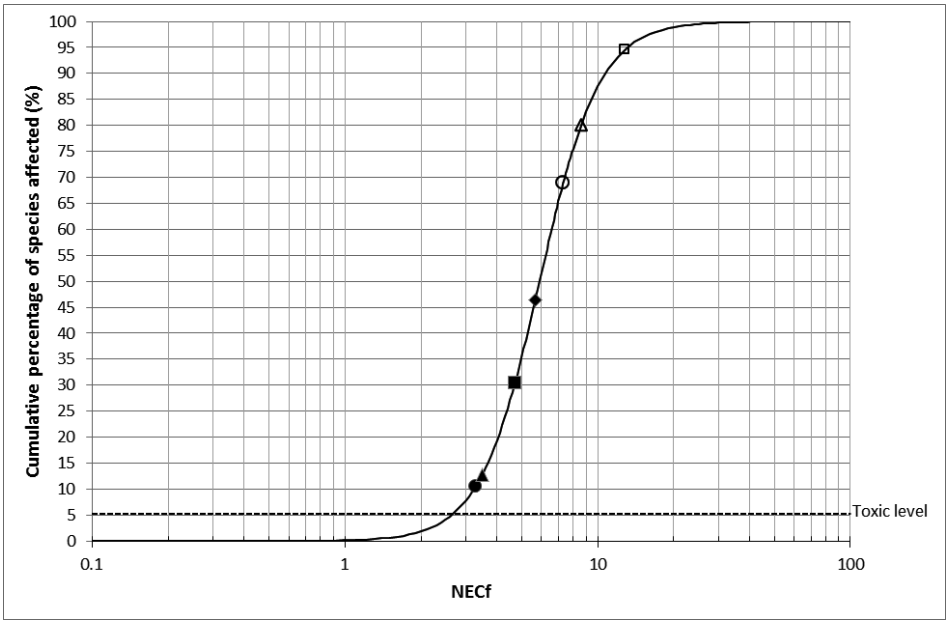

Figure 2.3: The cumulative distribution function of species sensitivity fitted (curve) to the no effect concentration factor (NECf) from bioassays, *Daphnia* IQ (△), Thamnotox (○), Rotox kit (□), *Raphidocelis* sp.Growth inhibition (●), *Raphidocelis* sp. PAM test (▲), *Scenedesmus* sp. Growth inhibition (◆) and Microtox® (■) of sample Lobith Sept 9 (98).

With the fitted function of each individual sample, the biological toxic pressure (pT), presented as the potential fraction of species affected by chemicals in non-concentrated samples (NECf =1), was calculated and corrected for the toxic pressure of blanks. The corrected biological toxic pressure values thus obtained for the different samples are presented in Table 2.7. The table also presents the average value of toxic pressure based on the outcomes for the individual sample.

Table 2.7: The biological toxic pressure (pT) presented as the fraction of species affected by chemicals in non-concentrated samples and the mean value ± s.d. as the general water quality of the River Rhine.

Sample date	pT sample (%)	pT blank (%)	pT sample corrected (%)
Lobith Jul 15 (98)	0.605	0.004	0.60
Lobith Sept 9 (98)	0.147	0.000	0.15
Lobith Nov 4 (98)	0.122	0.041	0.08
Lobith Feb 24 (99)	0.007	0.000	0.01
Lobith Apr 21 (99)	0.126	0.003	0.12
Lobith Jul 16 (99)	0.483	0.000	0.48
Mean value ± s.d.			0.25 ± 0.24

Based on the results from the seven bioassays used, the mean value for the potential affected fraction of organisms obtained for the six surface water samples amounts to 0.25 % ± 0.24 (Table 2.7). This is below the threshold of 5 %, at which samples are characterized as toxic (Van Straalen and Denneman, 1989).

The TU unit (based on chemical analysis and NOEC values) of the individual samples (Table 2.2) and the pT values (based on bioassays, Table 2.7) indicate that the non-concentrated water samples can be classified as non-toxic. With both type of values, a prediction can be made what level of NECf values is required before an effect can be observed. This enables a direct comparison of NECf's between chemical analysis and the results of the bioassays. Based on the chemical data, the required NECf values indicating the highest concentration factors that would result in toxicity, is the ratio between 1 and the total Toxic Units of a sample (Table 2.2). Based on the bioassay data, the required NECf values of the samples needed to enable detection of toxicity were calculated according to formula 1 by substituting the value of 0.05 (5 %) for the potential affected fraction of species. In Table 2.8, NECf values thus obtained for the six surface water samples are presented.

Table 2.8: Comparison of NECf values obtained by bioassay data and chemical data.

	NECf based on chemical data	NECf based on bioassay data
Lobith Jul 15 (98)	17	2.9
Lobith Sept 9 (98)	521	2.6
Lobith Nov 4 (98)	20	3.2
Lobith Feb 24 (99)	180	6.1
Lobith Apr 21 (99)	38	4.2
Lobith Jul 16 (99)	27	3.4

The NECf values obtained based on the bioassay data are lower than the NECf values obtained from the chemical data. This may be due to the fact that the chemical analyses are taking only a selected group of compounds into account. Other substances of potential toxicity and concern may not be detected and included in the analysis and thus in determination of the NECf values. It is concluded that the lower NECf values of the bioassay based analysis indicate that the overall toxicity measured with bioassays present better information about water quality of surface water than the chemical analysis of surface water only.

Based on the results presented in Table 2.3 it was already clear that a variation in toxicity detected for the six different surface water samples is observed. Next to the amount of chemicals released into the River Rhine (Table 2.2), the flow rate of the River Rhine might be a factor which influences the toxicity of a surface water sample. However, no relationship can be observed between the flow rate of the river and the results of the selected bioassays, *Daphnia* IQ, *Raphidocelis* sp.PAM test, Microtox® and Ames Ph7 +S9 (see Figure 2.4). A prediction of the (NO)(L)ECf_{50} based on dilution only is also presented in Figure 2.4. The (NO)(L)ECf_{50} value would increase from 50 to 500, presenting less toxicity, when the flow rate of the river increased from 1000 to 10000 m^3/s. However, with the bioassay data, high flow rates of the river showed the same level of toxicity compared to low flow rates. An explanation can be the additional release of chemicals from mainland, as runoff during high flow rates.

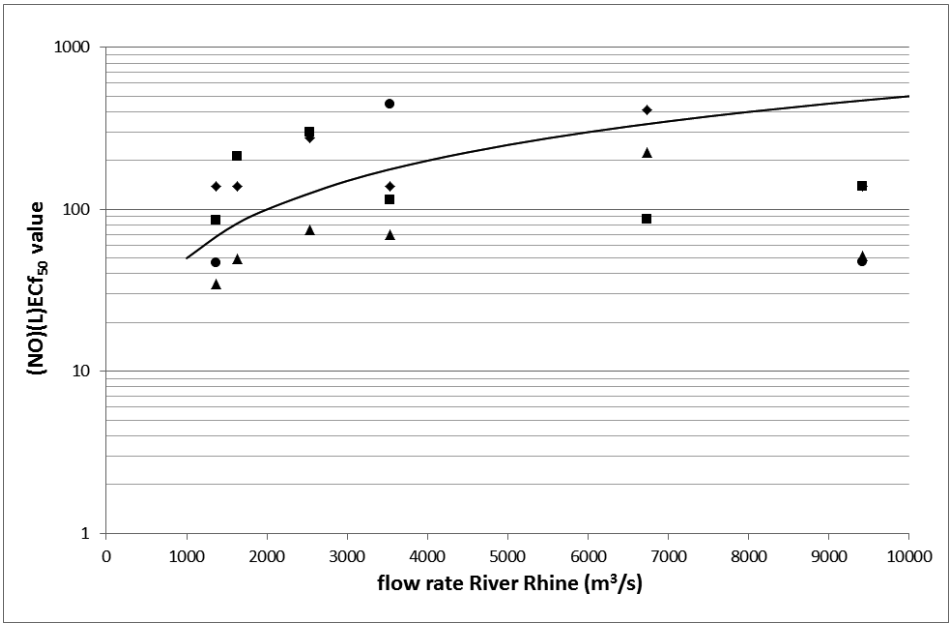

Figure 2.4: The relationship between flow rate of the River Rhine and the selected effect based assays (◆ = Ames TA98, pH=7+S9; ■ = *Daphnia* IQ; ▲= *Raphidocelis* sp. PAM; ● = Microtox®). The solid line presents the prediction of (NO)(L)ECf_{50} values based on dilution only.

2.4. Conclusions

The objective of the present paper was to evaluate the performance of a wide range of *in vitro* bioassays used in the previous years by RIWA for monitoring the toxicity as part of the quality assessment of surface water used for the production of drinking water and to define the optimal *in vitro* bioassay test battery, To this end the paper presents the data obtained in a variety of *in vitro* bioassays in several extracts of Rhine water and the evaluation of the different bioassays and genotoxicity tests in relation to sensitivity, selectivity and suitability for future routine monitoring programs. It was concluded that the best battery of bioassays for water quality monitoring of the River Rhine, which is sensitive, selective and suitable for routine monitoring programs, may include the *Daphnia* IQ assay, the *Raphidocelis* sp. PAM test and the Microtox® test as general toxicity tests, each being a representative for a specific trophic level, together with the Ames TA98 assay performed in the presence of metabolic activation to monitor genotoxicity. Based on data of different bioassays, using organisms with the same trophic level, a good correlation of data was observed. Therefore it is also concluded that the selection of only one bioassay per trophic level is sufficient to obtain reliable data for surface water quality monitoring.

Furthermore, it can be concluded that determination of the water quality by effect based *in vitro* bioassays and via a dose effect curve from a logistic response model is possible using a procedure in which sample water is concentrated using XAD solid phase extraction. The data obtained also indicate that the water quality of the River Rhine has improved over the years. The potentially affected fraction of organisms which is predicted to experience adverse effects of chemicals present in the surface water is lower than 5%.

All together, it was concluded that the overall toxicity measured with bioassays provides essential information about the quality of surface water that is not obtained by chemical analysis.

Acknowledgements

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Chapter 3

Increasing the sensitivity of online biological early warning systems for surface water quality control by integrating Solid Phase Extraction

This chapter is based on: E.J.M. Penders, G.M. Alink, I.M.C.M. Rietjens and W. Hoogenboezem “Increasing the sensitivity of online biological early warning systems for surface water quality control by integrating Solid Phase Extraction”, submitted.

Abstract

Online biological early warning systems have proven useful in the monitoring of water quality of surface waters. In their present form these systems, including the bbe Algaetoximeter and Microlan TOXcontrol system, monitor non-concentrated surface water samples. Other studies have however indicated that surface water quality has significantly improved during the last decades, whereas studies with *in vitro* bioassays have demonstrated the need for concentration of surface water samples to reach adequate detection limits. As a result there is a need for integrating concentration steps in the online biological early warning systems. The present paper describes the development and validation of Solid Phase Extraction (SPE) as an online concentration step in the bbe Algaetoximeter and Microlan TOXcontrol biological early warning systems. The results obtained provide a proof of principle for online bioassays with increased sensitivity, providing a suitable method for surface water quality control now indicating that water quality has been improved over the past decades, but still requires adequate online monitoring. The use of SPE connected to a biological early warning system will also prove a way to judge whether pollutants, chemically detected with ever increasing sensitivity, will still result in toxicity.

3.1. Introduction

Around 40% of the drinking water provided by the water companies in the Netherlands originates from surface water. The River Rhine is an important source for the production of drinking water. Around 4 million inhabitants are provided with drinking water produced from the River Rhine. In Nieuwegein, the drinking water company Waternet can abstract 150 million m^3 surface water per year from the Lekkanaal, an artificial side canal of the River Rhine. This water is treated on site with coagulation and rapid sand filtration before transport to the dunes area around Amsterdam. The water is infiltrated in the dunes with a detention time of ca. 100 days. The water is further treated by sand filtration, ozone and slow sand filters to generate safe drinking water (Hillegers *et al*., 2010). Due to the location of the intake (downstream the industrial and agricultural areas along the River Rhine), the quality of the surface water must be monitored frequently and thoroughly (Diehl *et al*., 2006). Before 1986, mainly chemical analytical techniques were used and water was monitored once a working day. Due to a major incident (fire at Sandoz, Basel) in 1986, a Rhine action program was started with the goal to improve the water quality of the River Rhine and to reduce the incidents of pollution (Anonymous, 1987). This program extended both, the number and frequency of chemical water analyses at the water laboratories. Due to the presence of thousands of chemicals in the river and the limited number of chemicals that can be detected by routine chemical analysis, biological early warning systems were also introduced. These systems monitor the water quality real-time, performing acute toxicity tests with aquatic organisms. When the water quality is below defined values for the biological early warning system, an alarm can be forwarded to the drinking water company. Following such a warning, the drinking water company can modify the water treatment process or the abstraction of river water may be interrupted. In 1987, several water companies implemented the Kerren fishmonitor as a biological early warning system, based on experiences presented by Kramer and Botterweg (2001) using *Leuciscus idus*. Since 1992, also biological early warning systems using other aquatic organisms were tested or implemented at the water intake Nieuwegein. The bbe Daphniatoximeter and Algaetoximeter are in operation since 2000 and 2001 respectively. In 2002, a biological early warning system using light emitting bacteria as biological sensor (TOXcontrol) was implemented. With the set of biological early warning systems at Nieuwegein, pollutants can be detected which have an effect on organisms from different trophic levels including consumers, producers and decomposers. During the first couple of years that biological early warning systems were implemented, several alarms were forwarded. However during the last decades, the quality of the River Rhine has improved, resulting in only a limited number of alarms triggered by the biological early warning systems in recent years. In 1997, The Association of River Water Works (RIWA) undertook an extensive study into the toxicological state of the River Rhine

(Penders *et al*., 2011). For that study, samples taken at Lobith and Nieuwegein were concentrated using solid phase extraction with XAD as absorbent and subjected to a number of different (geno)toxicity tests. The study revealed that detection of compounds by these *in vitro* bioassays required concentrationfactors of 59-126, depending on the organism used in the bioassay, to allow detection of the adverse effects, which was presented as 50% lethality or effect in the bioassay population. Since these levels of (geno)toxic compounds may still raise concern, there appears to be a need for integrating concentration steps in the online biological early warning systems. To improve the sensitivity of biological early warning systems, Solid Phase Extraction (SPE) might be used. The objective of the present study is to develop and validate the use of SPE as an online concentration step in the Algaetoximeter and TOXcontrol bioassays, using diuron and zinc as the reference compounds to express the toxicity observed in diuron respectively zinc equivalents. Diuron, (3-(3,4-dichlorophenyl)-1,1-dimethylurea), was selected as a reference compound, because of frequent elevated concentrations of diuron in the River Rhine (Anonymous, 2010a). Such an SPE procedure combined with the bbe Algaetoximeter or the Microlan TOXcontrol online early warning systems will enable sensitive detection of herbicides and other toxicants in surface water. The present paper describes the development and validation of the modified online biological early warning systems, as well as their actual performance in an online biological early warning system for surface water of the River Rhine.

3.2. Material and methods

Chemicals and media

Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA). All chemicals were of analytical grade and, if not stated otherwise, were obtained from Merck (Amsterdam, The Netherlands).

The algae cultivation medium consisted of 1.49 g $NaNO_3$, 1.25 g KH_2PO_4 (Acros organics, Geel, Belgium), 1.0 g $MgSO_4.7H_2O$, 22.12 mg $CaCl_2.2H_2O$, 22.84 mg H_3BO_3, 0.50 g EDTA, 0.5 g KOH, 88.1 mg $ZnSO_4.7H_2O$, 14.2 mg $MnCl_2.4H_2O$, 6.5 mg $NH_4Mo_7O_2.4H_2O$, 15.7 mg $CuSO_4.5H_2O$, 4.9 mg $Co(NO_3)_2.6H_2O$ and 49.8 mg $FeSO_4.7H_2O$ in one litre of Milli-Q water, adjusted to pH 7.0.

The medium for cultivating *Vibrio fischeri* bacteria was made by adding 30 g NaCl, 6.1 g $NaH_2PO_4.2H_2O$, 2.1 g $K_2HPO_4.3H_2O$, 0.204 g $MgSO_4.7H_2O$, 0.5 g $(NH_4)_2HPO_4$, 5 g Bacto-Pepton (OXOID, Badhoevedorp, The Netherlands), 0.5 g yeast extract (OXOID) and 3 ml Glycerol to one litre of Milli-Q water, adjusted to pH 7.0.

The 10x Elendt medium consisted of 28.5 mg H_3BO_3, 3.61 mg $MnCl_2.4H_2O$, 3.06 mg LiCl, 0.71 mg RbCl, 1.52 mg $SrCl_2.6H_2O$, 0.16 mg NaBr (Acros organics), 0.165 mg $CuCl_2.2H_2O$ (Boom, Meppel, The Netherlands), 0.13 mg $ZnCl_2$, 0.1 mg $CoCl_2.6H_2O$, 0.63 mg $Na_2MoO_4.2H_2O$, 0.0325 mg KI, 0.022 mg $Na_2Se_3.5H_2O$(J.T. Baker, USA), 0.006 mg NH_4VO_3, 25 mg EDTA, 9.95 mg $FeSO_4.7H_2O$, 2.94 g $CaCl_2.2H_2O$, 1.233 g $MgSO_4.7H_2O$, 57.5 mg KCl, 648 mg $NHCO_3$ (J.T. Baker), 75 mg $Na_2SiO_3.5H_2O$ (Sigma Aldrich, Zwijndrecht, The Netherlands), 2.74 mg $NaNO_3$, 1.43 mg KH_2PO_4, 1.84 mg K_2HPO_4, 0.75 mg Vitamin B1, 0.01 mg Vitamin B12 (Acros organics) and 0.0075 mg Vitamin H (Acros Organics) in one litre of Milli-Q water, adjusted to pH 8.4.

The Algaetoximeter biological early warning system

Figure 3.1A presents a picture of the bbe Algaetoximeter (biological biophysical engineering Moldaenke, Kiel, Germany), which analyses water for the presence of toxic substances by recording the activity of algae cells. The *Chlorella vulgaris* algae (culture SAG 211-11b, University Göttingen, Germany) are cultivated in the integrated fermentor (labelled B in Figure 3.1A), under continuous light and aeration. To this continuous algae culture, 30 mL per hour of algae cultivation medium (labelled A in Figure 3.1A) is provided for growth. For the measurement of toxicity, water samples from the River Rhine are pumped into the measuring sensor (labelled C in Figure 3.1A) and algae are added to this sample of the River Rhine but also to tap drinking water as a reference sample, followed by a 30 minute exposure time. After the 30 minutes incubation the algae concentration and their activity are determined fluorometrically. After a dark period, the steady ground level fluorescence Fo is measured. A single saturating flash is then applied and the maximal fluorescence Fm is measured. With the formula (Fm-Fo)/Fm, the Genty (Genty *et al*., 1989) is calculated as a relative mass of activity. The toxicity of the sample is expressed as a percentage that is calculated as 1 minus the ratio (Genty value of the Sample)/(Genty value of the Reference Sample) times 100%. After the measurement, all the tubing and the sensor are rinsed several times with reference water. The instrument provides a measurement for toxicity, displayed on the screen (labelled D in Figure 3.1A), every 45 minutes by analysing a newly collected sample. When the toxicity exceeds the defined limit value of 4% toxicity, an alarm is given. To ascertain adequate operation and sensitivity of the bbe Algaetoximeter, once every 9 sample measurement, a positive control solution of 2.5 or 5 µg/L diuron (Fluka Sigma Aldrich, Zwijndrecht, The Netherlands) is analysed. The sensitivity of the instrument, reflected by the concentration response curve for the toxicity of diuron is presented in Figure 3.1B.

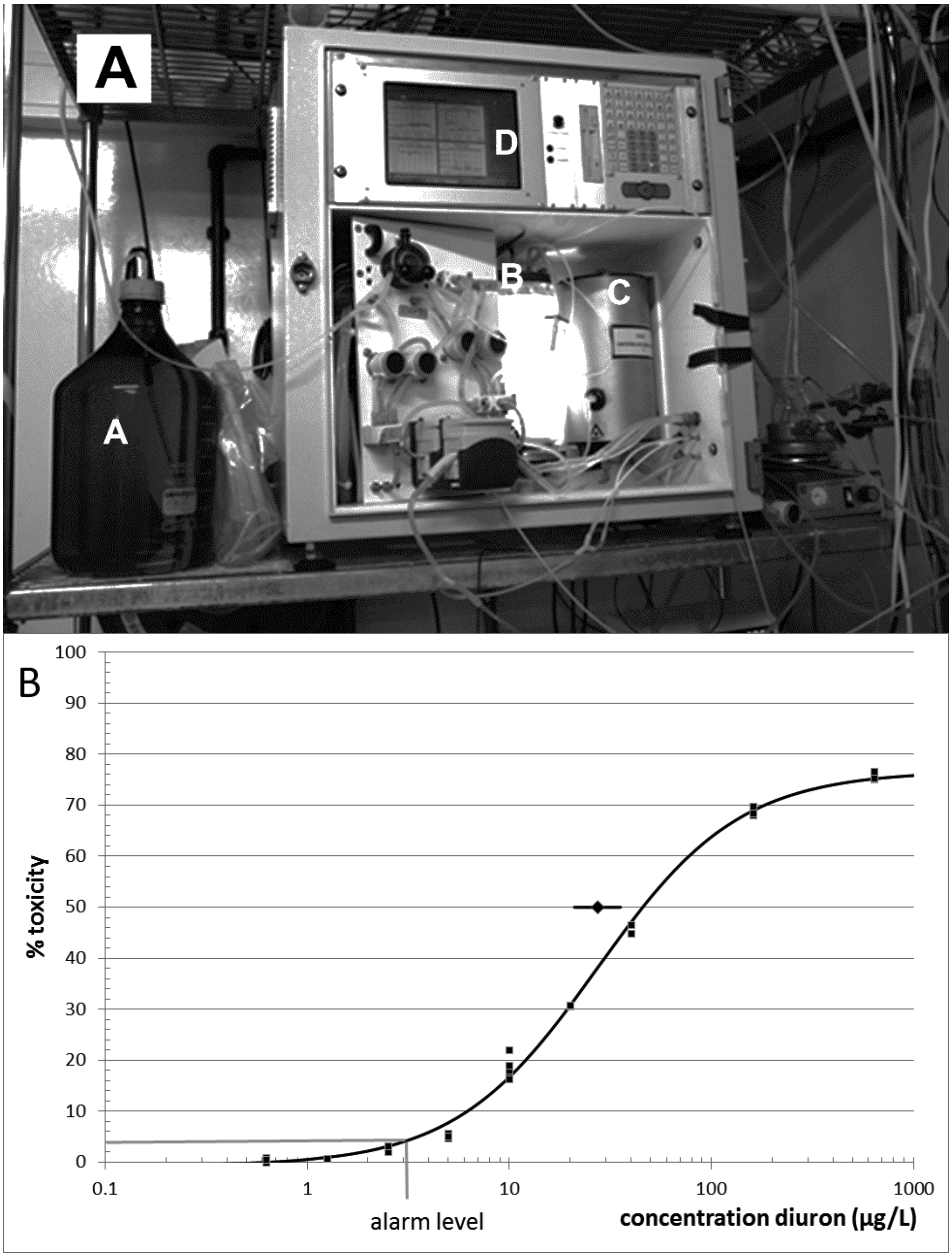

Figure 3.1: A) Picture of the bbe Algaetoximeter with labelling of some essential parts (A, B, C and D for details see text) and B) the concentration-response curve reflecting the response to increasing concentrations of diuron (■). Figure 3.1B also presents the alarm limit of diuron at 3 µg/L (causing 4% toxicity) as well as the EC_{50} value for diuron towards *Chlorella vulgaris* upon 20 minutes exposure as reported in Podola and Melkonian (2005) (◆; line = 95% confidence interval).

The TOXcontrol biological early warning system

Figure 3.2A presents a picture of the TOXcontrol system (Microlan BV, The Netherlands), which analyses water for the presence of toxic substances, by recording the light production of *Vibrio fischeri* bacteria. The used procedure is a modification of the ISO 11348-2 standard (Anonymous, 2007).The bacteria are present in the TOXcontrol in a storage vessel at 4 °C. The *Vibrio fischeri* bacteria are made by suspending freeze dried bacteria (Microlan, The Netherlands) in 50 mL *Vibrio fischeri* culture medium and incubating the solution for 24 hours at 20 °C. Before and during use in the TOXcontrol, the culture is kept at 4 °C. The bacteria in the TOXcontrol are renewed during the weekly maintenance of the instrument.

To activate the bacteria for an analysis to be performed, 40 µL bacteria from the storage vessel are mixed with 9 mL reference water and 1 mL 20% NaCl solution. After 5 minutes at 15 °C, the luminescence of the bacterial mix (I_O) is measured with a photomultiplier. Then, 5 mL of this bacterial mix is added to 4.5 mL of a sample of the River Rhine and 0.5 mL 20% NaCl solution. As a reference, 5 mL of the bacterial mix is added to 4.5 mL of tap drinking water and 0.5 mL 20 % NaCl. After 30 minutes exposure, the luminescence of the sample (I_S) and reference (I_R) incubations are measured. At first, a ratio $C_f = I_R/I_O$ is calculated reflecting the normal decline of luminescence of bacteria during the incubation. Secondly, the correct luminescence of sample I_{CS} is calculated as I_S/C_f. The toxicity of the sample is calculated as a percentage via formula $(I_O-I_{CS})/I_O*100$. The instrument provides a measurement of the toxicity of a newly obtained sample every 45 minutes. An alarm is given, when the toxicity value is above the defined limit value of 10 %. To ascertain adequate operation and sensitivity of the TOXcontrol, a control solution (20 mg zinc/L) is analysed once every 9 sample measurements. The concentration-response curve reflecting the response of the TOXcontrol to increasing zinc concentrations is presented in Figure 3.2B. The figure also presents the alarm level for zinc as well as the concentration-response curve obtained for 2,4-dichlorophenol, another positive control, and its alarm level.

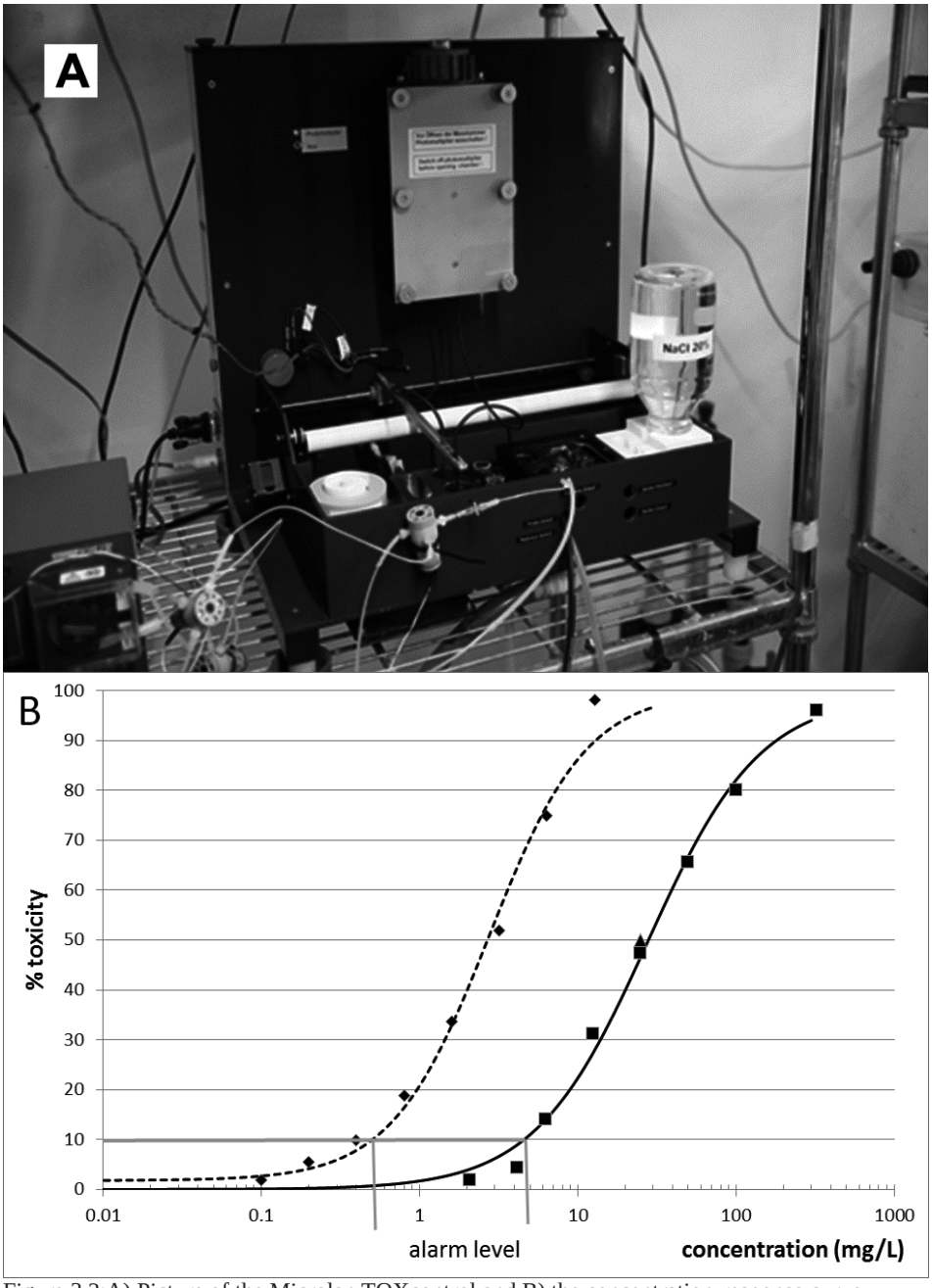

Figure 3.2:A) Picture of the Microlan TOXcontrol and B) the concentration-response curve reflecting the response to increasing concentrations of zinc (■) and 2,4-dichlorophenol (◆). Figure 3.2B also presents the alarm limit of zinc at 4.6 mg/L and the alarm limit of 2,4-

dichlorophenol at 0.5 mg/L (both causing 10% toxicity) as well as the EC_{50} value for zinc as reported in the ISO 11348 (▲).

SDB SPE BIO procedure

To show adequate implementation of a water concentration step in the Algaetoximeter and TOXcontrol biological early warning systems located at Nieuwegein, several requirements should be met. A first requirement is that the Algaetoximeter and the TOXcontrol systems must remain in operation as early warning systems to check the water quality at the intake location. For this reason, the results of the analysis of the extracted concentrated water samples must be excluded from the alarm evaluation routine. A second requirement to be met was that only small modifications in electronics or software were allowed in the TOXcontrol and Algaetoximeter. These modifications must have no effects on the reliability of the instruments. A third requirement was that the SPE instruments and SPE protocol used to perform the extraction as a concentration step must be in house validated procedures. Finally, given that the reference compound selected for converting the % toxicity to a concentration in equivalents/L in the water sample is diuron, which is a herbicide, the SPE BIO procedure applied should be able to concentrate herbicides.

The solid phase extraction is performed using a Gilson ASPEC XL instrument, together with a Gilson syringe diluter pump (type 401C) and a Gilson piston pump (type 307). An additional switch is used to provide nitrogen gas (Air Products, The Netherlands) to the needle for evaporation. The system is configured in such a way that it provides an extract once per day. The disposable extraction column, containing 200 mg of Styrene Divinyl Benzene Copolymer (SDB) (Avantor, USA), is preconditioned with 9 mL of 100 % methanol during 5 minutes. Avantor (Anonymous, 2010b) reported in an application note that with the SDB extraction column, recoveries of 106 % and 107 % were obtained for respectively isoproturon and diuron. After rinsing the SDB column with 6 mL of Milli-Q water, a volume of membrane filtered (Type F0285, Norit X Flow, The Netherlands) surface water is passed through the column with a flow rate of 10 mL/min. The volume of water sample for the automatic analysis is 5600 mL. A volume of 7600 mL surface water is used for the semi-automatic analysis. The column is flushed with nitrogen gas for 1 minute to remove all solvent. Subsequent elution of compounds from the column material is achieved by eluting the column with 6 mL of acetone at a flow rate of 6 mL/min. Because acetone is toxic for algae and bacteria, the acetone is removed from the eluate by treatment with a gentle flow of nitrogen during 30 minutes at 45 °C. From this step onwards, two different procedures are followed: 1) For the automatic online analysis of the extract, the extract is resuspended in 5 mL Milli-Q water and transported to a glass vessel connected to the biological early warning systems. The glass tube is rinsed several times with Milli-Q water (62.5 mL) into the glass vessel mixed with 7.5 mL of 10x Elendt medium to prevent toxic stress

from the Milli-Q water. The final volume of the extract for the instruments is 75 mL, resulting in a concentration factor of 74.7. When this process is finalised the Gilson instrument provide a signal to the electronic interfaces that the extract is ready for automatic measurement. 2) For the semi-automatic measurements, the tubes are closed and stored at 4 °C before analysis. A volume of 6 mL Milli-Q is added to the tube and mixed, resulting in a concentration factor of 1267. A volume of 3 mL of the extract is analysed with the TOXcontrol. A volume of 3 mL of the extract is diluted 10 times with drinking water before analysis in the Algaetoximeter.

The removal of organic solvent by a gentle flow of nitrogen and suspension in water are extra steps next to the analytical chemical SPE procedure. Therefore the whole procedure to obtain an extract for a biological early warning system is called the SDB SPE BIO procedure.

Connection of SDB SPE BIO procedure to Biological Early Warning Systems

For the automatic analysis of the extract by the TOXcontrol, an electronic programmable interface (Wilke technologies, Germany) is used, fitted with several relays. These relays are connected to a tube pump and four selection valves. Two sensing switches are placed in the TOXcontrol, for obtaining information on when the TOXcontrol can be provided with an extract for measurement. When a signal is obtained from the Gilson SPE unit and after the fifth measurement following the measurement of the zinc control solution, the interface switch on a valve that redirects the surface water sample from the River Rhine into the drain. The remainder of the surface water in the sample unit of the TOXcontrol is pumped out into the drain. The interface then directs 15 ml of the extract into the sample chamber and generates a signal to the TOXcontrol, that an extract measurement will be performed. After 30 minutes, surface water from the River Rhine is redirected again to the TOXcontrol.

For the automatic analysis of the extract by the Algaetoximeter, a second electronic programmable interface is used. When a signal is obtained from the Gilson SPE unit and when a valve for the diuron control solution is activated by the Algaetoximeter for control measurement, the interface directly activate a valve, which switch from the control diuron solution feeding tube to the extract feeding tube. The interface provide a signal to the Algaetoximeter that an extract measurement will be performed. After 45 minutes, the interface provide a signal to the TOXcontrol interface for cleaning and rinsing the extract glass vessel and the valve is deactivated, switching back to the control solution feeding tube for the next control measurement.

Data processing

The measured toxicity by the Algaetoximeter is calculated in diuron equivalents (µg/L) using the concentration-response curve presented in Figure 3.1B. During the operation of the Algaetoximeter, the sensitivity of the algae to organics may vary. To check and calibrate the sensitivity of the algae and thus the instrument, a control standard sample with diuron is used with known concentration and measured regularly. The ratio between the measured diuron equivalents/L of the control standard sample and the diuron concentration of this solution reflects the change in sensitivity of algae in the system, and is used to correct the measured diuron equivalent value obtained for the corresponding water extract. Thus, the amount of diuron equivalents in the non-concentrated surface water was calculated, by dividing the diuron equivalents/L obtained for the extract by both the ratio that corrects for the sensitivity of the algae and by the concentration factor used to prepare the extract.

The measured toxicity by the TOXcontrol is expressed in zinc equivalents (mg/L) based on the sensitivity curve presented in Figure 3.2B. During the operation of the TOXcontrol, the sensitivity and viability of the bacteria may vary. To check the sensitivity of the bacteria and thus the instrument, a control standard with zinc is used with known concentration and measured regularly. The ratio between the measured zinc equivalents/L of the control standard sample and the zinc concentration of this solution reflects the change in sensitivity of bacteria in the system, and is used to correct the measured zinc equivalent value obtained for the corresponding water extract. Thus, the amount of zinc equivalents in the non-concentrated surface water was calculated, by dividing the zinc equivalents/L obtained for the extract by both the ratio that corrects for the sensitivity of the bacteria and by the concentration factor used to prepare the extract.

3.3. Results and discussion

Validation of the SDB SPE BIO procedure

The extract volume required for the automatic analyses by the Algaetoximeter and TOXcontrol was 75 mL. As reported earlier, median ECf_{50} values, that is the median concentration factors of the water resulting in 50% effect in the respective bioassay, of more than 59 were obtained when detecting the toxicity of compounds in surface water by algae toxicity tests. In the present study a concentration factor around 75 was used to perform the validation of the SDB SPE Bio procedure. The required volume of surface water or spiked water which had to be concentrated was 5600 mL. Tap water was spiked with diuron to a concentration of 0.5 µg/L to obtain the spiked water sample. A recovery of 92.6 % was measured (Table 3.1, Test 1) when the SPE extract was measured by organic analysis. The period that a sample was passed to the SDB SPE column, the filtrate of the column was collected and measured by organic analysis. The filtrate contained less than 0.03 µg/L diuron, which indicated that less than 6 % of the total amount of diuron loaded on the column was not bound.

Table 3.1: Recoveries of diuron with the SDB SPE BIO procedure

	Test 1	**Test 2**	**Test 3**
Concentration diuron in spike solution (µg/L)	0.5	0.5	5
Volume filtrate collected from SPE SDB (mL)	5600	5600	8600
Volume of extract obtained (mL)	75.8	75	1.35
Concentration factor	73.9	74.7	6370
Expected value concentration diuron in extract (ug/L)	36.6	37.3	31852
Dilution used for measurement bbe Algaetoximeter	1.0	1.0	636.9
Concentration provided to bbe Algaetoximeter	36.6	37.3	50.0
Toxicity measured		40.6	33.5
Toxicity of diuron (50 µg/L)			37.1
Toxicity of diuron (35 µg/L)		41.8	
Toxicity of diuron (5 µg/L)		12.2	14.1
Concentration diuron EQ (µg/L) in extract measured by organic analyses	33.9		
Concentration diuron EQ (µg/L) in extract measured by bbe Algaetoximeter		33.3	43.0
Recovery	92.6	87.9	85.9

The elution of the SDB is performed with the use of 100 % acetone. The evaporation step in the SBD SPE BIO procedure must be able to decrease the amount of acetone to a level that does not result in any toxicity in order to ascertain that the toxicity of the resulting extract would not (in part) be due to residual acetone. To this end, the SDB SPE BIO procedure was performed with tap water and the extract was automatically measured with the Algaetoximeter and TOXcontrol. The toxicity of this control extract measured by the Algaetoximeter and the TOXcontrol assay was similar to that of non-concentrated control water from which it can be concluded that traces of acetone in the extract after the evaporation step do not lead to toxicity.

To validate the SDB SPE BIO procedure in combination with the Algaetoximeter, 5600 mL tap water spiked with 0.5 µg/L diuron was concentrated to an extract volume of 75 mL. The extract resulted in 40.6 % toxicity (Table 3.1, Test 2) which is close to the toxicity of 41.8 % for this 35 expected µg/L diuron solution. Converting the toxicity observed to µg/L diuron equivalents resulted in a value of 33.3 µg/L diuron equivalents, indicating a recovery of 87.9 %.

Because the SDB column is known to have a limited capacity for binding organics from surface water and no breakthrough values for organics were available, the recovery of diuron was measured in an experiment in which 8600 instead of 5600 mL spiked sample was loaded on the SDB column (Table 3.1, Test 3). In this experiment a spiked concentration of 5 µg/L diuron was used, which is 10 times the concentration used in test 1 and 2, and higher than the alarm level of the Algaetoximeter for untreated surface water (4 % toxicity corresponds to 3 µg/L diuron equivalents). In this test 3, performed with a higher sample volume and with a 10 fold higher concentration of diuron, the recovery was 85.9 %, which is comparable to the recovery measured in test 1 and 2. Based on this result it is concluded that, under the conditions applied, no breakthrough of diuron occurs in the SDB column up to a total amount of 43 µg of diuron.

Based on the high recoveries measured by organic analysis (Test 1, Table 3.1) and the Algaetoximeter (Test 2 and 3, Table 3.1), it is concluded that the SDB SPE BIO procedure is valid to provide concentrated water samples for automatic and semi-automatic measurements with the Algaetoximeter and TOXcontrol.

It is important to note that the time required to obtain an amount of extract of the SDB SPE BIO system sufficient for analysis by both instruments appeared to be 9.3 hours. Therefore, it is concluded that at the moment, it is not possible to use SPE extract samples as early warning sample since this requires a sample to be analysed every 45 minutes. However, with this automated SDB SPE BIO system connected to the Algaetoximeter and TOXcontrol, the sensitivity of both biological early warning systems is improved with a factor of 75 for the detection of organic micropollutants present in the river and it would allow daily measurements enabling detection of increasing or decreasing trends in the water quality over longer periods of time.

The most time consuming step is providing several litres of surface water to the column with a flow rate of 10 mL/min. Further research should be directed at defining ways to reduce this loading time by increasing the flow rate and/or by reducing the extract volume needed to perform an analysis with the respective biological early warning systems. The latter aspect could involve for example a reduction in the measuring volume of the sensor of the instrument, without compromising the sensitivity and reliability of the sensor itself.

Evaluation of the SDB SPE BIO procedure when coupled to automatic measurements by the Algaetoximeter and TOXcontrol

In a next step the performance of the SDB SPE BIO procedure was tested in a real life situation. To this end, during several weeks in 2007, the SDB SPE BIO unit was in operation at Waternet in Nieuwegein. Extracts were automatically analysed by the Algaetoximeter and TOXcontrol on a daily basis (once each day) while the normal water sampling of the River Rhine and the measurement of the quality control solution also continued. Figure 3.3 and Figure 3.4 present the result obtained for respectively the Algaetoximeter and the TOX control system.

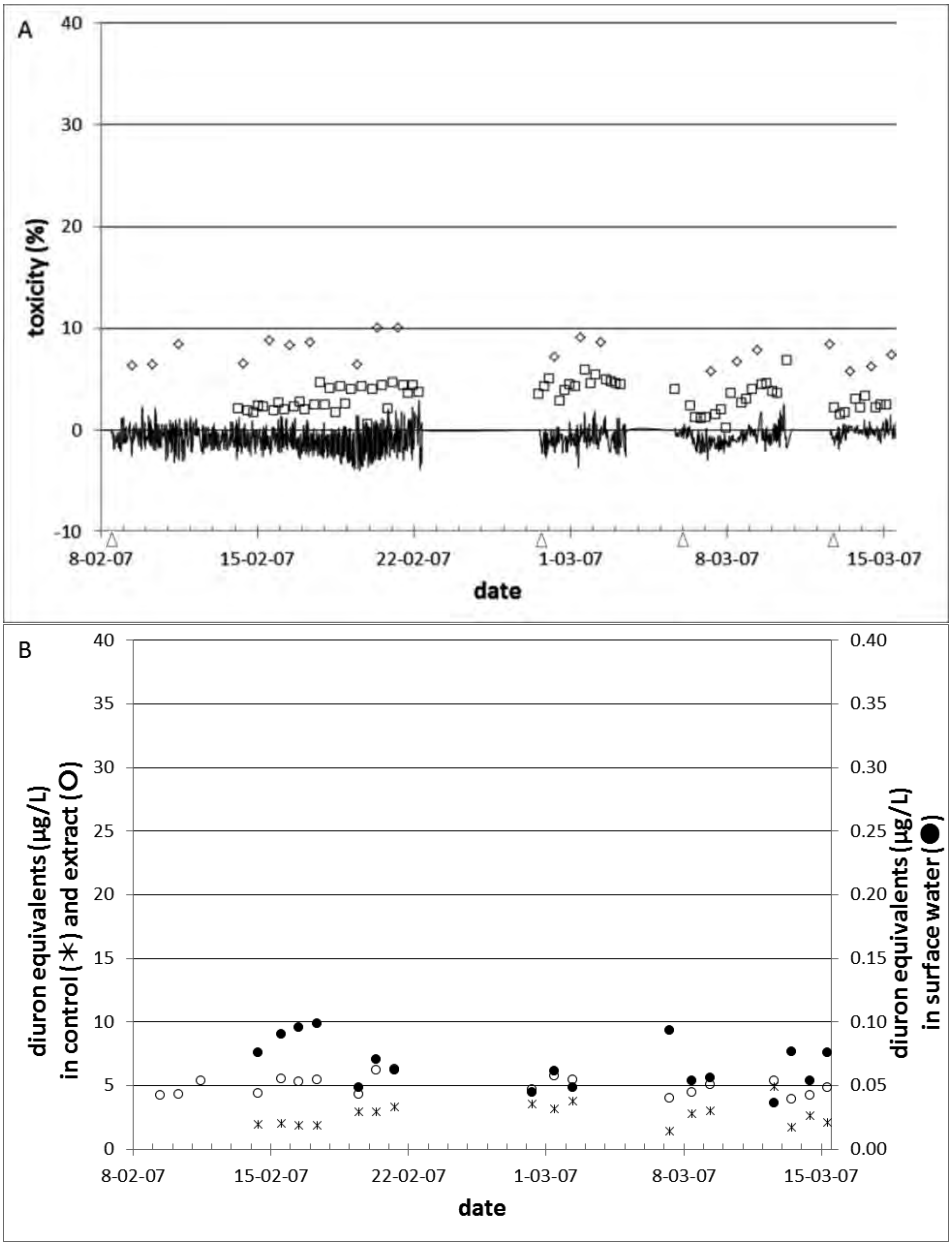

Figure 3.3:A) Time-dependent online SDB SPE BIO extract measurements with the bbe Algaetoximeter of 75 times concentrated River Rhine surface water expressed in % toxicity (◊) and of the corresponding 2.5 µg/L positive diuron control solution measured before the extract (□). The solid line presents the results from the online analysis of non-concentrated River Rhine surface water. Interruption of the series of daily data for the concentrated extracts occurred when

the algae concentration in the fermentor was below 850 µg chlorophyl/L, making the analysis unreliable, with data collection starting again upon renewal of the *Chlorella vulgaris* culture, indicated in the figure with a △ on the x-axis. B) Time dependent diuron equivalents (µg/L) of 75 times concentrated River Rhine surface water (○) and of the corresponding 2.5 µg/L positive diuron control solution measured before the extract (∗). The diuron equivalents in non-concentrated surface water (●) is corrected for the variation in sensitivity of the algae.

Figure 3.3A reveals that in the Algaetoximeter the toxicity of the extract obtained varied between 5.8 % and 10.1 %. The mean concentration detected for the diuron positive control samples, analysed immediately before the extract measurements (Figure 3.3B), was 2.70 ± 0.9 µg/L (n=17). Converting the % toxicity to concentrations expressed in diuron equivalents/L, resulted in values for the 75 times concentrated extracts of the River Rhine that varied between 2.73 and 7.40 µg/L diuron equivalents (Figure 3.3B). This is equal to 0.036 and 0.10 µg/L for the non-concentrated surface water samples and these values are also presented in Figure 3.3B. From the results presented it is clear that concentration enables detection of toxicity that could not be detected in the water sample when analysed without a pre-concentration step.

It is also important to note that toxicity detected for the non-concentrated water samples analysed just after analysis of the concentrated extract sample was not unexpectedly high (Figure 3.3A). This indicates that carry-over of the toxic extracts to the non-concentrated surface water samples did not occur in the instrument. This conclusion is corroborated by the fact that results from measurements of the non-concentrated water before and after the extract measurements were similar. Altogether it is concluded that it is possible to use the SDB SPE BIO unit generating extracts for automatic measurement, in parallel to the normal operation of the Algaetoximeter, measuring toxicity of non-concentrated water samples.

The level of toxicity of the SDB SPE BIO extract samples was lower than what would be expected based on the RIWA study performed in 1997 which presented a median ECf_{50} value of 59 for *Raphidocelis subcapitata* algae (Penders *et al*., 2011), because in the present study a 75 fold and thus higher concentration step resulted in less than 50% toxicity. One reason for this discrepancy might be a higher binding of organics to XAD4/8, used in the RIWA study in 1997 than to SDB used in the present study for the concentration step. However, the most probable reason for the difference might be the improvement of the water quality of the River Rhine. This explanation would be in line with the results of Dirven *et al*. (2008) who presented toxicity results of XAD extracts of River Rhine samples collected at Lobith in 2007, reporting a median ECf_{50} value for the PAM algae test of 200, indicating that a 200 times concentration step is needed to obtain 50% toxicity. It is concluded that the automated SDB SPE SDB, which can

present a 75 time concentrated water sample to the Algaetoximeter, provides a method to increase the sensitivity of the Algaetoximeter for detection of herbicides in surface water.

The results obtained with the TOXcontrol system are presented in Figure 3.4A en 3.4B. In the TOXcontrol system the toxicity of the SDB SPE BIO extracts varied between 7.6 % and 18.6% (Figure 3.4A).

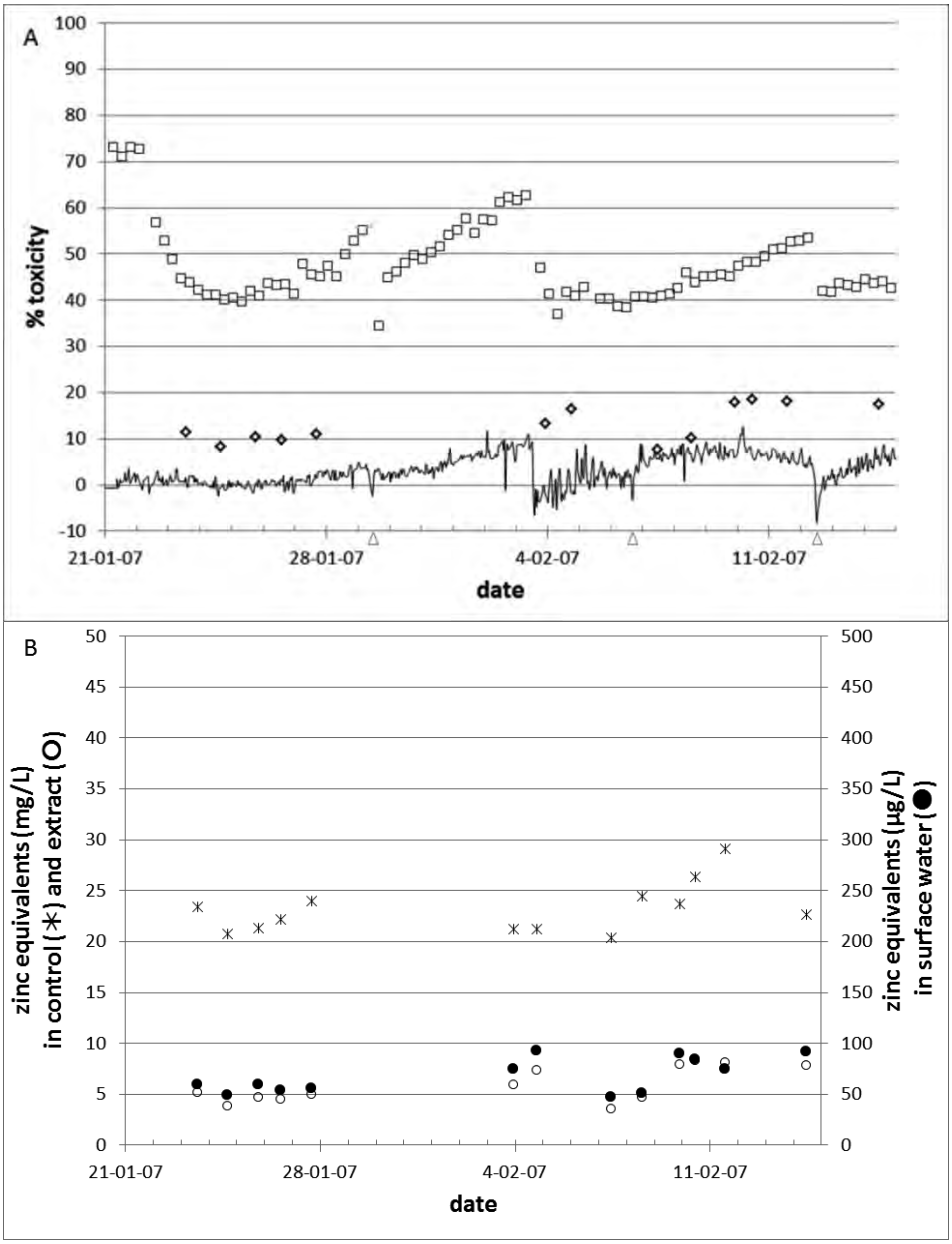

Figure 3.4:A) Time-dependent online SDB SPE BIO extract measurements with the Microlan TOXcontrol of 75 times concentrated River Rhine surface water expressed in % toxicity (◊) and of the corresponding 20 mg/L positive zinc control solution measured before the extract (□). The solid line presents the results from the online analysis of non-concentrated River Rhine surface water. Renewal of the *Vibrio fischeri* is indicated in the figure with a △ on the x-axis. B) Time

dependent zinc equivalents (mg/L) of 75 times concentrated River Rhine surface water (○) and of the corresponding 20 mg/L positive zinc control solution measured before the extract (∗). The zinc equivalents (µg/L) in non-concentrated surface water (●) is corrected for the variation in sensitivity of the bacteria.

The toxicity detected for the zinc control samples, which were analysed each day just before analysis of the extracts, varied between 40.5 and 51.2 %. The mean measured zinc concentration derived from these toxicity values for the positive zinc control samples was 23.5 ± 2.5 mg/L (n=13). The zinc equivalents of the 75 times concentrated extracts of the River Rhine varied between 3.51 and 6.93 mg/L equal to 46.9 and 92.4 µg/L for the non-concentrated surface water samples.

Based on the same reasoning as presented above for the data obtained with the Algaetoximeter also the results obtained with the TOXcontrol did reveal that carry-over of toxic extracts to normal surface water did not occur in the instrument since measurements of the normal water samples just before and after the extract measurement were similar. Thus, the use of the SDB SPE BIO unit preparing extracts for automatic measurement in parallel to the normal operation of the TOXcontrol measuring toxicity of non-concentrated water samples is possible.

As with the results of the extracts measured with the Algaetoximeter, the level of toxicity of the SDB SPE BIO extract measured with the TOXcontrol was lower than expected based on the RIWA study in 1997 which presented a median ECf_{50} value of 47.7 for the Microtox test (Penders *et al*., 2011). Dirven *et al*. (2008) presented toxicity results of XAD extracts of the River Rhine collected at Lobith in 2007, in which the median ECf_{50} value for the Microtox test was 262. This indicates that for obtaining toxicity results of 50%, surface water may have to be concentrated about 250 to 300 times.

SBD SPE BIO extract measurements 2010

The level of toxicity measured in 2007 in 75 times concentrated water samples of the River Rhine, was around 5.8 to 10.1 % for the Algaetoximeter (Figure 3.4) and 7.6 to 18.6 % for the TOXcontrol (Figure 3.5). These levels were just above the alarm level of the instruments, which were defined at 3% for the Algaetoximeter and 10% for the TOXcontrol system, and they fall in the less accurate lower part of the sigmoid concentration response curves for the reference compounds diuron and zinc (Figure 3.1B and 3.2B) that are used to convert the percentage of toxicity to respectively diuron or zinc equivalents per litre of water. To further improve the accuracy of the measurements, and generate data with a response preferentially around 50% toxicity, a further increase in the concentration factor is required. With a maximum volume of 8600 mL tap water delivered to the 200 mg SDB SPE column (see Table 3.1 test 3), the only option to increase the concentration factor was to decrease the volume in which the extracted material is dissolved before it is provided to the instruments. The minimal required extract volume needed for analysis is 30 mL for the Algaetoximeter and 3 mL for the TOXcontrol system. The maximum volume of surface water that could be provided to the SDB column before the SDB column clogged due to the presence of particles in the surface water was around 8000 mL. A slightly lower volume of 7600 mL of surface water was loaded on the SDB column and upon elution and evaporation of the eluens the extract was dissolved in the minimum volume needed for analysis in either the Algaetoximeter (3 mL extract + 27 mL drinking water) or the TOXcontrol system (3 mL). In this way, the maximum concentration factor that could be achieved was 127-fold for semi-automatic measurement with the Algaetoximeter and 1266-fold for that with the TOXcontrol system. In 2010, the SPE unit was in operation providing daily extracts with these concentration factors and using the same settings as in 2007.

Figure 3.5 presents the results of the new 2010 series of toxicity measurements of SDB SPE BIO extracts obtained with the Algaetoximeter.

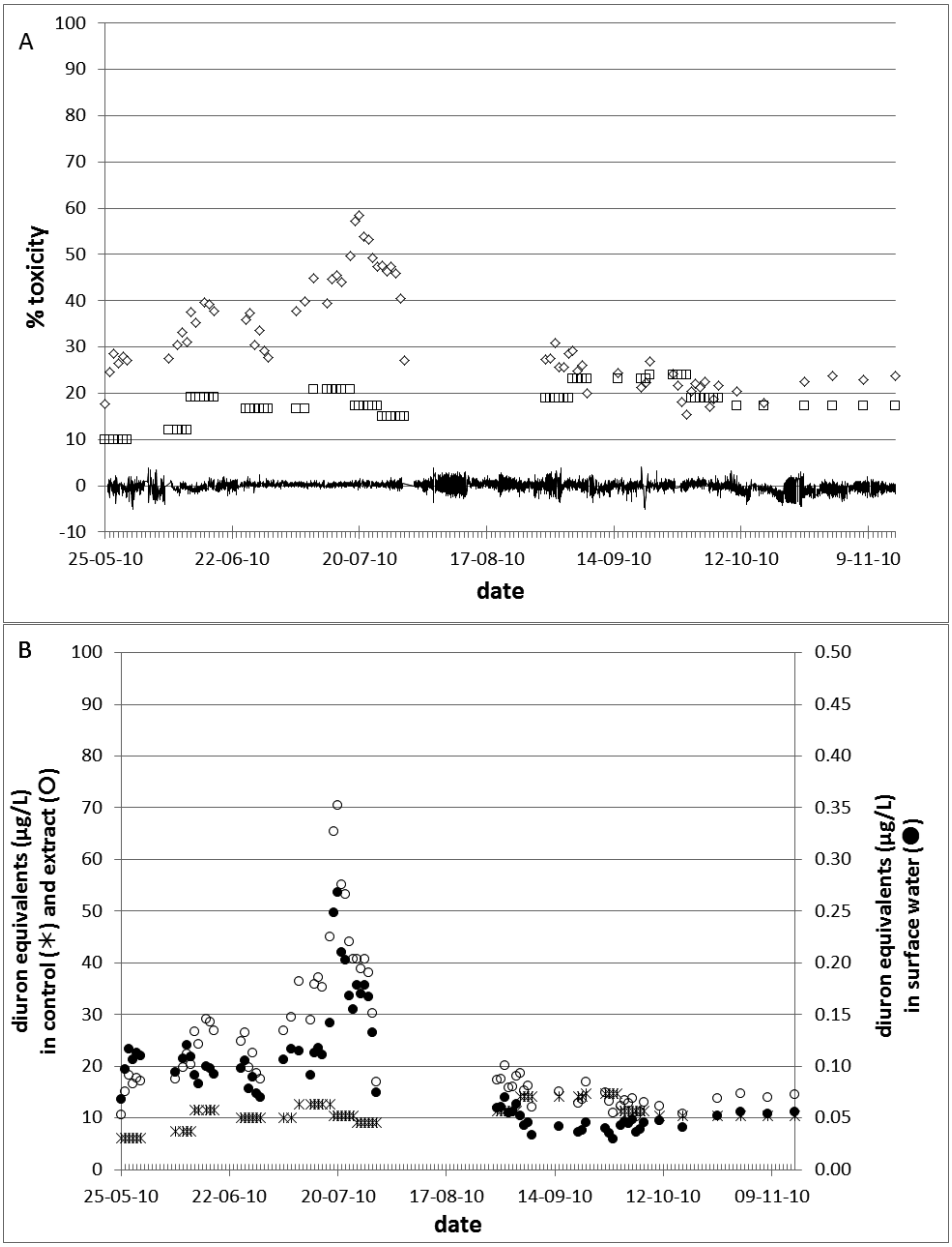

Figure 3.5:A) Time-dependent online SDB SPE BIO extract measurements with the bbe Algaetoximeter of 127 times concentrated River Rhine surface water expressed in % toxicity (◊) and of the corresponding 5 µg/L positive diuron control solution measured before the extract (□). The solid line presents the results from the online analysis of non-concentrated River Rhine surface water. B) Time dependent diuron equivalents (µg/L) of 127 times concentrated River

Rhine surface water (○) and of the corresponding 5 µg/L positive diuron control solution measured before the extract (∗). The diuron equivalents in non-concentrated surface water (●) is corrected for the variation in sensitivity of the algae.

The increase in the concentration factor from 75 to 127, resulted in an increase of the toxicity measured by the Algaetoximeter from a range of 5.8 - 10.1 % to a range of 15.4 - 58.4%. In this new series of measurements the toxicity of the positive control solution of 5 µg diuron/L towards the *Chlorella vulgaris* algae varied between 9.9 % and 23.9 %.Conversion of the toxicity values for the positive diuron control measurement to diuron equivalents resulted in a mean value of 10.7 ± 2.5 µg diuron equivalents/L (n= 12), which is higher than the nominal concentration of 5 µg diuron/L. The variation in the data obtained for the standard diuron solution was lower compared to the variation obtained in the experiment performed in the 2007. Based on these toxicity values, the diuron equivalents of the 127 times concentrated extracts of the River Rhine were calculated to vary between 3.72 and 33.9 µg/L equal to 0.03 and 0.27 µg/L for the non-concentrated surface water samples. It is of interest to note that the data presented in Figure 3.5 reveal a peak of diuron equivalents in the analysed River Rhine surface water extracts in the period between July 16th and July 30th 2010, with diuron equivalent concentrations in the non-concentrated water starting from 0.11 µg/L diuron equivalents to a maximum of 0.27 µg/L on July 20th 2010 and back to 0.07 µg/L on July 20th 2010. This peak in diuron concentration in the River Rhine surface water was not detected in the online measurements of the non-concentrated samples (solid line in Figure 3.5), which is in line with the fact that the detection limit of the Algaetoximeter for diuron in non-concentrated watersamples is around 2 µg/L. Thus, the peak between July 16th and July 29th 2010, presenting lower water quality of the River Rhine, was detected when using the SBD BIO extract procedure. However, this could not be detected with the Algaetoximeter when analysing non- concentrated water.
Figure 3.6 presents the results of the toxicity measurement of SDB SPE BIO extracts obtained with the TOXcontrol system in the same period in 2010 and the corresponding calculated zinc equivalents, together with results from toxicity measurements done with a positive control standard of 20 mg/L zinc.

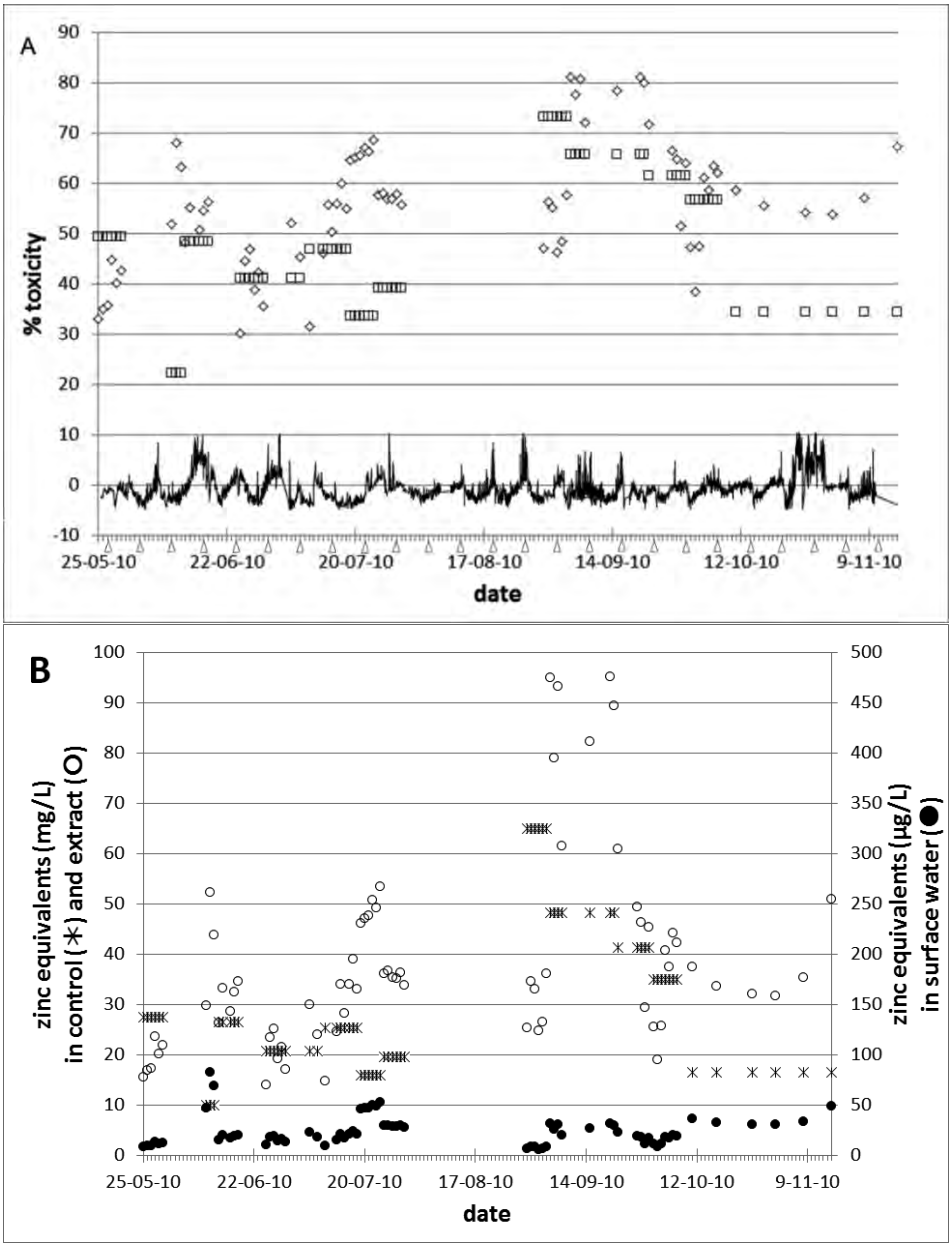

Figure 3.6: A) Time-dependent online SDB SPE BIO extract measurements with the Microlan TOXcontrol of 1266 times concentrated River Rhine surface water expressed in % toxicity (◊) and of the corresponding 20 mg/L positive zinc control solution measured before the extract (□). The solid line presents the results from the online analysis of non-concentrated River Rhine surface water. Renewal of the *Vibrio fischeri* is indicated in the figure with a △ on the x-axis. B)

Time dependent zinc equivalents (mg/L) of 1266 times concentrated River Rhine surface water (○) and of the corresponding 20 mg/L positive zinc control solution measured before the extract (∗). The zinc equivalents (µg/L) in non-concentrated surface water (●) is corrected for the variation in sensitivity of the bacteria.

The increase in concentration factor from 79 to 1266 resulted in an increase of toxicity from 7.6 - 18.6 % to 22.3 - 73.3 % of the SDB SPE Bio extracts measured by the TOXcontrol system. The calculated corresponding zinc equivalents of the 1226 times concentrated extracts of the River Rhine varied between 14.0 and 95.2 mg/L equal to 6.0 and 83 µg/L for the non-concentrated surface water samples.

The data presented in Figure 3.6 also reveal a rather large variation in the response of the TOXcontrol system to the positive control sample of 20 mg zinc/L, showing low toxicity of 22 % for the control standard in June 10th – 12th 2010 and high toxicity values of 60 to 73 % in August 30th – September 30th 2010. This may be due to a variable quality and sensitivity of the *Vibrio fischeri* culture used in the TOXcontrol at that time.

To further investigate whether the peak in toxicity observed for concentrated River Rhine surface water samples in the period from July 16th till July 29th 2010 when analysed by the Algaetoximeter in combination with the SDB SPE BIO procedure could be due to lower flow rates. The flow rate of the River Rhine was also measured at location Hagestein (several kilometres upstream the water intake in Nieuwegein)(Figure 3.7) and compared to the diuron and zinc equivalents of surface water as detected by the SDB SPE BIO biological early warning systems.

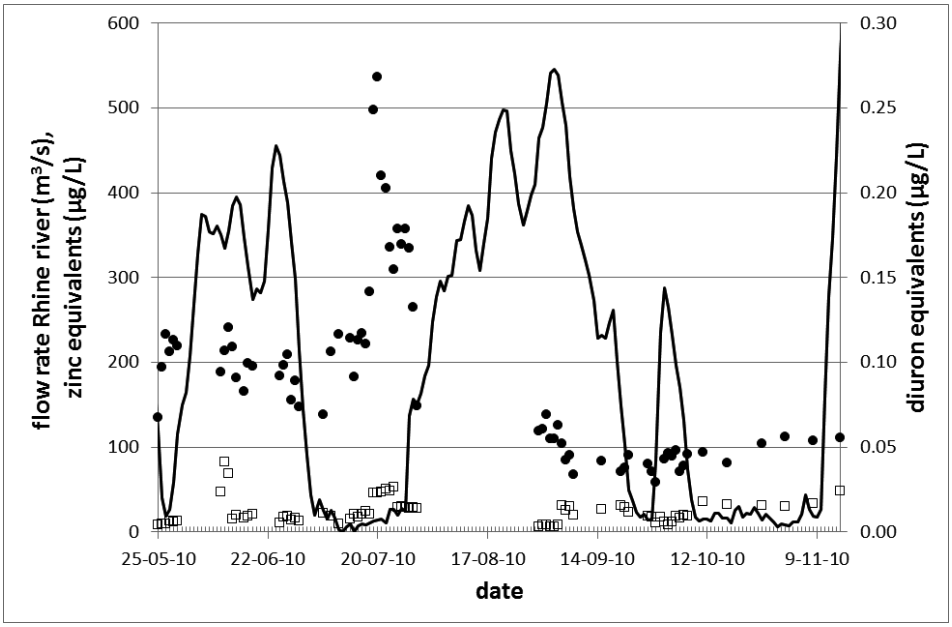

Figure 3.7: Calculated diuron equivalents in µg/L (•) and zinc equivalents in µg/L (□) in surface water, as detected in 2010 by SDB SPE BIO extract measurements using biological early warning systems, compared to the flow rate of the River Rhine at Hagestein (solid line, data from RIWA Rhine Database Nieuwegein (Anonymous, 2011).

The flow rate of the River Rhine at Hagestein did not change much during the 13 day period, varying from 8 m^3/s to 28 m^3/s and was low compared to the average flow rate of 280 m^3/s of the river for the year 2010. The pattern of the diuron equivalents displayed in Figure 3.7 together with the low constant flow rate of the river Rhine, reveals a clear peak indicative for a discharge of organic pollutants.

With the use of the SDB SPE BIO procedure, a poor water quality alarm can be given by the Algaetoximeter when the diuron equivalents in surface water is exceeding a 0.1 µg/L limit value (Figure 3.7). The limit value is a factor 5 lower compared to the limit described in the Dutch Water Supply act (Anonymous, 2001) in which the summated concentration of pesticides must not exceed the 0.5 µg/L level. The different pesticides that can be present in surface water, present different levels of toxicity to the algae. To check if the SDB SPE BIO Algaetoximeter alarm level is comparable with the 0.5 µg/L limit of the Dutch Water Supply act, additional information by chemical analyses of the surface water is required. The measurement of the extracts with the Algaetoximeter

provides water concentrations of organic pollutants expressed in µg/L diuron equivalents in surface water, representing the effect of the mixture of organic pollutants with a similar mode of toxicity as diuron in the river. The results of the present study indicate that the Algaetoximeter can reliable detect these organic pollutants in surface water at a level of 0.025 µg/L when surface water is concentrated with a factor of 127 by the SDB SPE BIO procedure. This level of detection is comparable to the detection limit for analytical organic analysis of diuron. For the combination TOXcontrol and SDB SPE Bio procedure a limit value cannot yet be set, due to the relatively large variation in the sensitivity of the system as reflected in the relatively large variation in the values obtained for the zinc control measurements.

It is concluded that especially the results obtained with the Algaetoximeter provide a proof of principle for online bioassays with increased sensitivity using the SDB SPE BIO procedure, providing a suitable method for surface water quality control now that water quality has been improved over the past decades, but still requires adequate online monitoring. The approach for using the SDB SPE BIO procedure coupled to the biological early warning system provides additional information to the drinking water manager to better judge whether pollutants, chemically detected with ever increasing sensitivity, will still result in toxicity.

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Chapter 4

Genotoxic effects in the Eastern mudminnow (*Umbra pygmaea* L.) after exposure to Rhine water using the SCE and Comet assay; a comparison between 1978 and 2005

This chapter is based on: G.M. Alink, J.T.K. Quik, E.J.M. Penders, A. Spenkelink, S.G.P. Rotteveel, J.L. Maas and W. Hoogenboezem “Genotoxic effects in the Eastern mudminnow (*Umbra pygmaea* L.) after exposure to Rhine water using the SCE and Comet assay; a comparison between 1978 and 2005”, Mutation Research 631:93-100.

Abstract

Surface water used for drinking water preparation requires continuous monitoring for the presence of toxic compounds. For monitoring of genotoxic compounds fish models have been developed, such as the Eastern mudminnow (*Umbra pygmaea* L.) because of its clearly visible 22 meta-centric chromosomes. It was demonstrated in the late seventies that Rhine water was able to induce chromosome aberrations and sister chromatid exchange in this fish species. Although *in vitro* mutagenicity studies of the RIWA (Association of River Water Works, The Netherlands) have shown that the genotoxicity of the river Rhine steadily decreased during the last decades, there is still concern about the presence of some residual mutagenicity. In addition, in most studies the water samples have been tested only in *in vitro* test systems such as the *Salmonella*-microsome test.

For this reason, and in order to be able to make a comparison with the water quality 27 years ago, a study was performed with the same experimental design as before in order to measure the effect of Rhine water on the induction of SCE in the Eastern mudminnow. As a new test system the single cell gel electrophoresis assay (Comet assay) was performed.

Fish were exposed to Rhine water or to groundwater for 3 and 11 days in flow-through aquaria. Fish exposed for 11 days to Rhine water had a significantly higher number of SCE and an increased comet tail-length compared with the control fish exposed to groundwater. After exposure for three days to Rhine water there was no difference in SCE and a slightly increased comet tail-length compared with the control. It was concluded that genotoxins are still present in the river Rhine, but that the genotoxic potential has markedly decreased compared with 27 years ago. Furthermore, the Comet assay appears to be a sensitive assay to measure the genotoxic potential of surface waters in fish.

4.1. Introduction

The river Rhine is an important source for drinking-water production and for this reason continuously monitoring for quality parameters is necessary. Studies by the RIWA (Association of River Water Works, The Netherlands) show that the quality of the river water is slowly improving (Penders and Hoogenboezem, 2001; Penders and Hoogenboezem, 2003; Pieters *et al*., 2004). However, there is still concern because of increasing levels of new chemical species as estrogens and because residual activity of possible genotoxic compounds is still present. In the last decades, much attention has been paid to the genotoxic potential of water of the river Rhine. Already in 1978, it was shown that fish exposed to Rhine water developed chromosome aberrations in their gill cells (Prein *et al*., 1978). Other studies also showed the genotoxic potential of water of the river Rhine and other Dutch rivers (Maas-Diepeveen *et al*., 1991; Alink *et al*., 1980; Hooftman and Vink, 1981; Van der Gaag *et al*., 1982; Maas *et al*., 1994). Although different animal species have been used for genotoxic monitoring of surface and waste waters (Wrisberg and Van der Gaag, 1992; Gauthier *et al*., 1993), fish models are still very useful. As a vertebrate model, fish is the best available to estimate possible human risks, because they can metabolize and accumulate pollutants (Kligerman *et al*., 1975, Kligerman, 1979; Kligerman and Bloom, 1976; Kligerman and Bloom, 1977; Grisolia and Cordeiro, 2000; Van der Hoeven *et al*., 1982; Hooftman, 1981; Hooftman and De Raat, 1982; Alink, 1982; Van der Gaag and Van de Kerkhoff, 1985, Van de Kerkhoff and Van der Gaag, 1985; Diekmann *et al*., 2004). So far the different studies indicated that there is a steady decrease of genotoxic potential of Rhine water. However these studies used bioassays that measured effects in non-vertebrate cells or tested specific fractions of the surface water (Hoogenboezem and Penders, 2003) (Figure 4.1). So there was no certainty about the presence of compounds exerting health risk for vertebrate cells *in situ*.

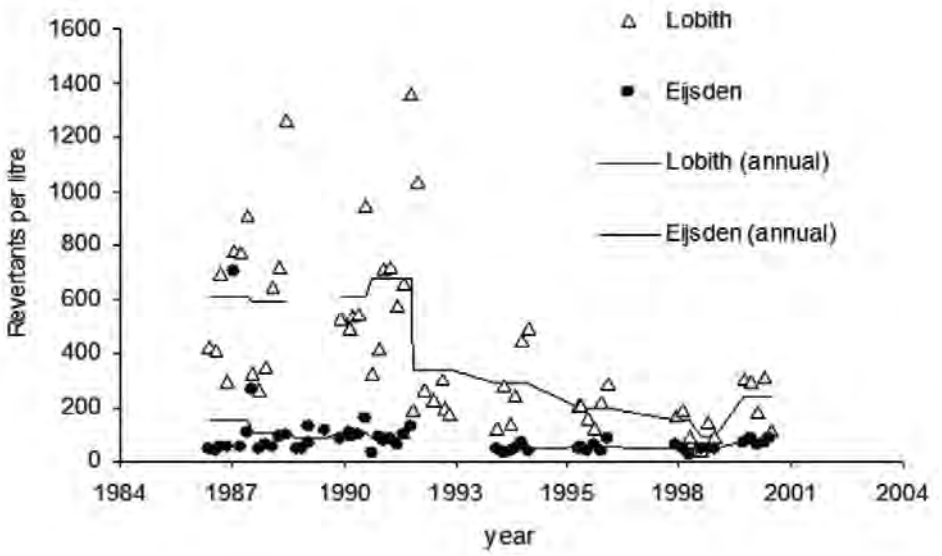


Figure 4.1: Mutagenicity of Rhine water near Lobith and of river Meuse water near Eijsden, two different locations in The Netherlands, measured in the period 1981-2001 in the *Salmonella*-microsome test (data from Hoogenboezem and Penders, 2003).

This question could only be answered by using a fish model appropriate to measure genotoxic effects. Such a fish model is the Eastern mudminnow (*Umbra pygmaea*) as this fish species has a karyotype with a small number (22) of large meta-centric chromosomes which makes cytogenetic analysis very feasible. In addition, this fish species is a non-native species for The Netherlands and bears ambient exposure to river water very well (Prein *et al*., 1978; Alink *et al*., 1980; Hooftman and Vink, 1981; Van der Gaag and Van de Kerkhoff, 1985). For this reason and in order to make a comparison possible with the quality of Rhine water 27 years ago, Eastern mudminnows (*Umbra pygmaea*, Figure 4.3A) were exposed to water of the river Rhine. Gill cells were then studied for genotoxic effects by means of the sister chromatid exchange (SCE) test. For comparison and to study other genetic endpoints the single-cell gel electrophoresis assay (Comet assay) was included in the present study as a new and rapid test (Lee, 2003; Schnurstein and Braunbeck, 2001; Rojas, 1999; Besten *et al*., 2006).

4.2. Materials and methods

Chemicals

All chemicals were of pro-analysis quality. Ethyl methanesulphonate (EMS) was obtained from Fluka (Buchs, Switserland). Collagenase, bovine serum albumin (BSA), lauroyl sarcosine, bromodeoxyuridine (BrdU), colchicine, Hoechst 33258, phosphate-buffered saline (PBS), Giemsa, normal melting point (NMP) agar and low melting point (LMP) agar were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). KCl, NaCl, acetic acid, phosphate and citrate salts for buffers, Triton X-100 and DMSO were from Merck (Amsterdam, The Netherlands) and EDTA was purchased from Baker (Deventer, The Netherlands).

Fish husbandry and exposure to Rhine water

Fifty Eastern mudminnows (*Umbra pygmaea*) were collected from small ponds in the National Park "De Groote Peel", a nature preserve in the south of The Netherlands, in collaboration with the Dutch Forest Service, and after permission of the Animal Welfare Committee of the Wageningen University. The fish were transported to the 'Waterlaboratorium' at Nieuwegein, located at the river Rhine, so that the fish could be exposed directly to Rhine water. As the peat water of the ponds had a pH of 3, fish were adapted slowly to the higher pH and the conditions of Rhine water and control water (defined below) in order to prevent stress. Prior to the experiment, the fish were placed for 14 days in mixtures of peat and control water of which the pH was gradually increased from about 3.0 to 7.0. Fish were fed standard feed consisting of frozen mosquito larvae (*Chironomid*) alternated with live feed (*Tubifex*) before and during the experiment.

Fish were exposed to Rhine or control water in the same way and at approximately the same location as in a previous experiment in 1978 (Alink *et al*., 1980). The control water was natural groundwater of drinking-water quality. It has been retained in deep aquifers for over 100 years. This water, which is not treated with chlorine or any other disinfectant, is aerated and rapidly filtered through sand before distribution. The lack of influence from infiltrating river water makes this groundwater ideal for control experiments. NaCl was added until the conductivity was the same as that of Rhine water (about 700 µS/cm). So only the pH and the conductivity were adjusted and there was no other relation between the controlwater and the Rhinewater. The control can, thus, be considered as a laboratory control.

In March 2005, two groups of 12 fish, 8 fish for the SCE-test and 4 fish for the Comet assay, were exposed to Rhinewater for 3 and 11 days, respectively. Two groups of 12 fish were kept in control water for the same periods. Fish for the SCE-test and Comet assay were kept in separate 100 L all-glass flowthrough aquaria with a flow rate of 216 L/day and continuous aeration. A temperature of 12 °C was maintained by heating the

incoming water. Silt was removed from the Rhine water by a 120-min sedimentation period and by a serial filtration with cotton candle filters (50, 10, 3 and 1 µm)(Figure 4.2). By removing the large particles, only dissolved substances and substances adsorbed to particles <1 µm are considered in this experiment. As a positive control, 5 fish were exposed in a 5-L aquarium to ethyl methanesulfonate (120 mg/L) for 3 days.

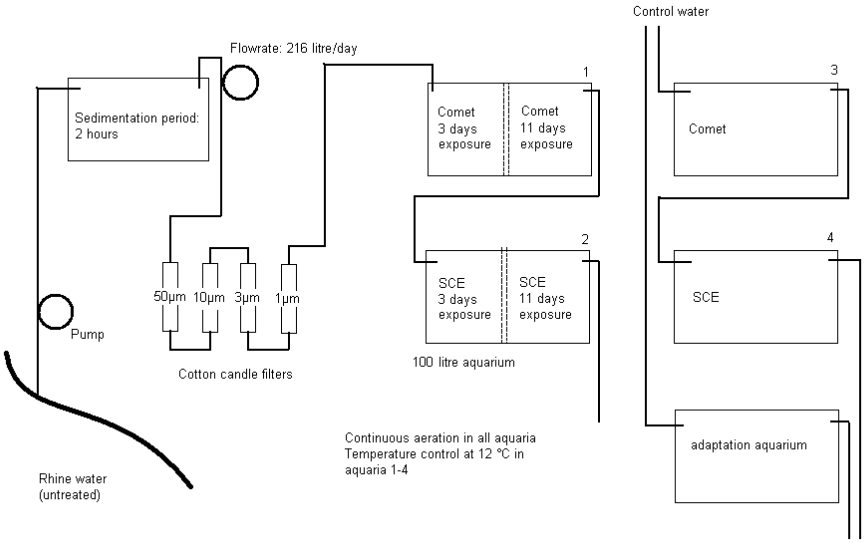


Figure 4.2: The experimental design for exposure of fish to Rhine and control water.

Sister chromatid exchange test

The sister chromatid differentiation technique *in vivo*, as described by Kligerman and Bloom (1976) was used with slight modifications. Fish were injected once intraperitoneally (i.p.) with 0.5 mg BrdU/g fish and were exposed to this base analogue for 10 days (2 cell cycles). Fish to be exposed for 3 days to Rhine water were first kept in control water for 7 days after injection with BrdU. At the end of the exposure period the fish were injected i.p. with 0.25 mg colchicine/g fish and killed 5–19 h later. After decapitation of the fish, the gills were removed and placed in a 0.4% hypotonic solution of KCl for 30 min. The tissues were then fixed in methanol-acetic acid (3:1). Cell preparations were made by the solid-tissue technique (Kligerman and Bloom, 1977). The cells were dried for at least 24 h, and then stained according to a modified fluorescence-plus-Giemsa method (Perry and Wolff, 1974). Preparations were first treated with Hoechst 33258 (50 µg/mL) in Sorensen's buffer (pH 7.0) for 10 min in the dark, rinsed in distilled water and then exposed to UV radiation (HPW 125W-T, Philips, Belgium) for 4 h in a phosphate–citrate buffer (pH 7.0). Subsequently, preparations were heated in 2×SSC at 60 ◦C for 40 min and stained in 5% Giemsa in Sorensen's buffer (pH 6.8) for 10 min. The preparations were dried for at least 48 h and the SCEs were scored double blind by two different persons, in metaphases of at least eight chromosomes. Mean values and standard errors were determined. For differences between means, Student's t-test was used with significance levels at $P < 0.05$.

Comet assay

The Comet assay, a technique that allows to detect and quantify chromosome damage in single cells, was a modification of the standard method for zebra mussels, *Dreissena polymorpha* (Osman *et al*., 2004), adapted according to a procedure described for Zebrafish (Schnurstein and Braunbeck, 2001). In short, the procedure was as follows.

After preparation of the gills a cell suspension was obtained by treatment with a collagenase solution for 20 min. After filtration, which was needed to get rid of undigested tissue, and centrifugation the pellet was resuspended in PBS with 0.1% BSA. The cell suspension was then mixed with LMP agarose and transferred to a slide pre-coated with NMP agarose. Subsequently, the slide was coated with 1:1 LMP agar in PBS with 0.1% BSA. Per fish 4 slides were prepared.

Subsequently, the cells – not the nuclei – were lysed in a lysis buffer (2.5M NaCl, 0.1M EDTA, 0.01M Tris, 1% sodium lauroyl sarcosine, 1% Triton X-100 and 10% DMSO, pH 10) at 4 °C for at least 1 h. Single-strand DNA was prepared by unwinding the DNA in electrophoresis buffer (0.3M NaOH, 1mM EDTA, pH 13) for 30 min. Then DNA fragments were separated during micro-electrophoresis (Hoeffer supersub, Pharmacia biotech) for 20 min at 25V and 400 mA.

After staining with ethidium bromide (20 µg/mL) for 10 min, the tail-length was measured by means of an Olympus BH-2 fluorescence microscope

(excitationwavelength, 515–560 nm) equipped with image-analysis software (Perceptive Instruments, Haverhill, UK). Per slide the tail-length of 50 comets was measured. Scoring was done in a double blind fashion.

Statistics

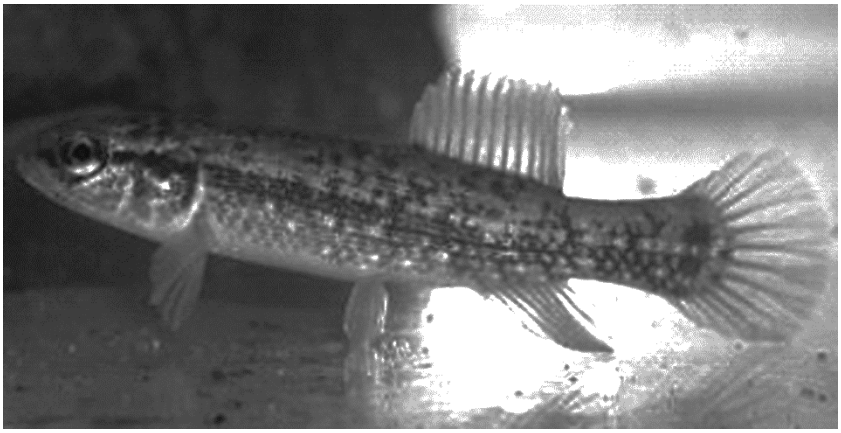
Differences between groups were studied using the Student's t-test with significance levels at $P < 0.05$. Each fish was considered as a test unit as described by others (Hartmann *et al*., 2003; Tice *et al*., 2000).

4.3. Results

Sister chromatid exchange test

In Figure 4.3B, a typical example is given of a metaphase of a gill cell of the Eastern mudminnow showing sister chromatid differentiation and exchange. Not all the fish showed sister chromatid differentiation.

A)



B)

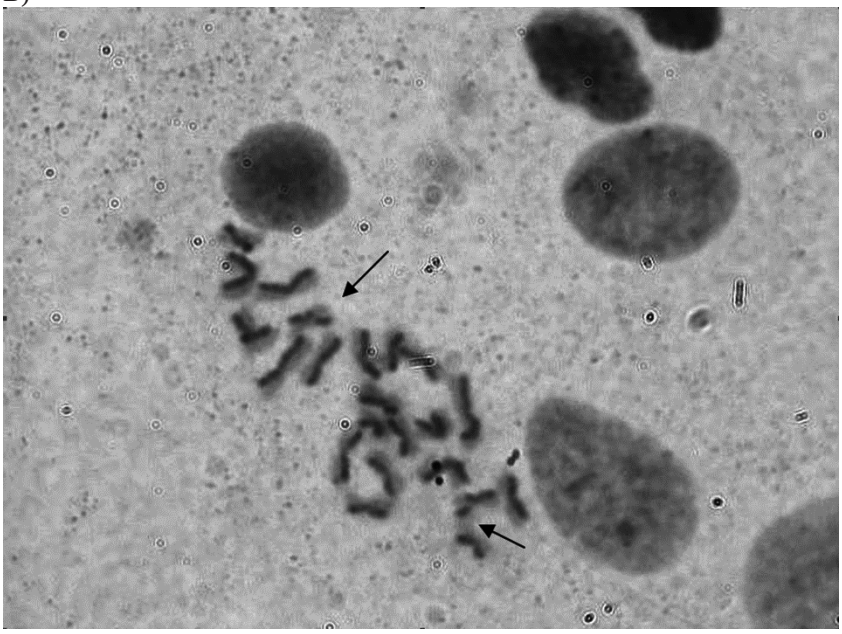


Figure 4.3: A) Eastern mudminnow (*Umbra pygmaea*); (B) metaphase image of a gill cell of the Eastern mudminnow showing two sister chromatid exchange events (see arrows).

For the control group six fish and for the experimental groups five fish were used for calculating the data. After 11 days of exposure to Rhine water there was a significant increase, almost a doubling, in the number of SCEs per chromosome compared with the control (P = 0.013) and also compared with the 3-day exposure group (P = 0.023). No induction in SCEs was seen after 3 days of exposure (Table 4.1, Figure 4.4).

Table 4.1: Number of sister chromatid exchange events per chromosome in gill cells of *Umbra pygmaea* after exposure to Rhine water (R) or groundwater (C) for 3 or 11 days, compared with data from 1978 (Alink *et al*., 1980); mean±S.D., n is number of fish or chromosomes

	1978			**2005**		
	SCEs/chromosome	**n_{chr}**	**n_{fish}**	**SCEs/chromosome**	**n_{chr}**	**n_{fish}**
C	0.045 ± 0.012	1149	5	0.044 ± 0.012	1260	6
R3	0.128 ± 0.023	2416	6	0.043 ± 0.017	913	5
R11	0.155 ± 0.021	2317	5	0.072 ± 0.016	917	5

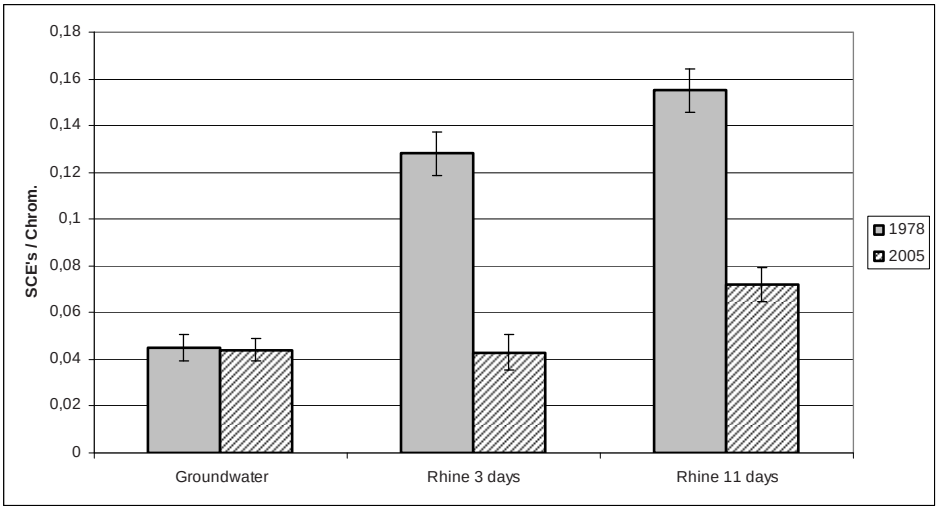


Figure 4.4: Effect of Rhine water on sister chromatid exchange in gill cells of the Eastern mudminnow after exposure for 3 and 11 days in 1978 and 2005; mean ± S.E.M., n = 5–6.

Comet assay

In order to differentiate between DNA damage due to cytotoxicity or genotoxicity, cell viability was assessed using the trypan blue assay. The viability of the cell suspensions of all fish was between 88 and 96%. Validation studies have not been conducted to identify acceptable cytotoxicity levels for *in vivo* Comet assays (Tice *et al*., 2000). However, cell viability below 70–80% of that in the control animals may be considered excessive (Tice *et al*., 2000). The viability of cell suspensions used in this study was above these values and therefore considered acceptable. Thus, it is unlikely that cytotoxicity of the Rhine water or of the positive control had an effect on the outcome of the experiments. In Figure 4.5, comets are shown of gill cell DNA derived from fish exposed to Rhine water for 11 days.

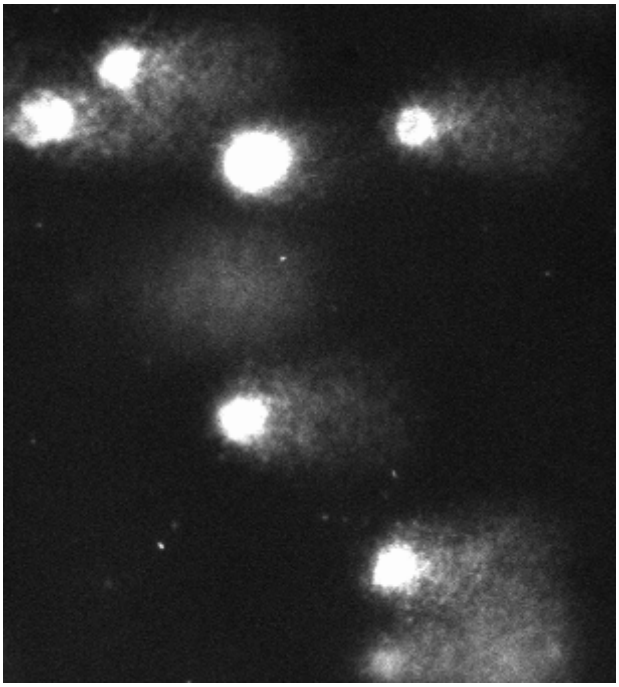


Figure 4.5: Comets after single cell electrophoresis of gill cell DNA from Eastern mudminnows exposed to Rhine water.

As shown in Figure 4.6 and Table 4.2, there was a significant difference between the comet tail-length of fish exposed for 11 days to Rhine water compared with the groundwater control ($P < 0.05$). After 3 days of exposure to Rhine water the comet tail-length was slightly longer than in the control, but the difference was not significant. Exposure to EMS significantly ($P < 0.05$) increased the comet tail-length.

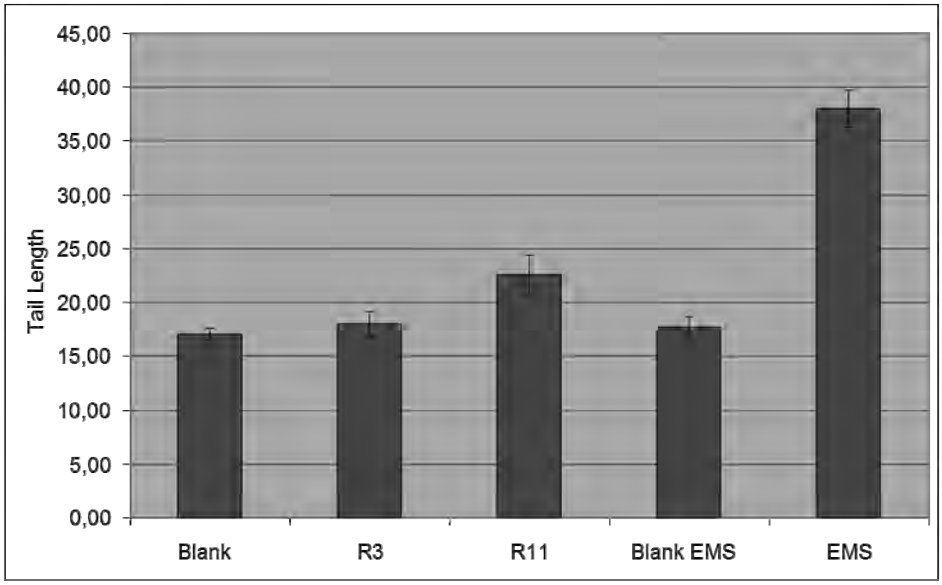


Figure 4.6: Effect of 3 and 11 days of exposure to Rhine water on chromosome breaks in gill cells of the Eastern mudminnow, measured as comet tail-length after single-cell gel electrophoresis; positive control: 120 mg/L EMS; mean±S.E.M., n=4.

Table 4.2: Significant differences between EMS-exposed and Rhine water exposed fish compared with their controls in the Comet assay; one-tailed P-values in Student's t-test.

Differences between		**P one-tailed**	**Significance**
Blank EMS	EMS	0.000394	++
Blank R	R 11	0.013356	+
Blank R	R 3	0.482744	-

-: not significant, +: $P < 0.05$, ++: $P<0.001$

4.4. Discussion

The main conclusion of the present study is that Rhinewater still contains genotoxins that are able to induce sister chromatid exchange and single-strand DNA breaks, measured with the Comet assay, in gill cells of fish exposed for 11 days to Rhine water. After 3 days of exposure no effect was seen on SCE frequency and a slight, but not significant increase of DNA damage in the Comet assay. This suggests that there is a dose- and time-dependent effect. Twenty-seven years ago, our group observed an increase in SCEs in the same fish species, the Eastern mudminnow, already after a 3-day exposure to Rhine water, and a much higher SCE frequency after an 11-day exposure than in the present study. The SCE frequency in the fish exposed to groundwater was exactly the same as before (Alink *et al*., 1980). Based on the SCE assay, it can be concluded that the quality of the water of the river Rhine with respect to the presence of genotoxic compounds has improved during the last decades. Furthermore, the present study shows that the Comet assay can be applied successfully in fish, for genotoxic monitoring of surface waters. As far as we know this is the first time that the Comet assay is used for genotoxic monitoring in the Eastern mudminnow. The Eastern mudminnow, *Umbra pygmaea*, a non-native species, is abundantly present in pools in the south of The Netherlands. In previous studies this fish appeared to be an appropriate model for cytogenetic studies, because of the restricted number of well-visible metacentric chromosomes, and because this fish species can be kept easily under laboratory conditions (Prein *et al*., 1978; Alink *et al*., 1980; Kligerman *et al*., 1975, Kligerman, 1979; Kligerman and Bloom, 1976; Kligerman and Bloom, 1977). As the sister chromatid differentiation (SCD) technique is a laborious method, especially *in vivo*, because it needs dividing cells, the Comet assay was applied in order to compare the results with the SCE test. The Comet assay has many advantages, such as being independent of chromosome number, not requiring animal pre-treatment with BrdU and colchicine, and being less time-consuming due to automatic scoring of the comets by use of image-analysis software (Schnurstein and Braunbeck, 2001; Tice *et al*., 2000; Rank, 2003). For most compounds both assays exhibit a similar sensitivity, although there are some differences in types of DNA damage detected (Hartmann and Speit, 1995). SCEs reflect a DNA-repair process and they may occur spontaneously in normal cycling cells, suggesting a link between SCE and DNA replication. Although their molecular basis still remains obscure, homologous recombination may be one of the principal mechanisms responsible for SCEs in vertebrate cells (Sonoda *et al*., 1999). The Comet assay detects primary DNA lesions such as DNA strand breaks, but may also detect genomic instability, repair of double-strand breaks, DNA-adduct formation and DNA cross-links (Hartmann and Speit, 1995). The widespread applicability of the Comet assay is reflected in the wealth of data that appeared in the last few years. In this study, the data of the SCE test and the Comet assay are in agreement. A comparison of

the recent results with data obtained in the previous study 27 years ago (Alink *et al*., 1980) shows a marked decrease in SCE frequency, suggesting the presence of less genotoxins. This corresponds with recent studies of the RIWA and the 'Waterlaboratorium' showing a decrease in mutagenicity ofwater extracts measured in the *Salmonella*-microsome test (Hoogenboezem and Penders, 2003). Other studies show also an improvement of the water quality of the river Rhine (Pieters *et al*., 2004). The observed correspondence in sensitivity between the Comet and SCE assays in this study is also in agreement with the literature, where for most chemicals tested a similar sensitivity was found for the two assays (Hartmann and Speit, 1995). As the Comet assay measures primary DNA lesions and the SCE test reflects DNA-repair processes, our data suggest that compounds present in Rhine water still display a broad spectrum of genotoxic effects.

During the exposure period of the fish in this study, Rhine water was also collected and tested in the *Salmonella*-microsome test (tester strain TA98, with S9) as described earlier (Hoogenboezem and Penders, 2003). A doubling of the number of revertants per litre of Rhine water was seen, indicating a weak mutagenic response in the bacterial mutagenicity assay (data not shown). Therefore, it appears that the residual mutagenicity observed in bacterial assays also induces cytogenetic changes in fish. So far the compounds causing these effects are unknown. A list of organic contaminants that have recently been measured in Rhine water is presented in Table 4.3.

Table 4.3 Organic contaminants present in the river Rhine during the experimental period.

Substance	CAS-number	Use	Mutagenic[a]	Average Concentration (µg/L)
Diethyleneglycol dimethylether (diglyme)	111-96-6	Solvent	No	0.12
Azo-bis-isobutyronitrile (AIBN)	78-67-1	Plasticizer	No	0.04
2-(Trifluoromethyl)aniline	88-17-5	Pesticide	No[b]	0.08
Dipropyleneglycol methylether	13429-07-7	Additive in Paint	No	0.03
Triethylphosphate (TEP)	78-40-0	Flame retardant; Plasticizer	No	0.06
3,5,5-Trimethylcyclohex-2-enone (isophorone)	78-59-1	Solvent	No	0.03
Triethyleneglycoldimethylether (triglyme)	70992-85-7	Solvent	No	0.37
Diethyl-methyl-carbamodithioaat	7-1-8018	Herbicide	Possible	0.11
Surfynol 104	104-76-7	Surfactant	Yes	0.48
1,1,3,5-Tetramethyl-cyclohexane	4306-65-4	Personal Care Product	No	0.03
Tetra-ethyleneglycol dimethylether (tetraglyme)	143-24-8	Solvent	No	0.11
Tri-isobutylfosfaat	126-71-6	Pesticide	No[c]	0.05
Tetra-acetylethylenediamine (TAED)	10543-57-4	Detergent	No	0.09
Benzophenone	119-61-9	Personal Care Product	No	0.04
N-Ethyl-p-toluenesulfonamide	80-39-7	Herbicide	No	0.05
N-Butylbenzenesulphonamide	1907-65-9	Herbicide	-	0.05
Tri (2-chloro-isopropyl) phosphate (Fyrol PCF)	13674-84-5	Flame retardant	No	0.11
Galaxolide (HHCB)	1222-05-5	Personal Care Product	No	0.08
Dimethylpropylphenol	80-46-6	Pesticide	Possible	0.11
Hexakis(methoxymethyl)melamine (HMMM)	3089-11-0	Adhesive	No	0.30
Xylene	104-76-7	Pesticide	No	0.13
tert-Butylmethylether (MTBE)		Fuel Additive	No	0.16
Ethylbenzene	100-41-4	Fuel Additive, Basic Chemical	No	0.11
Carbamazepine	298-46-4	Pharmaceutical	No	0.15

The frequency of sampling was daily. Only compounds determined frequently (>50%) are given.
[a] From: " Chemical Safety Information from Intergovernmental Organizations" http://www.inchem.org) or the "European chemical Substances Information System" (http://ecb.jrc.it/esis/), unless otherwise stated.

[b] From: Andenen *et al*., 1972.
[c] Toxicological Evaluation of triisobutylphosphate; BG Chemie, 2000.

Although in this study testis cells were not included, it may be supposed, based on previous data (Alink *et al*., 1980), that the male reproductive system of the fish is still at risk. As this study and others showthat exposure during a period of 11 days increases genotoxic effects, it cannot be excluded that long-term exposure to low doses of genotoxins in the surface water leads to marked genotoxic effects in somatic and reproductive cells of fish and other aquatic organisms. Whether bioaccumulation contributes to this effect is unknown and should be a subject of future research. Although at present no mutagenicity can be detected in drinking-water samples derived from Rhine water, it cannot be excluded that small, undetectable amounts of genotoxins are still present in drinkingwater. As long as the identity and the actual presence of these genotoxins in drinking-water is unknown, and as long as there is no information on whether these compounds can accumulate in vertebrate organisms, further studies are needed to investigate (a) the presence of genotoxic micropollutants in drinking-water and (b) the long-term genotoxic effects in vertebrates, in order to conclude whether or not chronic exposure of humans to drinking-water derived from surfacewater requires extra purification steps.

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chapter 5

Genotoxic effects in the Eastern mudminnow (*Umbra pygmaea*) after prolonged exposure to River Rhine water, as assessed by use of the *in vivo* SCE and Comet assays

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Abstract

The production of drinking water from river water requires a certain minimal river water quality. The association of Rhine Water Works (RIWA), therefore, operates a monitoring network. *In vitro* mutagenicity studies have shown that the genotoxicity of the River Rhine water steadily decreased from 1981 until 2001. Compared to a study in 1978, a decrease in genotoxicity was also observed in an *in vivo* genotoxicity study in 2005, in which Eastern mudminnows (*Umbra pygmaea*) were exposed to Rhine water, and gill cells were used for the Sister Chromatid Exchange (SCE) test and the Comet assay. In this 2005 study, the *in vivo* genotoxicity increased upon extending exposure of the fish from 3 to 11 days. Therefore, the objectives of the present study were to investigate i) whether further prolonged exposure results in a further increase in *in vivo* genotoxicity, ii) whether new data corroborate that *in vivo* genotoxicity of Rhine water is at present lower than in 1978, and iii) whether the Comet assay is a suitable alternative to the SCE assay. Prolonging the exposure time of Eastern mudminnows to River Rhine water from 11 days to 42 days, did not give a significant increase in SCEs and DNA damage (Comet assay) in gill cells. The new data corroborate that *in vivo* genotoxicity of River Rhine water is at present lower than in 1978. The Comet assay is a useful addition but does not provide a substitute for the SCE endpoint in these *in vivo* genotoxicity studies.

5.1. Introduction

The production of drinking water from river water requires a certain minimal river water quality. The Association of Rhine Water Works (RIWA) operates a monitoring network to obtain information about this water quality. Next to frequent analytical chemical analyses, which provide direct information about the level of organics or other chemical impurities present in the surface water, genotoxicity studies have also been incorporated in this network of test batteries. In 1978, it was shown that fish exposed to River Rhine water developed chromosome aberrations in their gill cells (Alink *et al.*, 1980). In the period from 1981 to 2001, the *in vitro* Ames test was used to monitor the genotoxicity of the River Rhine water. A decline of genotoxicity in Rhine water was shown (from a maximum level of 600-700 revertants per litre in 1981 to 200-300 revertants per litre in 2001 using concentrated water samples) (Hoogenboezem and Penders, 2003). In order to monitor the genotoxicity in vertebrate cells, a study was presented in 2005 on the *in vivo* genotoxicity of River Rhine water using the gill cells of the Eastern mudminnows, the Sister Chromatid Exchange (SCE) test and the Comet assay (Alink *et al.*, 2007). The latter test was used for the first time for genotoxicity monitoring in the Eastern mudminnows. The main conclusion of this study was that unconcentrated Rhine water still contains genotoxins that are able to induce SCEs and DNA damage, the latter as measured with the Comet assay, in gill cells of fish that were exposed for 11 days to Rhine water. After 3 days of exposure no effect was seen on SCE frequency and a slight, but not significant increase of DNA damage was seen in the Comet assay. This suggests a dose- and/or time-dependent increase in *in vivo* genotoxicity upon prolonged exposure. Such an increase in *in vivo* genotoxicity up in prolonged exposure to genotoxic compounds may pose a risk for environmental species as well as for humans who are exposed to drinking water prepared from surface water. In that case extra purifications steps may be required to reduce this risk. The consequences of prolonged exposure to organics present in low concentrations can be studied using a fish model with a long term exposure time. In this study, the Eastern mudminnow (*Umbra pygmaea*) was used, from which exposed gill cells were studied with the SCE test and the single-cell gel electrophoresis assay (Comet assay). In our previous study an increase in *in vivo* genotoxicity was observed in this model system when exposure time was increased from 3 to 11 days. In the present study the effect of extending the exposure from 11 to 42 days was investigated. All together the aims of the present study were to investigate i) whether prolonged exposure from 11 to 42 days would result in a further increase in genotoxicity, ii) whether the new *in vivo* genotoxicity data obtained would corroborate the conclusion from the 2005 study that *in vivo* genotoxicity of River Rhine water is at present lower than that detected in 1978, and iii) whether the Comet assay would provide a suitable alternative or addition to the SCE assay as an *in vivo* endpoint.

Eastern mudminnows were exposed to River Rhine water for 11 and 42 days in flow-through aquaria and gill cells were used for the Sister Chromatid Exchange (SCE) test and the Comet assay.

5.2. Materials and Methods

Chemicals

All chemicals were of pro-analysis quality. Ethyl methanesulphonate (EMS) was obtained from Fluka (Buchs, Switserland). Sodium N-lauroylsarcosine, collagenase, bovine serum albumin (BSA), phosphate-buffered saline (PBS), normal melting point agarose (NMP), low melting point agarose (LMP), HEPES, ethidium bromide, bromodeoxyuridine (BrdU), colchicine, Hoechst 33528 and Giemsa were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Potassium chloride (KCl), sodium chloride (NaCl), acetic acid, EDTA, sodium hydroxide (NaOH), phosphate and citrate salts for buffers, Triton-X-100, methanol, and DMSO were obtained from VWR International B.V. (Amsterdam, The Netherlands). Tris was obtained from Invitrogen (Breda, The Netherlands).

Experimental setup and exposure to Rhine water

After permission of the Animal Welfare Committee of Wageningen University and in collaboration with the Dutch Forest Service, ninety Eastern mudminnows (*Umbra pygmaea*) were collected from small ponds in the National Park "De Groote Peel", a nature reserve near Ospel in The Netherlands. The fish were transported to the intake station for the city of Amsterdam water works "Waternet" at Nieuwegein, located at the River Rhine. In order to prevent stress, the fish were adapted gradually to the conditions of Rhine water and control water. During a period of 14 days "De Groote Peel" water was slowly diluted with River Rhine water or ground water for the control. Fish were fed daily with frozen red mosquito larvae (*Chironomids*) until the end of the exposure.
Fish were exposed to Rhine or control water in the same manner and at the same location as in the previous experiment in 2005 (Alink *et al.*, 2007). The control water was natural groundwater of drinking-water quality. The groundwater has been retained in deep aquifers for over 100 years, thus considered to be free of contaminants. 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. For comparison with previous studies, sodium chloride (NaCl) was added at the exposure site to the control water to increase the conductivity to the same level as River Rhine water (approximately 700 µS/cm). The pH of both waters was around 8. There were no other relations between the control water and the Rhine water, thus the ground water control can be considered as a laboratory blank.

Starting on December 4th 2007, 8 fish for the Comet assay and 12 fish for the SCE test were exposed to Rhine water for 42 days. Starting on January 4th 2008, 4 fish for the Comet assay and 6 fish for the SCE test were exposed to Rhine water for 11 days. Two

groups of fish, 8 fish for the Comet and 12 fish for the SCE test, were kept in control water for 42 days.
The fish were kept in 100 L all-glass flow through aquariums (Figure 5.1), with a flow rate of 800 L per day and continuous aeration. Silt was removed from the Rhine water, using a sedimentation tank and filtration unit with four serially interconnected cotton candle filters (30, 10, 3 and 1 µm pore size respectively). Only dissolved substances and substances absorbed to particles smaller than 1 µm are considered to be able to pass through the filters. A temperature of 12 °C was maintained by heating the incoming Rhine and control water.

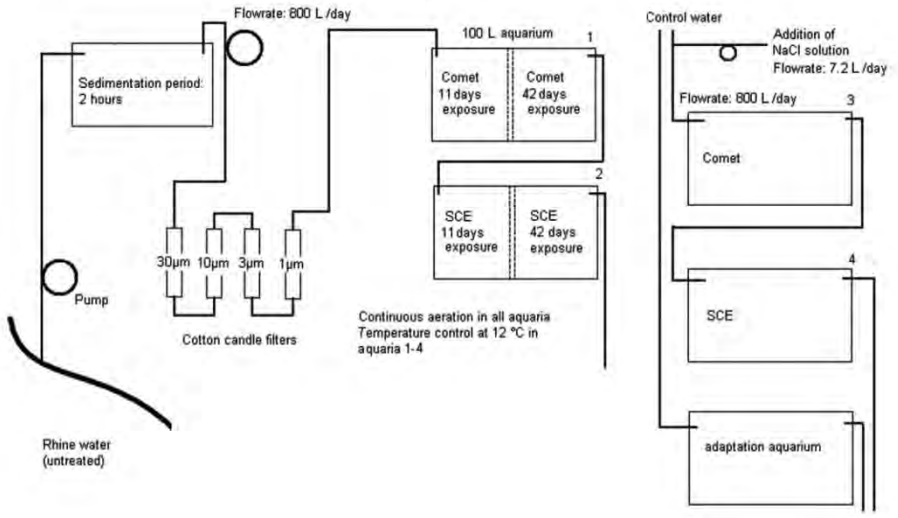

Figure 5.1: The experimental design for exposure of Eastern mudminnow fish to Rhine and control water.

As a positive control, 6 fish for the SCE test and 4 fish for the Comet assay were exposed in separate 5-L aquariums to ethyl methanesulfonate (25 mg/L) for 3 days.

Sister chromatid exchange (SCE) test

The sister chromatid differentiation technique *in vivo*, as described by Kligerman and Bloom (1976) was used with slight modifications. For the experiments, 9 fish were used for the control group, 5 fish were used for the group with 11 days exposure to River Rhine water, and for the River Rhine group exposed for 42 days 8 fish were used. All the fish for the SCE test were injected intraperitoneally (i.p.) with 0.5 mg BrdU/g fish and were exposed to this DNA base analog for 10 days (2 cell cycles). At the end of the exposure period, the fish were injected i.p. with 0.25 mg colchicine/g fish. 10-12 Hours

later, the fish were decapitated and the gills were removed and placed in a 0.4% hypotonic solution of KCl for 30 minutes. The tissues were then fixed in a methanol-acetic acid (3:1) solution. Cell preparations were made by the solid-tissue technique (Kligerman *et al.*, 1977). The cells were dried for at least 24 hours, and then stained according to a modified fluorescence-plus-Giemsa method (Perry and Wolff, 1974). The preparations were first treated with Hoechst 33528 (50 µg/mL) in Sorensen's buffer (pH 7.0) for 10 minutes in the dark, rinsed in distilled water and then exposed to UV radiation (HPW 125 W-T, Philips, Belgium) for 4 hours in a phosphate-citrate buffer (pH 7.0). Subsequently, the preparations were heated in 2X Saline-Sodium Citrate buffer at 60 °C for 40 minutes and stained in 5 % Giemsa in Sorensen's buffer (pH 6.8) for 10 minutes. The preparations were dried for at least 48 hours and the SCEs were scored. Scoring was done in a double-blind fashion.

Comet assay

The alkaline Comet assay was a modification according to a procedure described for zebrafish (*Danio rerio*) (Schnurstein and Braunbeck, 2001) of the standard method for zebra mussels (*Dreissena polymorpha*) (Osman *et al*., 2004). After the exposure period, four fish were decapitated. For the Comet assay, the number of fish used, was according to the recommendation described by Hartmann *et al.* (2003). The gills of the fish, and subsequent the cell suspensions and comet slides were kept away from strong lights to avoid photolysis. The gills were removed and placed in cold PBS buffer with 5 mM HEPES and 0.65 mM EDTA. A cell suspension of the gills was obtained by treatment with a collagenase solution for 15 minutes. After filtration (150 µm pore size) and centrifugation (2000 rpm, 5 °C, 5 minutes), the pellet was re-suspended in cold PBS buffer with 5 mM HEPES, 0.65 mM EDTA and 0.1 % BSA. The cell suspension was mixed with 1 % LMP agarose (37 °C) and transferred to a slide pre-coated with 1 % NMP agarose. A top layer of LMP agarose (1:1 diluted with PBS and 0.1% BSA) was added to the slide to fill in any residual holes in the second agarose layer. Per fish 4 slides were prepared.

The slides were placed for at least 1 hour in cold lysis buffer (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 1 % sodium N-lauroylsarcosine, 1 % Triton-X-100 and 10 % DMSO, pH 10). To produce single-stranded DNA, the slides were placed on a horizontal electrophoresis unit and covered with electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13). After 30 minutes incubation time, electrophoresis was performed for 20 minutes at 25 V and 400 mA (Hoeffer supersub, Pharmacia biotech). Subsequently, the slides were rinsed using neutralization buffer (0.4 M Tris, pH 7.5, 5 minutes) and agarose gels were dehydrated by immersing the slides in absolute ethanol for 1-2 minutes. The DNA was stained for 10 minutes using ethidium bromide solution (20 µg/mL). The equipment used for scoring was an Olympus BH-2 fluorescence microscope (excitation wavelength 515-560 nm) with image-analysis software Comet II

(Perceptive Instruments, Haverhill, United Kingdom). The software provides information about the Comet like tail length, percentage DNA in tail and tail moment (product of the tail length and the fraction of total DNA in the tail). When very low levels of DNA damage are present, tail length is most informative (Collins *et al*., 2008). Per slide the tail length of 50 comets were measured. Scoring was done in a double blind fashion.

Chemical analysis

The chemical analysis of water samples from the sampling location Nieuwegein were performed at Het Waterlaboratorium, Haarlem, The Netherlands. Using solid phase extraction, HPLC and GCMS, a large array of organics and other micro-pollutants were measured. Specific information about the performed analysis can be obtained from the RIWA Annual Reports 2007 and 2008, available at www.riwa.org under Publications or from the first author.

Statistics

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

5.3. Results

Water conditions

The temperature of the water in both groups (control and Rhine group) was adjusted to 12 °C during the exposure time (Figure 5.2). The conductivity of the water for the control group was adjusted frequently depending on the change in conductivity of the River Rhine water. The conductivity of the River Rhine water decreased with increasing discharge of the River Rhine. The pH value remained constant over the entire period. The total hardness of the control water was 202 mg/L $CaCO_3$.

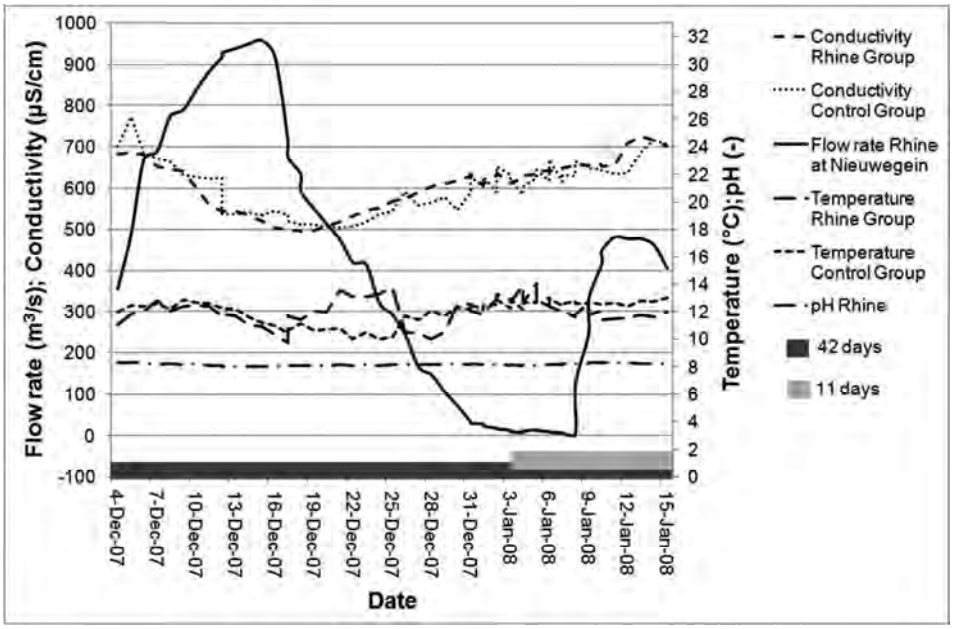

Figure 5.2: Flow rate and pH of the River Rhine water during exposure. Controlled temperature and conductivity of Rhine and Control group during different exposure periods (bars).

Physical chemical quality parameters of River Rhine water

The general physical chemical quality parameters of the River Rhine water in December 2007 and January 2008 are presented in Table 5.1 together with annual means of 2007 and 2008.

In addition to the regular chemical analyses within the framework of the RIWA monitoring program, additional analyses were conducted under the surveillance screening program in operation at the Nieuwegein intake for drinking water production. A selected set of results for quantification of organics from this program is presented in Table 5.2, in which the reporting level is 0.010 µg/L. As far as the analysed compounds are concerned, no significant change in the overall water quality between the 42 days exposure group and the 11 days exposure group was observed. Table 5.2 also indicates which compounds analysed are compounds listed as carcinogenic or mutagenic (Anonymous, 2011) as well as which compounds are listed as carcinogenic, genotoxic or reprotoxic (Pflaumbaum, 2010). From this it follows that River Rhine water used for the study contains a variety of compounds of concern because of genotoxicity albeit in low concentrations.

Table 5.1: The general physical chemical quality parameters of River Rhine water in December 2007 and January 2008, with annual means of 2007 and 2008.

	Unit	**December 2007**	**Annual mean 2007 (n=13)**	**January 2008**	**Annual mean 2008 (n=13)**
Temperature	°C	2.1	13.2	8.1	13.2
Oxygen	mg/L	12.3	9.45	12	9.63
Turbidity	FTE	36	30.7	40	23
Suspended solids	mg/L	32	24.7	23.1	28.4
Total hardness	mg/L $CaCO_3$	197	219	240	221
Total Organic Carbon	mg/L	4.2	3.53	3.3	3.12
Biological Oxygen Demand	mg/L	<1	<1	NA	*1.52*
Chemical Oxygen Demand	mg/L	15	12.8	NA	*9.75*

NA=Not Available, *number*=annual mean (n=4)

Table 5.2: Organic contaminants (average concentration in µg/L) in the River Rhine water (location Nieuwegein; 42 days exposure time compared to 11 days exposure time).

Compound name	CAS-number	period 42 days		period 11 days		
		average concentration (µg/L)	number of data	average concentration (µg/L)	number of data	increase decrease
o-xylene	95-47-6	0.028	3	0.016	1	-
m- and p-xylene	108-38-3 and 106-42-3	0.040	3	0.027	1	-
1,3,5-trimethyl benzene	108-67-8	0.010	3	0.016	1	+
2-methylaniline [@,#] and 4-methylaniline [#]	95-53-4 and 106-49-0	0.013	3	0.000	1	-
2-(benzenesulfonyl)aniline	4273-98-7	0.010	3	0.000	1	-
aminomethylphosphonic acid	1066-51-9	0.268	4	0.270	1	+
aniline [#]	62-53-3	0.059	3	0.075	1	+
adsorbable organic halogens		12.500	3	11.600	1	-
benzene [@,#]	71-43-2	0.038	3	0.000	1	-
bromacil	314-40-9	0.010	2	0.011	1	+
carbamazepine	298-46-4	0.036	14	0.026	4	-
3-(3-chloro-4-methyl-phenyl)-1,1-dimethyl-urea (chlorotoluron) [@,#]	15545-48-9	0.035	14	0.012	4	-
2-[2-[(2,6-dichlorophenyl)amino]phenyl]acetic acid (dichlofenac)	15307-86-5	0.044	2	0.058	1	+
dichloromethane [#]	75-09-2	0.011	3	0.023	1	+
1-methoxy-2-(2-methoxyethoxy)ethane (diglyme)	70992-86-8	0.128	2	0.173	1	+

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Table 5.2 - continued from previous page

Compound name	CAS-number	period 42 days		period 11 days		increase decrease
		average concentration (µg/L)	number of data	average concentration (µg/L)	number of data	
2-[2-[2-(bis(carboxymethyl)amino)ethyl-(carboxymethyl)amino]ethyl-(carboxymethyl)amino]acetic acid (DTPA)	67-43-6	5.197	2	8.050	1	+
dipotassium 2-[2-(carboxylatomethyl-(carboxymethyl)amino)ethyl-(carboxymethyl)amino]acetate (EDTA)	60-00-4	5.233	2	6.475	1	+
ethenylbenzene	100-42-5	0.009	3	0.014	1	+
ethylbenzene	100-41-4	0.027	3	0.000	1	-
sodium 2-[(hydroxy-oxido-phosphoryl)methylamino]acetic acid (glyphosate)	1071-83-6	0.058	4	0.080	1	+
N2,N2,N4,N4,N6,N6-hexakis(methoxymethyl)-1,3,5-triazine-2,4,6-triamine (HMMM)	3089-11-0	0.417	12	0.412	3	-
sodium 2-[4-(2-methylpropyl)phenyl]propanoate (ibuprofen)	15687-27-1	0.022	2	0.031	1	+
1,1-dimethyl-3-(4-propan-2-ylphenyl)urea (isoproturon) [#]	34123-59-6	0.094	14	0.029	4	-
toluene [@,#]	108-88-3	0.141	3	0.000	1	-
2-methoxy-2-methyl-propane (MTBE)	1634-04-4	0.039	3	0.058	1	+
2-(bis(carboxymethyl)amino)acetic acid (NTA)	139-13-9	1.579	2	2.053	1	+
diphenylphosphorylbenzene	791-28-6	0.022	12	0.014	3	-

@ = Compound listed as carcinogenic or mutagenic (Anonymous, 2011)

= Compound listed as carcinogenic, genotoxic or reprotoxic (Pflaumbaum, 2010)

Sister chromatid exchange (SCE) test

All fish showed sister chromatid differentiation. As shown in Figure 5.3, after 11 days of exposure to Rhine water there was a significant increase in the number of SCEs per chromosome compared to the control (P = 0.007). A significant increase in the number of SCEs per chromosome was also present in gill cells of fish exposed to Rhine water during 42 days compared to the control (P = 0.005). No significant difference in numbers of SCEs was observed between the 11 days and 42 days groups (P = 0.92). From this it was concluded that no increase of SCEs occurred after a further prolonged exposure to River Rhine water.

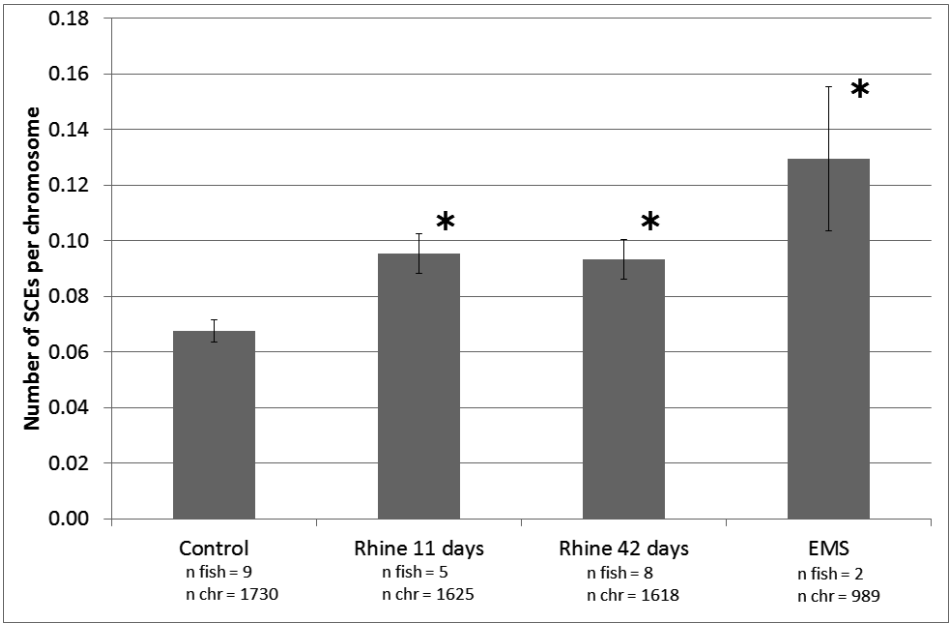

Figure 5.3: Number of SCEs per chromosome in gill cells of Eastern mudminnow after exposure of the fish to River Rhine water for 11 days and 42 days and groundwater for 42 days and EMS; mean ± SEM, n fish = number of fish tested, n chr = number of chromosomes observed.=, ✱= significantly different compared to control ($p<0.05$).

Comet assay

Using the trypan blue assay, the viability of the gill cell suspension varied between 80 and 98 % for all fish used. The cell suspensions used were therefore considered acceptable for use in the Comet assay.

As shown in Figure 5.4, there was a tendency towards an increase in the tail length for the fish exposed to Rhine water during 11 (P = 0.21) or 42 days (P = 0.43) compared to the groundwater 42 days, but these effects were not statistically significant. No significant difference was found between the 11 days and 42 days groups (P = 0.29).

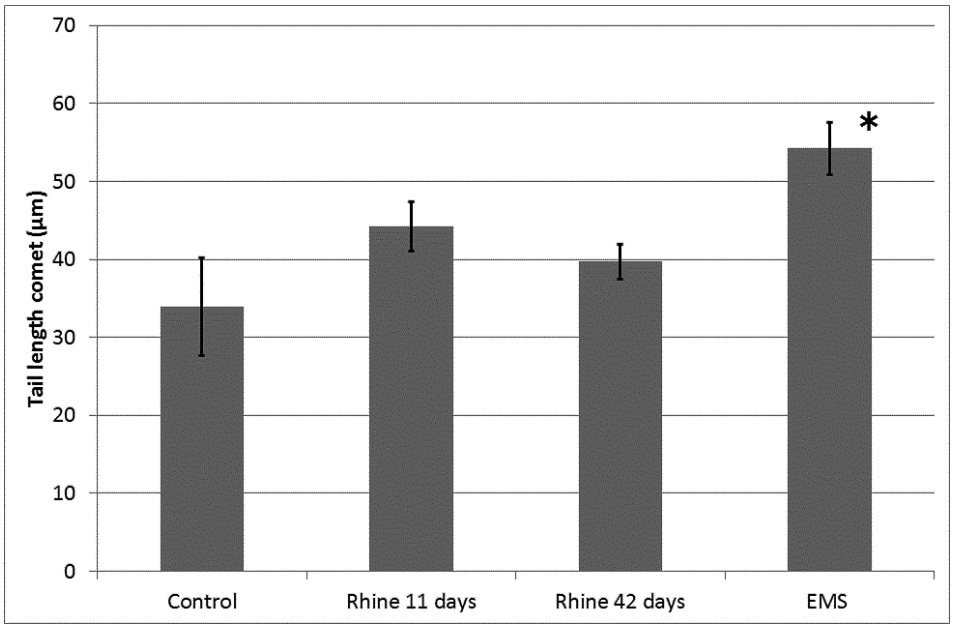

Figure 5.4: Effect of 11 and 42 days of exposure to Rhine water on DNA damage in gill cells of the Eastern mudminnow, measured as comet tail length; mean ± SEM, n = 4. ∗= significantly different compared to control ($p<0.05$).

5.4. Discussion

The main conclusion of the present study is that prolonging the exposure time to River Rhine water from 11 days to 42 days, does not result in a significant increase in SCEs and DNA damage (Comet assay) in gill cells of the Eastern mudminnow. Evaluation of chemical results showed no significant change in the overall water quality between the 42 days exposure group and the 11 days exposure group (Table 5.2). It is concluded that only minor chemical variations occurred during the exposure period. Therefore, no marked changes in overall water quality will have influenced the results of the genotoxicity tests. So far the compounds causing these effects are still unknown. Further studies are needed to investigate the presence of genotoxic micropollutants in surface water and in surface water derived drinking-water, in order to conclude whether or not extra purifications steps are required.

The data of the present study can be compared to those obtained in similar *in vivo* bioassays performed in 1978 (Alink *et al*., 1980) and 2005 (Alink *et al*., 2007). Whereas the 1978 study did not include the Comet assay, the results obtained in 1978 for the SCE test amounted to 0.045 $\pm$ 0.012 SCEs/chromosome for the control, 0.128 $\pm$ 0.023 SCEs/chromosome upon 3 day exposure and 0.155 $\pm$ 0.021 SCEs/chromosome upon 11 day exposure of the fish to River Rhine water. Comparison of these values to the data of the present study reveals that the 2.8- and 3.4-fold increase in the number of SCEs/chromosome for the 3 and 11 day exposed fish as compared to the control was higher than the 1.4-fold increase now observed for the 2008 water samples upon 42 day exposure and the 1.6-fold increase observed for the 2005 water samples upon 11 days exposure (Alink *et al*., 2007). It is concluded that the new data corroborate the conclusion form the 2005 study that *in vivo* genotoxicity of River Rhine water is at present lower than in 1978.

The Comet assay was applied in this study to compare the results with the SCE test. The Comet assay is a rapid, sensitive and inexpensive method for measuring DNA strand breaks (Lee and Steinert, 2003) and has been used in several environmental studies in organisms living in rivers (Ohe *et al*., 2004; Whitehead *et al*., 2004; Keiter *et al*., 2006; Liney *et al*., 2006). Compared to the SCE test, the Comet assay has many advantages, such as being less time-consuming (in the preparations of slides and the microscopic scoring of the DNA damage) and not requiring fish pretreatment with BrdU and colchicine. Due to the lower costs, the lower labour intensity and the possibility to use other fish species, the use of the Comet assay is preferred over the SCE assay. In the present study the Comet assay gave a similar result for the positive control EMS, and a same tendency as the SCE assay pointing at increased DNA damage upon exposure of the fish to River Rhine water. However, the increase in the Comet assay data obtained

for gill cells of fish exposed to River Rhine water, were not statistically significantly increased, whereas the SCE scores were statistically significantly higher than those in controls. This suggests the Comet assay to be less sensitive, than the SCE test. This may be due to the nature of the chemicals present and the fact that the two biomarkers may not be detecting the same class of pollutants. Increasing the number of fish may also prove to solve this issue, although it is noted that for the Comet assay, the number of fish used was according to the recommendation described by Hartmann *et al.* (2003). Given all this, it is concluded that the Comet assay is a useful addition but does not provide a substitute for the SCE endpoint in these *in vivo* genotoxicity studies. Future investigations and studies with other pollution types may be required to ascertain that also in these cases test results between the Comet assay and the SCE assay are comparable.

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Chapter 6

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 *in vivo* Comet assays and an *in vivo* SCE test

This chapter is based on: E.J.M. Penders, A.J. Martijn, A. Spenkelink, G.M. Alink, I.M.C.M. Rietjens and W. Hoogenboezem “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 *in vivo* Comet assays and an *in vivo* SCE test.”, submitted.

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. Genotoxicity was tested *in vitro* using the Ames and Comet assay. All samples showed negative results in both assays. Samples were also tested in *in vivo* genotoxicity tests in Eastern mudminnow fish (*Umbra pygmaea*). Genotoxicity was analyzed by a Sister Chromatid Exchange (SCE) and a Comet assay performed using isolated gill cells. 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 before distribution. 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.

6.1. 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 the Lake IJsselmeer which receives water from the River Rhine. Figure 6.1 presents the location of this PWN water treatment plant Andijk.

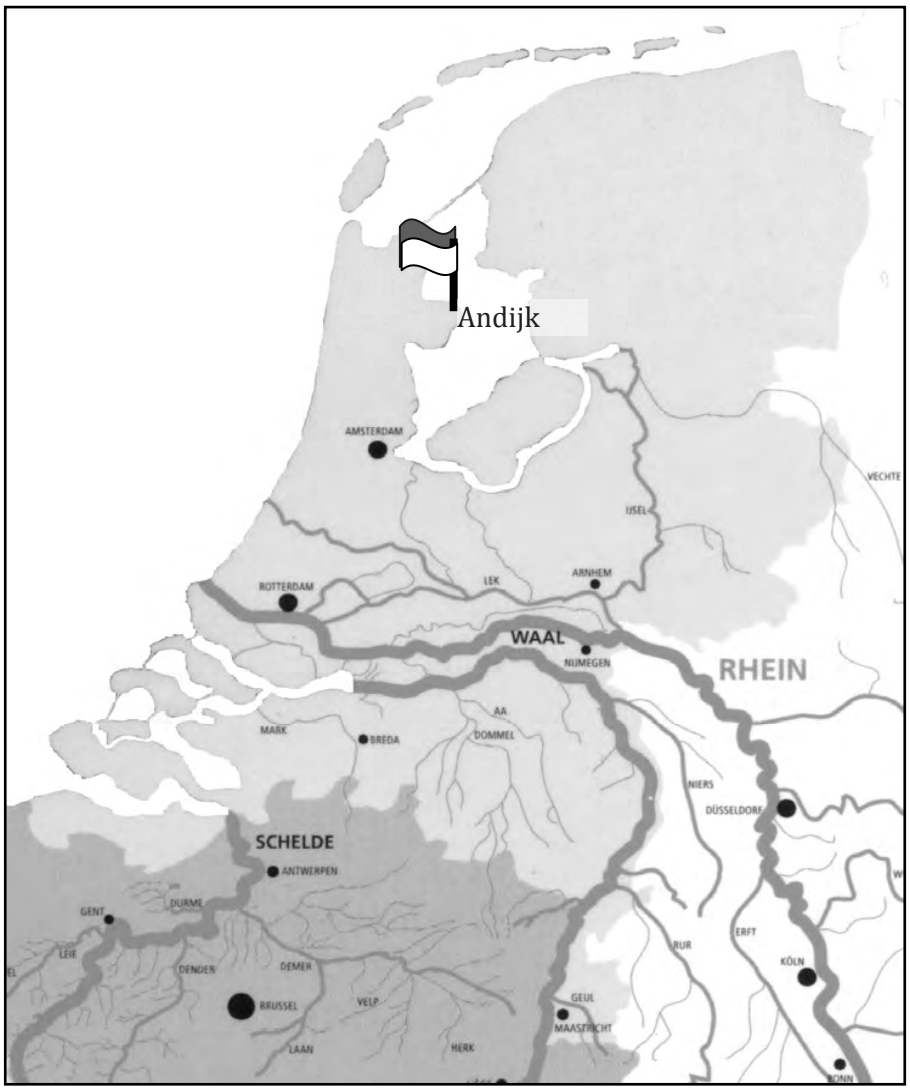

Figure 6.1: Location PWN water treatment plant Andijk

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 (Von Gunten, 2003; Kurokawa *et al.*, 1990), PWN does not apply an ozone based AOP but has selected an UV/H_2O_2 based AOP. 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). Besides degrading the organic micro pollutants present in the water, AOPs also oxidize 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 filtration (GAC) 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 (Ames and Comet) with and without metabolic activation. In addition, the water samples were also tested in an *in vivo* Sister Chromatid Exchange (SCE) test and an *in vivo* Comet assay in Eastern mudminnow fish (*Umbra pygmaea*). Genotoxicity was analyzed by performing an SCE test and a Comet assay in isolated gill cells obtained from fish 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, because the fish are directly exposed to water samples *in vivo* and no concentration procedure is required (Alink *et al.*, 1980, 2007). The water samples tested include (Figure 6.2): 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 microstaining, 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 Granular Activated Carbon (GAC) filtration (sample GAC filtration), also representing the water for distribution as drinking water.

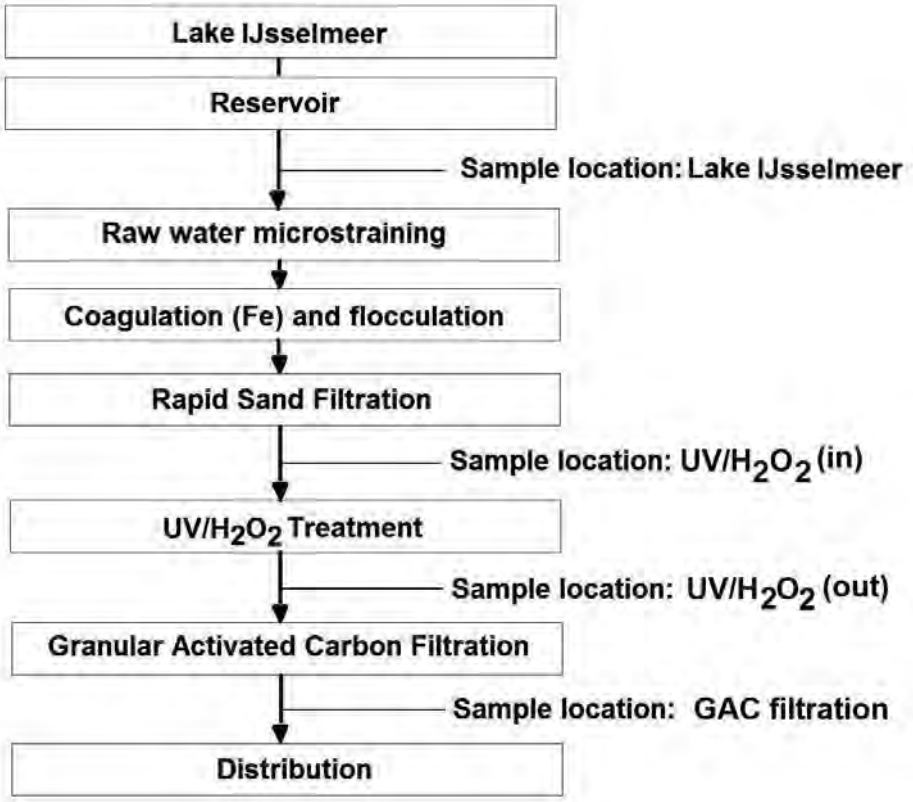

Figure 6.2: Water treatment process scheme of water treatment plant Andijk and sample locations.

6.2. Material and Methods

Site description PWN drinking water production at Andijk

The genotoxicity study was performed at PWN's water treatment plant Andijk (Figure 6.1). At this location, surface water from the Lake IJsselmeer is treated to produce drinking water. The water treatment process consists of conventional pretreatment followed by an UV/H_2O_2 based AOP followed by a subsequent granular activated carbon (GAC) filtration (Figure 6.2). For the study four samples at critical steps in the treatment process were taken as illustrated in Figure 6.2. These four water samples included 1) raw water of lake IJsselmeer (sample Lake IJsselmeer), 2) the influent for the UV/H_2O_2 treatment (sample UV/H_2O_2(in)), 3) the effluent of the UV/H_2O_2 treatment (sample UV/H_2O_2(out)), and 4) the water of the effluent of the UV/H_2O_2 treatment after a subsequent Granular Activated Carbon (GAC) filtration (sample GAC filtration). 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 process conditions of the UV/H_2O_2 treatment are set to meet 80% reduction in the concentration of atrazine (as a model compound for organic pollutants) resulting in a maximum required electrical energy dose of 0.54 kWh/m^3 and a H_2O_2 dose of 6 mg/L. 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 (seasonal variation) 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 assimilable organic carbon (AOC) by advanced oxidation process. 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 decompose this surplus H_2O_2. Biological activity in the GAC filters also converts nitrite into nitrate and decreases the AOC from approximately 150 µg/L after UV/H_2O_2 treatment to 15 µg/L after GAC filtration. 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 and 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 sterile phosphate buffer solution or in a metabolic activation solution containing S9. The S9 was obtained from Molecular Toxicology Incorporated, USA, which 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 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 metabolic activation solution S9 (Molecular Toxicology Incorporated, USA). After 5 minutes centrifugation at 3000 rpm and 4 °C, the cell pellet obtained was diluted in cold PBS. The cell suspension thus obtained was mixed with molten LMP agarose (0.8 %) and added on the GelBond® Film. The GelBond® Films were placed in cold lysis solution overnight at 4 °C. The GelBond® Films 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 slide at least 100 comets were measured. As a positive control, benzo(a)pyrene was used at a concentration of 25 µM when S9 was present and ethyl methanesulfonate at a concentration of 2.5 mM when S9 was not used.

***In vivo* genotoxicity tests**

After permission of 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 9-12 cm and the weight from 8-17 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. 45 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 samples sites described above (Figure 6.2) and including: sample Lake IJsselmeer, sample UV/H_2O_2(in), sample UV/H_2O_2(out) and sample GAC filtration. 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 6.3A 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 this sample contains 147 µM H_2O_2, and H_2O_2 is known to induce toxicity in fish itself. A safety data sheet (Solvay, 2010) presented an LC_{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). Figure 6.3B presents the extended experimental set-up for this treatment in which 3 hours of incubation time for catalase was used, resulting in a final concentration of 1.21×10^{-7} µM H_2O_2 in the water to which the fish were exposed. This residual concentration of H_2O_2 is not expected to induce genotoxicity 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).

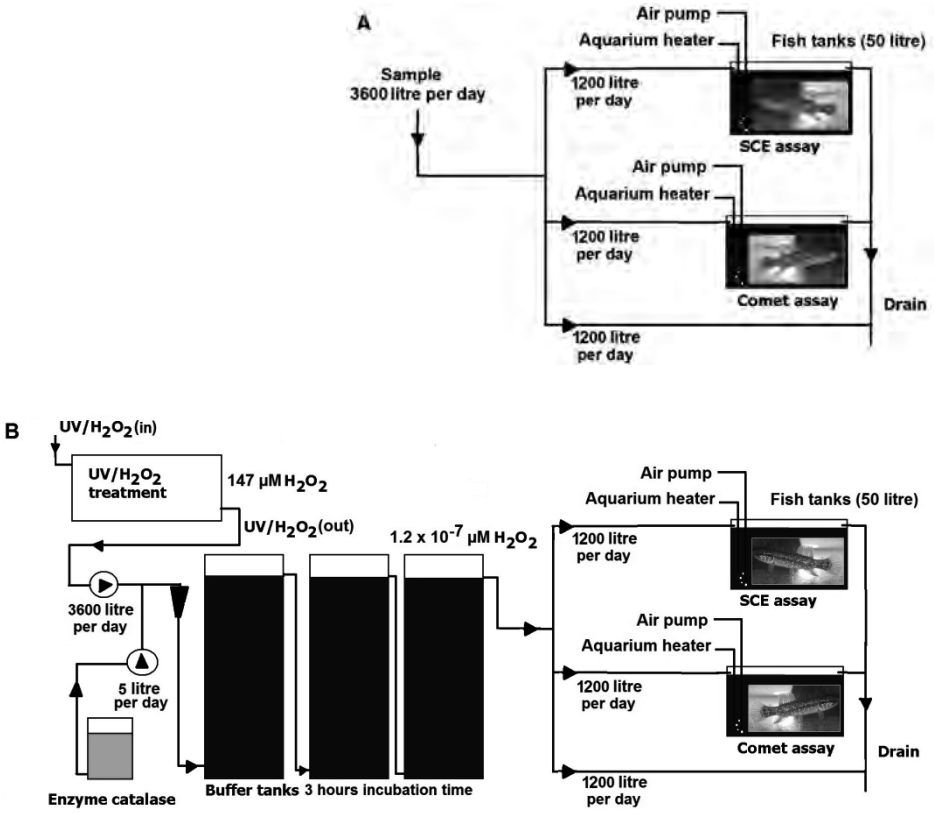

Figure 6.3 The general experimental design for exposure of the fish (Eastern mudminnow) to water (A) and to UV/H_2O_2(out) water (B).

Per assay and for each water sample to be tested, the mudminnows were kept in 50 litre all-glass flow through tanks, with a flow rate of 1200 litre 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, 6 fish for the SCE test and 4 fish for the Comet assay were exposed for 3 days in separate 5 litre aquaria to 25 mg/L ethylmethanesulfonate (EMS), obtained from Fluka (Buchs, Switserland). The gills of the exposed fish were used for the sister chromatid exchange (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 50 comets per slide 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 $P<0.05$.

6.3. 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 6.1), 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 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 6.1).

From the results presented in Table 6.1 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.

Table 6.1: 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 analyzed at a single day for each test.

Water sample	**Sampling date**	**S9 mix**	**Induction factor Ames TA98 Plate incorporation**	**% DNA Comet assay**
	dd-mm-yyyy			**Mean µm ± SEM (n=3)**
Negative control		-	1.00[@]	10.4 ± 0.6
Negative control		+	1.00[$]	8.8 ± 0.9
EMS positive control		-	NA	57.6 ± 3.8
B(a)P positive control		+	NA	33.2 ± 5.4
4-NQO positive control		-	5.92	NA
2-AA positive control		+	70.00	NA
Lake IJsselmeer	13-05-2008	+	1.23	14.4[#]
	01-12-2008	+	1.22	8.2 ± 2.0
	18-11-2008	-	1.19	6.5 ± 0.4
UV/H_2O_2(in)	13-05-2008	+	1.10	8.1 ± 1.3
	01-12-2008	+	1.22	8.2 ± 0.8
	18-11-2008	-	0.96	5.7 ± 0.9
UV/H_2O_2(out)	13-05-2008	+	1.31	6.8 ± 0.5
	01-12-2008	+	1.25	8.8 ± 1.9
	18-11-2008	-	1.31	7.5 ± 0.7
GAC filtration	13-05-2008	+	1.38	7.9 ± 2.9
	01-12-2008	+	1.07	7.8 ± 1.3
	18-11-2008	-	0.81	9.6 ± 3.0

NA= Not Available because not tested; @=24 revertants; $=29 revertants. # n=1.

***In vivo* genotoxicity**

In subsequent studies the water samples were also tested in an *in vivo* Sister Chromatid Exchange (SCE) test and an *in vivo* Comet assay in Eastern mudminnow fish (*Umbra pygmaea*) exposed to the respective water samples for 11 day. 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 6.4 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 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.

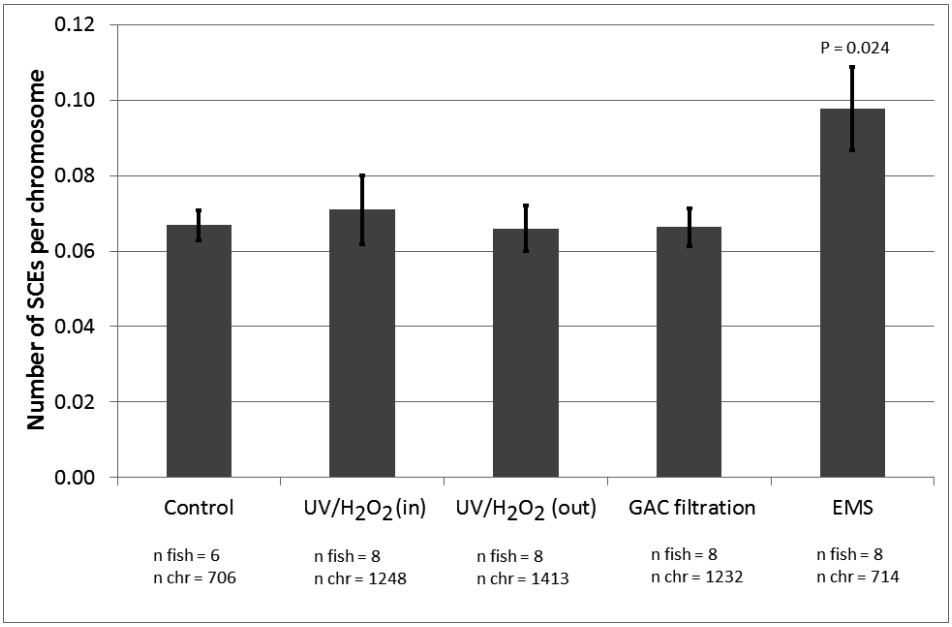

Figure 6.4: Number of SCEs in gill cells per chromosome of the Eastern mudminnow after 11 days exposure to control water, water containing the positive control EMS or water samples collected at the water treatment plant at Andijk including water samples UV/H_2O_2(in), UV/H_2O_2(out) and GAC filtration (Figure 6.2). mean ± SEM, n fish = number of fish used, n chr = number of chromosomes scored.

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 6.5 presents the results of the *in vivo* Comet assay. The results 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 form 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 (Penders *et al.*, submitted). A previous study (Alink *et al.*, 2007), in which 120 mg EMS per litre was used as a positive control, showed a 2.2-fold increase in tail length (P=0.00039). 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.

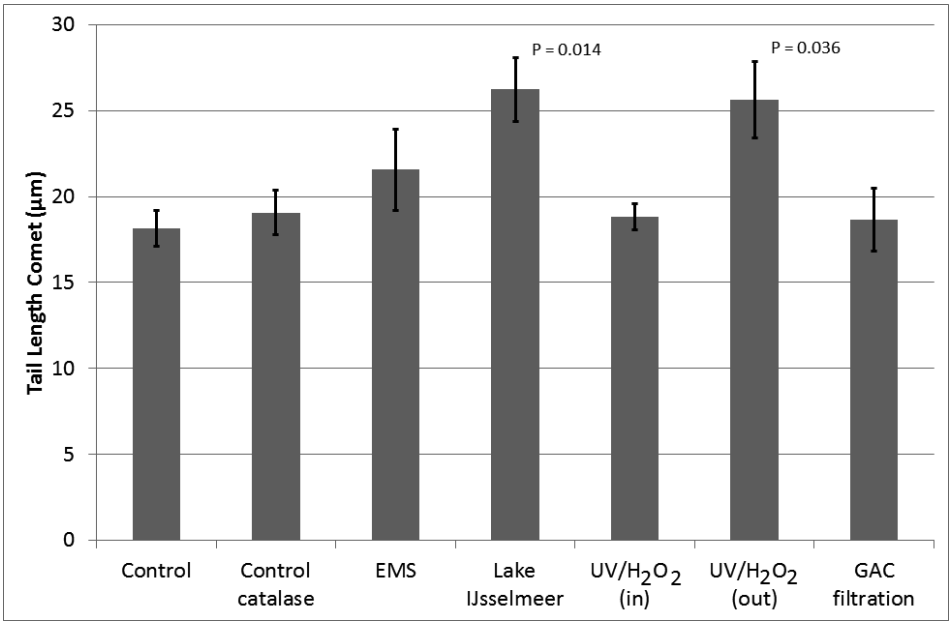

Figure 6.5: Comet tail length in gill cells of the Eastern mudminnow after 11 days exposure to control water (with or without catalase), water containing the positive control EMS, or water samples collected at the water treatment plant at Andijk including; water samples Lake IJsselmeer, UV/H_2O_2(in), UV/H_2O_2(out) and GAC filtration (Figure 6.2). mean ± SEM, n = 4 fish.

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 at 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 reveals 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 6.2 presents results from an analysis of 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 6.5.

Table 6.2: 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[#] µg/L Carbon	humic acids[#] µg/L Carbon	UV 254 ext/metre	Suspended solids mg/L
Lake IJsselmeer	6400	2940	10.5	23.96
UV/H_2O_2(in)	2120	954	3.8	<0.2
UV/H_2O_2(out)	2100	970	3.1	<0.2
GAC filtration	1500	438	1.4	<0.2

Based on 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 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.
This residual H_2O_2 concentration was found to be much lower than the concentrations of H_2O_2 reported in the literature to induce genotoxicity. Induction of genotoxicity *in vivo* in *Unio pictorum* requires H_2O_2 concentrations > 10 µM (Štambuk *et al.*, 2008), or 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).

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 absorbed 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 the difference between the negative results 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 and 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 when UV/H_2O_2(out) water extracts were tested with the *Salmonella typhimurium* TAmix, consisting of strains TA7001, TA7002, TA7003, TA7004, TA7005 and TA7006 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. Further risk/benefit analyses of usage of UV/H_2O_2 compared to use of ozone or chlorine as disinfectants are needed, and further research is required to identify the compounds which induced DNA damage formed during the UV/ H_2O_2 treatment.

6.4. 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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Chapter 7

Summary and future perspectives

Summary and discussion

Given the need for continued quality control of surface waters used for the production of drinking water by state-of-the-art bioassays and biological early warning systems, the objective of the present thesis was to validate and improve some of the bioassays and biological early warning systems used for quality control of surface water.

Although there is a decline in the (geno)toxicity of surface waters over the years as observed for example for the water from the River Rhine over last decades, there is still a need for continued quality control. Due to the lower (geno)toxicity, bioassays with increased sensitivity are needed because of i) the increasing number of chemicals present in surface water and ii) the fact that concentrations of chemicals of concern may be toxicologically relevant but below the level of detection of the currently applied protocols for bioassays or biological early warning systems.

The River Rhine was selected as a model river for our studies. The results obtained provide an overview of the trends in the quality of surface water from the River Rhine over the last decades and indicate how bioassays and biological early warning systems used for quality control of surface water can be further improved, optimised and used for future quality control in the process of producing drinking water from surface water. The present chapter first presents an overview of the major results obtained and conclusions derived from our studies and ends with an overview of future trends and perspectives in the field of quality control of surface water and its treatment for drinking water production.

In vitro bioassays that monitor the toxicity of surface water used for the production of drinking water provide an important addition to existing chemical, physical and biological parameters. The *in vitro* toxicity bioassays applied may detect general toxicity or genotoxicity. The Association of River Water Works (RIWA) includes the Ames test in its measuring program already for over a decade and the objective of chapter 2 was to evaluate the performance of additional *in vitro* assays in monitoring the toxicity as part of the quality assessment of surface water used for the production of drinking water and to define the optimal *in vitro* bioassay test battery. To this end the paper presents the data obtained in a variety of *in vitro* bioassays for several extracts of Rhine water and the evaluation of the different bioassays and genotoxicity tests in relation to sensitivity, selectivity and suitability for future routine monitoring programs. An ecological risk model evaluation based on the bioassay data obtained is included to judge the ability of the *in vitro* bioassays to detect effects on the organisms in the ecosystem as a whole. It is concluded that an optimal bioassay battery of *in vitro* tests for water quality monitoring of the River Rhine includes the *Daphnia* IQ assay, the *Raphidocelis* sp. PAM test and the Microtox® test as general toxicity tests, together

with the Ames TA98 test (with metabolic activation) to monitor genotoxicity. The results also reveal that the water quality of the River Rhine has improved over the years. The potentially affected fraction of organisms which is predicted to experience adverse effects of chemicals present in the surface water is lower than 5%. All together it was concluded that the results obtained measuring overall toxicity with bioassays as described above provide essential information about the quality of surface water that is not obtained by chemical analysis.

Next to the use of bioassays in the laboratory, the online biological early warning systems have proven useful in the monitoring of water quality of surface waters. In their present form these systems, including the bbe Algaetoximeter and Microlan TOXcontrol system, monitor non-concentrated surface water samples, whereas studies with *in vitro* bioassays have demonstrated the need for concentration of surface water samples to reach adequate detection limits (chapter 2). As a result there is a need for integrating concentration steps in the online biological early warning systems. Chapter 3 describes the development and validation of Solid Phase Extraction (SPE) as an online concentration step in the bbe Algaetoximeter and Microlan TOXcontrol biological early warning systems. The results obtained provide a proof of principle for online bioassays with increased sensitivity, providing a suitable method for surface water quality control now indicating that water quality has been improved over the past decades, but still requires adequate online monitoring. The use of SPE connected to a biological early warning system will also prove a way to judge whether pollutants, chemically detected with ever increasing sensitivity, will still result in toxicity.

As presented in chapter 2 and 3, surface water used for drinking water preparation requires continuous monitoring for the presence of toxic compounds. For monitoring of genotoxic compounds fish models have been developed, such as the Eastern mudminnow (*Umbra pygmaea* L.) because of its clearly visible 22 meta-centric chromosomes. It was demonstrated in the late seventies that Rhine water was able to induce chromosome aberrations and sister chromatid exchange in this fish species. Although *in vitro* mutagenicity studies of the RIWA showed that the genotoxicity of the River Rhine steadily decreased during the last decades, there is still concern about the presence of some residual mutagenicity. In addition, in most studies the water samples have been tested only in *in vitro* test systems such as the *Salmonella*-microsome test. For this reason, and in order to be able to make a comparison with the water quality 27 years ago, a study was performed with the same experimental design as before in order to measure the effect of Rhinewater on the induction of SCE in the Eastern mudminnow (chapter 4). As a new end point the single cell gel electrophoresis assay (Comet assay) was performed. Fish were exposed to Rhine water or to groundwater for 3 and 11 days in flow-through aquaria. Fish exposed for 11 days to Rhine water had a significantly higher number of SCE and an increased comet tail-length compared with control fish

exposed to groundwater. After exposure for three days to Rhine water there was no difference in SCE and a slightly increased comet tail-length compared with the control. It was concluded that genotoxins are still present in the river Rhine, but that the genotoxic potential has markedly decreased compared with 27 years ago. Furthermore, the Comet assay appears to be a sensitive assay to measure the genotoxic potential of surface waters in fish.

As in the 2005 study (chapter 4) the *in vivo* genotoxicity increased upon extending exposure of the fish from 3 to 11 days, the objectives of chapter 5 were to investigate i) whether further prolonged exposure results in a further increase in *in vivo* genotoxicity, ii) whether new data corroborate that *in vivo* genotoxicity of Rhine water is at present lower than in 1978, and iii) whether the Comet assay is a suitable alternative to the SCE assay. Prolonging the exposure time of Eastern mudminnows to River Rhine water from 11 days to 42 days, did not give a significant increase in SCEs and DNA damage (Comet assay) in gill cells. The new data corroborate that *in vivo* genotoxicity of River Rhine water is at present lower than in 1978. The Comet assay is a useful addition but does not provide a substitute for the SCE endpoint in these *in vivo* genotoxicity studies.

In addition to (geno)toxicity measurements to monitor the quality of the surface water that is used as a source for the production of drinking water, the quality of water retrieved from different processes during the drinking water treatment, can also be monitored by bioassays.
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. Chapter 6 investigates the genotoxicity of samples collected from the water treatment plant Andijk, applying UV/H_2O_2 treatment. Genotoxicity was tested *in vitro* using the Ames and Comet assay. All samples showed negative results in both assays. Samples were also tested in *in vivo* genotoxicity tests in Eastern mudminnow (*Umbra pygmaea*). Genotoxicity was analyzed by the Sister Chromatid Exchange (SCE) and the Comet assay using isolated gill cells. No significant increase in SCEs was 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 were 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 before distribution. 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.

Overall, it was demonstrated that the use and further optimisation of bioassays will strengthen the current state of the art in water quality assessment.

Future perspectives

Together with a water concentration procedure, a selection of three *in vitro* bioassays, each representing one trophic level in the ecosystem, and one *in vitro* genotoxicity test, was shown to be adequate for monitoring the toxicity of surface water. It is important to stress however, that this selection of the test battery is based on results from organisms exposed to a specific extract and thus to a selected organic fraction of the surface water. To evaluate the toxic pressure of all chemicals present in surface water, further research is needed to assess also the toxicity of the compounds which may have been less effectively extracted with the concentration procedures used, like polar organics, or those that were not extracted at all, like for instance metals. At the moment, other solid phase extraction procedures are available in routine analytical chemical analyses and these can possibly be adapted to the conditions required for the bioassays used for surface water quality control. These new concentration procedures may reveal new information on the toxicity of the mixture of chemicals present in surface water. Such new solid phase extraction procedures can also be implemented next to biological early warning systems, resulting in an increase of the sensitivity of the biological early warning systems for these compounds as well.

In addition it is of interest to note that the Ames TA98 assay with metabolic activation was selected in the bioassay battery to detect genotoxins in concentrated surface water. This assay detects gene mutations but does not detect compounds causing only chromosomal aberrations, which represent another type of genotoxic effect. Using the Ames TA98 assay a decline in genotoxicity in the River Rhine over the years was detected, which was corroborated by results obtained with the *in vivo* SCE assay. In chapter 6, negative Ames results are presented for other samples, like the samples obtained from the UV/H_2O_2 process used during the production of drinking water. In case of such negative results additional testing with other *in vitro* genotoxicity assays may be necessary to obtain information about other types of genetic damages which can occur when chemicals in water interact with DNA. In most guidelines, in which new or existing chemicals are assessed for their genotoxicity hazards, an initial battery of *in vitro* assays is selected, which provide information about small-scale genetic damage (e.g. point mutations) and larger-scale genetic alterations (e.g. chromosomal damage) (Dearfield *et al*., 2011). Also the *in vitro* Comet assay could be used, to obtain information about chromosomal damage. In our studies the *in vitro* Comet assay presented negative results (chapter 6) for concentrated water samples. Although this assay is useful as a screening test for the prediction of the outcome of the OECD 473 chromosomal aberration test, (Hartmann *et al*., 2003) or micro nucleus test (Hartmann *et al*., 2001), the question still remains if the chromosomal aberration test or the micro nucleus test should be included in the *in vitro* genotoxicity battery.

As described in most guidelines, requirements for genotoxicity testing for individual agents (pesticides, pharmaceuticals, etc.) are to generate data for use in genotoxicity risk assessments (Dearfield *et al*., 2002). 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 *in vivo* genotoxicity also when negative *in vitro* results are obtained (Becks *et al*., 2006) or to use the *in vivo* genotoxicity assay next to *in vitro* genotoxicity in a standard test battery (Anonymous, 1997 and 2008). The guidelines or recommendations are based on testing a single chemical compound to obtain information about its genotoxicity. With the complex mixture of chemicals present in water samples and for use in genotoxicity risk assessment, *in vivo* genotoxicity results should be included as well, as *in vitro* assays are applied on concentrated samples missing certain compounds that are present in the unconcentrated water (see chapter 6). However, the number of *in vivo* genotoxicity assays for testing water samples is limited, and there is a tendency towards the replacement of *in vivo* assays by other *in vitro* assays or models. In the future, new omics technology may contribute to better mechanistic understanding of (geno)toxic processes and could be included in the *in vitro* or *in vivo* (geno)toxicity battery. Comparison of gene expression profiles via micro array analysis may provide insights in the mechanisms and pathways altered after exposure to (geno)toxic compounds present in (extracts of) water samples and can probably provide information which (group of) (geno)toxic compounds are present in the complex and varying water matrix. (Snape *et al*., 2004). A number of micro arrays have been developed for fishes which have been used to study gene expressions involved in developmental processes and growth, host pathogen responses, ecotoxicology, and effects of foods (Douglas, 2006). Most of the arrays are available for model species such as zebra fish, or intensively studied species such as salmonids. Micro arrays for other fish species are also available (Hook, 2010). As an example, Moens *et al*. (2007) presented a study to investigate the mode of action of substances present in whole effluent using a microarray with a set of 960 carp gene fragments. In their study, micro array analysis showed that mainly molecular pathways were affected associated with “the energy balance of the fish, including changes in carbohydrate and lipid metabolism, as well as digestive enzyme activity”. It is preferred that the same model organisms are used in the omics technology as used in aquatic toxicology, enabling translation of the (geno)toxicity in molecular parameters or vice versa. An example is presented by Connon *et al*. (2008), who studied the molecular responses in *Daphnia magna* exposed to cadmium and linked them to population stress responses. Steinberg *et al*. (2008) presented an overview of micro arrays available with gene expression profiles of bacteria, plant species, invertebrate species and fish species used to study molecular responses of various environmental chemicals. At the moment, further research is required for regulatory implementation of omics technology (Van Aggelen *et al*., 2010).

Standardization and validation of omics analysis with ecologically relevant species is highly needed. Generation of profiles based on a defined set of chemicals with relevant modes of actions and linkage between omics responses to adverse alterations in survival, growth and development and reproduction are important for the validation of these new techniques.

Another aspect which requires more research is to define the concentration factor (or range) of water extracts that would not be of safety concern when generated by in *in vitro* or *in vivo* toxicity and genotoxicity tests. At the moment, no guideline values are given in the EU drinking water directive (Anonymous, 1998) or Dutch drinking water act (Anonymous, 2011a) or even in the EU water framework directive (Anonymous, 2000) about the maximum permitted (geno)toxicity of surface water for the aquatic environment. Guideline values for the toxicity of complex mixtures of several compounds, as in polluted surface water, that would not raise a safety concern, are difficult to establish. Apart from toxicity of surface water, it is still a matter of discussion whether guideline values for genotoxicity should or can be given for surface water or if surface water should not be genotoxic at all when measured with *in vivo* genotoxicity assays, or what the concentration factors that would not raise a safety concern will be, when *in vitro* genotoxicity assays are used.

A procedure that could be helpful to determine some guideline values for (geno)toxicity and the maximum level of the concentration factor can be the translation of the (geno)toxic response measured in a water extract to the response generated by a defined concentration of a reference substance. This would allow expression of the (geno)toxicity in equivalents, of the selected reference compound(s). This raises the question which reference compounds would best represent the classes of pollutants expected to be present in surface water samples. An example of a reference compound already used to quantify toxicity of surface water samples can be found in a water act for the production of drinking water from 1983 (Anonymous, 1983), in which a parametric value was given for a group of compounds which interact with the enzyme acetylcholine esterase and was expressed as paraoxon equivalents. In chapter 3, data obtained from the online algae-monitor were also expressed as diuron equivalents. This approach would be essential to allow evaluation of the actual risks and not only the hazards related to the toxicity and genotoxicity data obtained by allowing definition of more general future guideline values for judgement of surface water quality. Further research is required on the question which compounds and mechanisms can be used as relevant reference compounds to define equivalent based guideline values for the different bioassays in relation to judgement of the risks associated with the compounds present in the surface water. The use of (several) reference substance equivalents can also be helpful to evaluate and to compare data from the different *in vitro* and *in vivo* genotoxicity tests.

With the information of *in vitro* and *in vivo* (geno)toxicity assays, the future definition and use of reference equivalents, and of omics-data, the risk management of surface water quality can be taken to a higher level. A start with such a compound-based evaluation of drinking water was proposed by the World Health Organisation (WHO). Recently, the WHO released the fourth edition of drinking water guidelines with guideline values for chemicals in drinking water (Anonymous, 2011b). The guideline values are based on Tolerable Daily Intake (TDI) values, and/or NOAEL or LOAEL values in combination with uncertainty factors and chemical-specific adjustment factors, when chemicals are not considered to be genotoxic. Guideline values for genotoxic compounds are presented by WHO as "the concentrations in drinking-water associated with an estimated upper-bound excess lifetime cancer risk of 10^{-5}", i.e. one additional case of cancer per 100,000 of the population ingesting drinking-water containing the substance at the guideline value for 70 years. One can argue that such an approach requires not only identification of the relevant genotoxic carcinogens present in the water samples but also the presence of full data sets on their carcinogenicity. Often such information will not be available. In such cases one might consider application of the so-called Threshold of Toxicology Concern (TTC) approach (Kroes *et al*., 2005; Munro *et al*., 2008). The TTC approach assumes that one can define exposure levels that are insignificant from the toxicological point of view. This implies that a low level of exposure with a negligible risk can be identified for many chemicals, including those of unknown toxicity, based on knowledge of their chemical structures. If the expected intake of a chemical is below the TTC, the authorities could decide not to have any more safety evaluations carried out, or to give them very low priority. TTC values for non-genotoxic compounds as well as for compounds with an alert for genotoxicity were defined. Munro *et al*. (1996) defined TTC values for non-genotoxic compounds amounting to 1800, 540 and 90 µg/person/day for Cramer Class I, II and III compounds respectively. Kroes *et al*. (2004) defined a threshold for the endpoint of cancer by deriving a TTC value of 0.15 µg/person/day for substances containing a structural alert for potential genotoxicity, but not belonging to the class of aflatoxin-like, azoxy-, and *N*-nitroso- compounds. Although the TTC approach also requires characterisation of the relevant compounds, or at least of their chemical classes, it no longer requires cancer data to judge the safety of relatively low levels of contaminants. Melching-Kollmuß *et al*. (2010) already outlined that when using the lowest TTC value of 90 µg/person/day for non-genotoxic chemicals and the typical exposure assessment for drinking water contaminants (consumption of 2 litres of drinking water/person/day, and allocation of 10% of the tolerable daily intake to drinking water), a TTC-based upper concentration limit of 4.5 µg/l for "non-relevant metabolites" in ground/drinking water could be proposed. In their publication it has been evaluated, whether this value would cover all relevant toxicities (repeated dose, reproductive and developmental, and immune effects). They concluded, after evaluation of specific reproduction toxicity data from

chemicals and pharmaceuticals, a value of 1 µg/kg bw/day would cover developmental and reproduction toxicity, and a TTC value of 60 µg/person/day would represent a safe value. Based on these assumptions, a TTC-derived threshold of 3 µg/L in drinking water was derived. This would imply that when a non-genotoxic contaminant is present in a concentration below 3 µg/L, animal testing for toxicity is not considered necessary for a compound-specific risk assessment since the application of the TTC covers all relevant toxicities to be considered in such assessment and any health risk resulting from these exposures is very low (Melching-Kollmuß *et al*., 2010). Implementing such an approach in water quality assessment would make it possible to better judge the risks associated with low level contaminants.
In spite of these developments, in the EU drinking water directive 98/83/EG (Anonymous, 1998) or the Dutch drinking water act (Anonymous, 2011a), a limit value of 0.1 µg/l is used for a single pesticide, to protect ground water and drinking water against pesticide contamination, based on the principle of precautions (Anonymous, 1998; Bro-Rasmussen, 1999), which differs from the risk concept. The precautionary principle is bound to deal with uncertainties related to lack of knowledge and the unpredictable consequences when chemicals are used in an open society. However, using the TTC concept or other risk-based judgements of water quality would enable a better judgement of the consequences of contaminants detected with ever decreasing detection limits, avoiding risk management actions being directed at compounds present at levels that do not pose a real health risk.

Apart from the upper limit values for chemical and biological parameters, described in the EU drinking water directive and the Dutch drinking water act, the different treatments in drinking water production in The Netherlands are also evaluated using Quantitative Microbial Risk Assessment (QMRA), in which information is used from the different treatments about the elimination of micro-organisms in the process (De Roda Husman and Medema, 2005). With this QMRA, further optimisation of the different processes during drinking water treatment can be initiated. Generally in water treatment for the production of drinking water some type of advanced oxidation process (AOP) is applied as a general barrier for micro-organisms, but also 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 chapter 6, genotoxicity assays were used to obtain information about the quality of water retrieved from different processes, including UV/H_2O_2, during the drinking water treatment. With the results obtained, several processes could be characterized in the decrease or increase of toxicity. In the future, *in vitro* or *in vivo* (geno)toxicity data, next to chemical data may contribute to the characterisation of different treatment processes during drinking water treatment and like the risk

assessment in microbiology, can initiate further optimisation of the different water treatment processes.

All together it is concluded that quality control of surface water and drinking water treatment procedures should be further optimised and taken from the stage of hazard-based decision making to a stage of risk-based quality control and decision making.

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Samenvatting

Er is behoefte aan intensieve controle van de kwaliteit van de oppervlaktewateren die gebruikt worden als grondstof voor de productie van drinkwater. Het doel van dit proefschrift is de bruikbaarheid van state-of-the-art bioassays te onderzoeken en biologische bewakingssystemen verder te optimaliseren voor dit toepassingsgebied.

Hoewel er door de jaren heen een daling van de (geno)toxiciteit van oppervlaktewateren wordt waargenomen, bijvoorbeeld voor het water van de rivier de Rijn gedurende de laatste decennia, is er nog steeds behoefte aan regelmatige kwaliteitscontrole. Vanwege de geringe (geno)toxiciteit, zijn bioassays met verhoogde gevoeligheid nodig omwille van i) het toenemend aantal chemische stoffen dat aanwezig is in het oppervlaktewater en ii) het feit dat de concentraties van deze chemische stoffen toxicologisch relevant kunnen zijn, maar onder het niveau van detectie liggen bij de momenteel toegepaste protocollen voor bioassays en biologische bewakingssystemen.

De Rijn werd geselecteerd als een model rivier voor onze studies. De resultaten geven een overzicht van de trends in de kwaliteit van het oppervlaktewater van de Rijn in de afgelopen decennia en geven aan hoe de bioassays en biologische bewakingssystemen, die gebruikt worden voor de controle van de kwaliteit van het oppervlaktewater, verder werden verbeterd, geoptimaliseerd en gebruikt voor toekomstige kwaliteitscontrole in het productieproces van drinkwater uit oppervlaktewater. Deze samenvatting geeft een overzicht van de belangrijkste resultaten en conclusies die zijn afgeleid van onze studies.

In vitro bioassays, die de toxiciteit meten van het voor de bereiding van drinkwater ingenomen oppervlaktewater, bieden een belangrijke aanvulling op bestaande chemische, fysische en biologische parameters. De toegepaste *in vitro* bioassays kunnen de algemene toxiciteit of de genotoxiciteit meten. De vereniging van rivierwaterbedrijven (RIWA) heeft de Ames genotoxiciteitstest in het meetprogramma in de periode 1980 tot 2002 ingezet. De doelstelling van hoofdstuk 2 is om de prestaties van extra *in vitro* bioassays te evalueren als onderdeel van de kwaliteitsbeoordeling in toxiciteit van het oppervlaktewater, dat gebruikt wordt voor de productie van drinkwater, en hieruit de optimale testbatterij *in vitro* bioassays te bepalen. Hiertoe worden in dit proefschrift gegevens gepresenteerd die verkregen zijn door het gebruik van verschillende *in vitro* bioassays op een aantal extracten van Rijnwater en wordt de evaluatie weergegeven van de verschillende bioassays en genotoxiciteitstests met betrekking tot de gevoeligheid, selectiviteit en inzetbaarheid voor toekomstige routine meetprogramma's. Een ecologisch risicobeoordelingsmodel op basis van de verkregen bioassay gegevens is opgenomen om de toepasbaarheid van de *in vitro* bioassays te

beoordelen om effecten op de organismen in het gehele ecosysteem te kunnen bepalen. Geconcludeerd wordt dat een optimale *in vitro* bioassay batterij, ten behoeve van waterkwaliteitsmonitoring van de Rijn, bestaat uit de *Daphnia* IQ test, de *Raphidocelis* sp. PAM test en de Microtox ® test, om zo de algemene toxiciteit te kunnen bepalen, en de Ames TA98 test (met metabolische activering) om de genotoxiciteit te kunnen bepalen. Uit de resultaten blijkt ook dat de kwaliteit van het Rijnwater door de jaren heen is verbeterd. De potentieel aangetaste fractie aan organismen, die negatieve effecten kunnen ervaren van de chemische stoffen aanwezig in het oppervlaktewater, is lager dan 5%. Algemeen werd geconcludeerd dat de resultaten verkregen uit toxiciteitsmetingen met bioassays zoals hierboven beschreven, essentiële informatie verschaffen over de kwaliteit van het oppervlaktewater dat niet door chemische analyse verkregen wordt.

Naast het gebruik van bioassays in het laboratorium, hebben online biologische bewakingssystemen hun nut bewezen bij het meten van de waterkwaliteit van de oppervlaktewateren. In hun huidige uitvoering controleren deze systemen, met inbegrip van de bbe Algaetoximeter en het Microlan TOXcontrol systeem, ongeconcentreerde oppervlaktewatermonsters, daarbij overwegende dat studies met *in vitro* bioassays de noodzaak van het concentreren van oppervlaktewatermonsters hebben aangetoond om hiermee een adequate detectiegrens te kunnen bereiken (hoofdstuk 2). Daarom is er behoefte aan concentreringtechnieken die in de online biologische bewakingssystemen geïntegreerd kunnen worden. Hoofdstuk 3 beschrijft de ontwikkeling en validatie van vaste-fase-extractie (SPE) als een online concentreringstap in de bbe Algaetoximeter en Microlan TOXcontrol biologische bewakingssystemen. De verkregen resultaten bieden een experimenteel ontwerp voor online bioassays met verhoogde gevoeligheid, bieden een geschikte methode voor kwaliteitscontrole van oppervlaktewater en geven aan dat de kwaliteit van het water in de afgelopen decennia is verbeterd, maar waarbij nog steeds adequate online controle vereist wordt. Het gebruik van SPE gekoppeld aan een biologisch bewakingssysteem zal ook een manier zijn om te beoordelen of in de verontreinigingen, die chemisch gedetecteerd worden met steeds grotere gevoeligheid, nog steeds toxiciteit waargenomen wordt.

Zoals in hoofdstuk 2 en 3 weergegeven, wordt voor de drinkwaterbereiding gebruikte oppervlaktewater vereist dat er controle plaatsvindt op de aanwezigheid van toxische verbindingen. Voor het meten van genotoxische stoffen zijn er vismodellen ontwikkeld, zoals de Amerikaanse hondsvis (*Umbra pygmaea* L.) met zijn 22 duidelijk zichtbare metacentrische chromosomen. In de late jaren zeventig werd aangetoond dat Rijnwater chromosoomafwijkingen en uitwisseling van zusterchromatiden (SCE) in deze vissoort kon induceren. Hoewel bij het *in vitro* mutageniteitsonderzoek van de RIWA bleek dat de genotoxiciteit van Rijnwater tijdens de jaren 1980 tot 2002 gestaag daalde, is er nog steeds bezorgdheid over de aanwezigheid van enige resterende mutageniteit. Bovendien,

zijn in de meeste studies de watermonsters getest met alleen *in vitro* testen zoals de *Salmonella*-microsome Ames test. Om deze reden en om een vergelijking te kunnen maken met de waterkwaliteit van 27 jaar geleden, werd een studie uitgevoerd met dezelfde proefopzet als toen, om het effect van Rijnwater te kunnen meten als zijnde de inductie van SCE in de Amerikaanse hondsvis (hoofdstuk 4). Als nieuwe test werd de Comet assay uitgevoerd. Vissen werden blootgesteld aan Rijnwater of aan grondwater gedurende 3 en 11 dagen in doorstroomaquaria. Vissen, die werden blootgesteld gedurende 11 dagen aan Rijnwater, hadden een aanzienlijk groter aantal SCEs en een toename in Comet staartlengte vergeleken met vissen die gedurende 11 dagen werden blootgesteld aan controlewater (grondwater). Na blootstelling gedurende drie dagen aan Rijnwater was er geen verschil in de SCEs waarneembaar en maar wel een lichte toename in de Comet staartlengte in vergelijking met de controle. Geconcludeerd werd dat er nog steeds genotoxische verbindingen in de Rijn aanwezig zijn, maar dat het genotoxisch potentieel aanzienlijk is gedaald vergeleken met 27 jaar geleden. Bovendien, lijkt de Comet assay een gevoelige assay te zijn voor het meten van het genotoxische potentieel van oppervlaktewateren in de vissen.
Naar aanleiding van de 2005 studie (hoofdstuk 4), waarbij geconcludeerd werd dat de *in vivo* genotoxiciteit toenam als de blootstellingstijd van de vissen aan Rijn water werd verlengd van 3 naar 11 dagen, waren de doelstellingen van hoofdstuk 5 om te onderzoeken i) of verder verlengde blootstelling een verdere stijging in *in vivo* genotoxiciteit resulteert ii) of nieuwe gegevens bevestigen dat *in vivo* genotoxiciteit van Rijnwater op dit moment lager is dan in 1978 en iii) of de Comet assay een geschikt alternatief is voor de bepaling van de SCE. Verlenging van de blootstellingstijd van de hondsvissen aan Rijnwater van 11 naar 42 dagen, gaf geen significante verhoging in aantal SCEs en DNA schade (Comet assay) in kieuwcellen. De nieuwe gegevens bevestigen dat *in vivo* genotoxiciteit van Rijnwater op dit moment lager is dan in 1978. De Comet assay is een nuttige aanvullende assay, maar biedt geen vervanging voor de SCE-test in deze *in vivo* genotoxiciteit studies.

In aanvulling op (geno)toxiciteitsmetingen om de kwaliteit te meten van het oppervlaktewater, dat wordt gebruikt als een bron voor de productie van drinkwater, kan de waterkwaliteit ook met bioassays gemeten worden van water afkomstig van verschillende processen tijdens de productie van drinkwater. UV/H_2O_2 behandeling kan deel uitmaken van het proces waarbij oppervlaktewater wordt omgezet in drinkwater, maar zou een potentieel probleem kunnen opleveren ten aanzien van genotoxiciteit. Hoofdstuk 6 onderzoekt de genotoxiciteit van watermonsters afkomstig van de waterzuiveringsinstallatie Andijk, waar UV/H_2O_2 wordt toegepast. Genotoxiciteit werd *in vitro* getest met behulp van de Ames en Comet assay. Alle monsters vertonen negatieve resultaten in beide testen. Monsters werden ook getest met *in vivo* genotoxiciteitstesten in Amerikaanse hondsvissen (*Umbra pygmaea*). Genotoxiciteit

werd geanalyseerd met de Sister Chromatide Exchange (SCE) assay en de Comet assay waarbij geïsoleerde kieuwcellen werden gebruikt. Geen significante toename van SCEs werd waargenomen, maar kieuwcellen geïsoleerd van hondvissen blootgesteld aan water onmiddellijk na de UV/H_2O_2 behandeling en water van het IJsselmeer, laten een aanzienlijk verhoogde DNA-beschadiging zien bij de Comet assay. Alle andere geteste monsters waren negatief in deze Comet assay. Dit geeft aan dat DNA schadelijke verbindingen mogelijk ontstaan tijden de UV/H_2O_2 behandeling, maar ook dat deze verbindingen efficiënt kunnen worden verwijderd met granulaire actieve kool (GAC) behandeling voordat het water wordt gedistribueerd. Geconcludeerd wordt dat in combinatie met deze GAC behandeling, het UV/H_2O_2 proces voor de productie van drinkwater uit oppervlaktewater geen reden tot zorg is met betrekking tot de genotoxiciteit.

Algemeen werd aangetoond dat het gebruik en de verdere optimalisering van bioassays, de huidige technieken ten aanzien van de waterkwaliteitsbeoordeling zal versterken.

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Curriculum vitae

Eric Penders werd op 5 mei 1968 geboren te Geulle. Na de lagere school, behaalde hij in 1984 het MAVO-D diploma. In 1988 behaalde hij het diploma aan de Zuid-limburgse laboratoriumschool te Sittard met als studierichting M.L.O. laboratoriumtechniek/amanuensis, in 1992 gevolgd door een getuigschrift van de Hogere laboratorium Opleiding te Delft met als studierichting Biotechnologie. In 1994 startte hij als medewerker bioalarmering bij de Watertransportmaatschappij Rijn-Kennemerland (WRK) en was hiermee de interesse in het gebied van aquatische toxicologie en ecotoxicologie geboren. Naar aanleiding van verschillende projecten in de Vereniging van Rivierwaterbedrijven (RIWA) verband, begon hij in 2007 aan zijn promotieonderzoek, getiteld: “De ontwikkeling van aquatische biomonitoring model voor oppervlakte wateren dat gebruikt wordt voor de drinkwaterbereiding”, wat heeft geresulteerd in dit proefschrift. Thans is hij werkzaam als senior adviseur biologie bij Het Waterlaboratorium.

Eric Penders was born on May 5, 1968 in Geulle. After elementary school, he obtained the MAVO-D diploma in 1984. In 1988 he obtained a diploma from the Intermediate Vocational Education at Sittard (M.L.O.) in study laboratory technics and physics/chemistry/biology laboratory assistant (amanuensis), in 1992 followed by a certificate of the higher Vocational Education (H.L.O.) in Delft with a study in biotechnology. In 1994 he started at the Water transport company Rhine Kennmerland (WRK) as researcher in biomonitoring and biological early warning systems and the interest in the area of aquatic toxicology and ecotoxicology was born. As a result of several projects in the Association of River water companies (RIWA), he started in 2007 his doctoral research, entitled: "The development of aquatic biomonitoring model for surface waters used for drinking water preparation", which has resulted in this thesis. He is currently working as senior consultant biology at Het Waterlaboratorium in Haarlem.

Overview of completed training activities

SENSE

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C E R T I F I C A T E

The Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment
(SENSE), declares that

Eric Jacobus Maria Penders

born on 5 May 1968 in Geulle, The Netherlands

has successfully fulfilled all requirements of the
Educational Programme of SENSE.

Wageningen, 12 December 2011

the Chairman of the SENSE board

Prof. dr. Rik Leemans

the SENSE Director of Education

Dr. Ad van Dommelen

The SENSE Research School has been accredited by the Royal Netherlands Academy of Arts and Sciences (KNAW)

KONINKLIJKE NEDERLANDSE
AKADEMIE VAN WETENSCHAPPEN

The SENSE Research School declares that Mr. Eric Iacobus Maria Penders has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 60 ECTS, including the following activities:

SENSE PhD courses

- Research Context Activity: Preparation of a press release "Verbeterde waterkwaliteitsbewaking door inzet van bioassays "

Postgraduate Education in Toxicology (P.E.T.I. courses)

- Ecotoxicology
- Epidemiology
- Pathobiology
- Risk Assessment
- Mutagenesis and Carcinogenesis
- Molecular Toxicology
- Cell Toxicology
- Food Toxicology and Food Safety
- Medical, Forensic and Regulatory Toxicology
- Laboratory Animal Science
- Organ Toxicology
- Reproductive Toxicology

Presentations

- Poster presentation: Biological early warning systems in drinking water production, 9th International Symposium on Toxicity Assessment, 26 September – 1 October 1999, Pretoria, South Africa
- Poster presentation: Early Warning in Drinking Water Intake Protection: Correlating Chemical Data and Biological Warning Systems, SETAC, 22nd Annual Meeting, 11 – 15 November 2001, Baltimore, USA
- Poster presentation: Toxicity based assessment of water quality, 9th International Specialised IWA Conference on River Basin Management, 11 – 13 September 2002, Edinburgh, Scotland
- Poster Presentation: Biological Early Warning Systems in the production of drinking water from surface water: Modifications to increase sensitivity of BEWS, SETAC Europe, 14th Annual Meeting, 18 – 24 April 2004, Prague, Czech Republic
- Oral Presentation: Genotoxicity Study on UV/H_2O_2 Treated Surface Water Using Comet and SCE Assay, International IUVA world congress, 21 – 23 September 2009, Amsterdam, The Netherlands
- Poster Presentation: Pre-investigation to assess the requirements for the water framework directive based on the experience of the waterworks at the river Rhine, 3rd International Symposium, Genotoxicity in aquatic systems: Causes, effects and future needs, 22 – 24 September 2010, Freiburg im Breisgau, Germany

SENSE Coordinator PhD Education and Research

Mr. Johan Feenstra

List of publications

Peer-reviewed articles

Penders E.J.M., Rietjens I.M.C.M., Alink G., and Hoogenboezem W. “Evaluation of existing *in vitro* bioassays for toxicity testing of surface water”, submitted to Aquatic Toxicology.

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Addendum

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