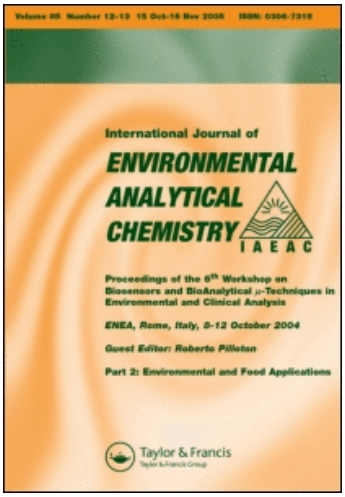


International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Biosensors for unattended, cost-effective and continuous monitoring of environmental pollution: Automated Water Analyser Computer Supported System (AWACSS) and River Analyser (RIANA)

Jens Tschmelak[a]; Guenther Proll[a]; Johannes Riedt[a]; Joachim Kaiser[b]; Peter Kraemmer[b]; Luis Bárzaga[c]; James S. Wilkinson[d]; Ping Hua[d]; J. Patrick Hole[d]; Richard Nudd[e]; Michael Jackson[e]; Ram Abuknesha[f]; Damià Barceló[g]; Sara Rodriguez-Mozaz[g]; Maria J. López de Alda[g]; Frank Sacher[h]; Jan Stien[h]; Jaroslav Slobodník[i]; Peter Oswald[i]; Helena Kozmenko[i]; Eva Korenková[i]; Lívia Tóthová[j]; Zoltan Krascsenits[j]; Guenter Gauglitz[a]

[a] Eberhard-Karls-University of Tuebingen, Institute of Physical and Theoretical Chemistry (IPTC), 72076 Tuebingen, Germany [b] Siemens AG, CT PS 6, 91050 Erlangen, Germany [c] Siemens AG, CT SM ICA, 81730 Munich, Germany [d] Optoelectronics Research Centre, Southampton University, Southampton, SO17 1BJ, UK [e] Central Research Laboratories Limited, Hayes, Middlesex, UK [f] Division of Life Sciences, King's College London, London, SE1 9NN, UK [g] IIQAB, Department of Environmental Chemistry, 08034 Barcelona, Spain [h] DVGW-Technologiezentrum Wasser, 76139 Karlsruhe, Germany [i] Environmental Institute, 97241 Kos, Slovak Republic [j] Water Research Institute, 81249 Bratislava, Slovak Republic

To cite this Article Tschmelak, Jens , Proll, Guenther , Riedt, Johannes , Kaiser, Joachim , Kraemmer, Peter , Bárzaga, Luis , Wilkinson, James S. , Hua, Ping , Hole, J. Patrick , Nudd, Richard , Jackson, Michael , Abuknesha, Ram , Barceló, Damià , Rodriguez-Mozaz, Sara , de Alda, Maria J. López , Sacher, Frank , Stien, Jan , Slobodník, Jaroslav , Oswald, Peter , Kozmenko, Helena , Korenková, Eva , Tóthová, Lívia , Krascsenits, Zoltan and Gauglitz, Guenter(2005) 'Biosensors for unattended, cost-effective and continuous monitoring of environmental pollution: Automated Water Analyser Computer Supported System (AWACSS) and River Analyser (RIANA)', International Journal of Environmental Analytical Chemistry, 85: 12, 837 — 852
To link to this Article: DOI: 10.1080/03067310500149619
URL: http://dx.doi.org/10.1080/03067310500149619

Intern. J. Environ. Anal. Chem.
Vol. 85, No. 12–13, 15 October–15 November 2005, 837–852

Biosensors for unattended, cost-effective and continuous monitoring of environmental pollution: Automated Water Analyser Computer Supported System (AWACSS) and River Analyser (RIANA)

JENS TSCHMELAK†*, GUENTHER PROLL†, JOHANNES RIEDT†, JOACHIM KAISER‡, PETER KRAEMMER‡, LUIS BÁRZAGA§, JAMES S. WILKINSON¶, PING HUA¶, J. PATRICK HOLE¶, RICHARD NUDD‖, MICHAEL JACKSON‖, RAM ABUKNESHA††, DAMIÀ BARCELÓ‡‡, SARA RODRIGUEZ-MOZAZ‡‡, MARIA J. LÓPEZ DE ALDA‡‡, FRANK SACHER§§, JAN STIEN§§, JAROSLAV SLOBODNÍK¶¶, PETER OSWALD¶¶, HELENA KOZMENKO¶¶, EVA KORENKOVÁ¶¶, LÍVIA TÓTHOVÁ‖‖, ZOLTAN KRASCSENITS‖‖ and GUENTER GAUGLITZ†

†Eberhard-Karls-University of Tuebingen, Institute of Physical and Theoretical Chemistry (IPTC), Auf der Morgenstelle 8, 72076 Tuebingen, Germany
‡Siemens AG, CT PS 6, Paul-Gossen-Str. 100, 91050 Erlangen, Germany
§Siemens AG, CT SM ICA, Otto-Hahn-Ring 6, 81730 Munich, Germany
¶Optoelectronics Research Centre, Southampton University, Highfield, Southampton, SO17 1BJ, UK
‖Central Research Laboratories Limited, Dawley Road, Hayes, Middlesex, UB3 1HH, UK
††Division of Life Sciences, King's College London, 150 Stamford St., London, SE1 9NN, UK
‡‡IIQAB, Department of Environmental Chemistry, CID-CSIC, c/Jordi Girona 18, 08034 Barcelona, Spain
§§DVGW-Technologiezentrum Wasser, Karlsruher Str. 84, 76139 Karlsruhe, Germany
¶¶Environmental Institute, Okružná 784/42, 97241 Kos, Slovak Republic
‖‖Water Research Institute, Nábr. arm. gen. L. Svobodu 5, 81249 Bratislava, Slovak Republic

(*Received 13 January 2005; in final form 14 March 2005*)

This work describes our recent progress and achievements in the field of fully automated biosensors (Automated Water Analyser Computer Supported System (AWACSS) and River Analyser (RIANA)) for unattended, cost-effective and continuous monitoring of environmental pollution. We report on ultra-sensitive immunoassays for the hormones progesterone, testosterone and estrone and the pesticides propanil and isoproturon as examples of the outstanding progress made on biosensors in the field of environmental monitoring and water analysis. Most of the bio-active organic pollutants (estrone, progesterone, propanil and isoproturon) were detected at levels as low as 1.0 pg mL^{-1} or even below. In fact, the reported limits of detection (LOD) were between 0.2 and 6.0 pg mL^{-1}. For the first time, commercially available derivatives and antibodies were incorporated into immunoassays (progesterone and

*Corresponding author. Fax: +49-7071-295490. Email: jens.tschmelak@ipc.uni-tuebingen.de

International Journal of Environmental Analytical Chemistry
ISSN 0306-7319 print: ISSN 1029-0397 online © 2005 Taylor & Francis
DOI: 10.1080/03067310500149619

testosterone) for fully automated biosensors. To verify the assay performance for quantifying testosterone, progesterone, and isoproturon in real-world samples using our immunosensors, we spiked river and drinking water at six different levels from 0.9 pg mL^{-1} to 90 ng mL^{-1}. Nearly all recovery rates could be obtained between 70 and 120% as the AOAC International recommends it chiefly for water analysis.

Keywords: Automated water analyser computer supported system (AWACSS); River analyser (RIANA); Optical immunosensor; Environmental analysis; Bio-active organic pollutants; Continuous monitoring

1. Introduction

Pollution of water sources, aquifers and wetland systems caused by industry, agriculture, and municipally treated wastewater is a pan-European problem with extreme conditions existing in newly associated European states. The European Community Water Directives [1, 2] have been implemented to review and implement strategies and measures to control pollution from diverse sources and to establish practical rules. However, complications arise in implementing the directives because there is at present a wide gulf between the demands set by the Directives and the currently available analytical capabilities because, for instance, measurements for an ever-expanding list of pollutants are demanded. In addition, true enforcement demands more frequent monitoring of water catchment areas. At the same time, little technology currently exists that can monitor water sources quickly and at a reasonable cost. The automated water analyser computer supported system (AWACSS) (No. EVK1-CT-2000-00045) project is intended to help meet the needs of both today's and tomorrow's water managers. The goal of this EU funded consortium was to develop a cost-effective, on-line, water-monitoring device that will measure a variety of pollutants quickly with remote control and surveillance.

The AWACSS project gathered expertise from nine groups in four different European nations. Only with this critical mass of technical expertise in instrument design along with the environmental monitoring experience of the water monitoring groups, were we able to produce a state-of-the-art immunosensor that caters to the needs of water managers on a European-wide scale. The instrument is based on immunochemistry technology with detection via evanescent field excitation of labeled biological recognition elements (e.g. antibodies). The immunochemistry utilized in this project takes advantage of a binding inhibition test that requires antibodies directed against specific analytes and analyte derivatives that can be covalently bound to the transduction unit. Antibodies directed against a variety of small organic water contaminants have been produced along with the corresponding analyte derivatives. Once they were purified and labeled, they were developed into immunoassays and used to detect their targets in the lower nanogram per litre range in aqueous samples. The water monitoring groups have catalogued contaminant levels at various European water sites. In so doing, they have developed new testing procedures for a variety of interesting small organic contaminants. In addition, they are addressing to what extent and to what effect water matrices, such as variable pH changes, temperature, and turbidity, have on the immunochemistry. These partners have had extended training on the systems RIANA and AWACSS, to become familiar with immunosensors and their strengths and weaknesses. This training has been aided by the development of a web-based multimedia tutorial developed within the project.

Having in mind actual needs of water-sector managers related to the implementation of the Drinking Water Directive (DWD) (98/83/EC, 1998) [1] and Water Framework Directive (WFD) (2000/60/EC, 2000) [2], drinking, ground, surface, and waste waters were major media used for the evaluation of the system performance [3, 4]. The instrument was equipped with remote-control and surveillance facilities. The system's software allows for the internet-based networking between the measurement and control stations, global management, trend analysis, and early-warning applications [4]. The experience of water laboratories has been utilized at the design of the instrument's hardware and software in order to make the system rugged and user-friendly. Several market surveys were conducted during the project to assess the applicability of the final system. A web-based AWACSS database was created for automated evaluation and storage of the obtained data in a format compatible with major databases of environmental organic pollutants in Europe. A market survey has been conducted within the project, accessing the most relevant organic compounds, their measurement frequency, measurement costs, and the overall market volume [3].

The ultra-sensitive, fully automated and robust biosensors RIANA and AWACSS are capable of detecting multiple organic targets rapidly and simultaneously at heterogeneous assay formats (solid phase: glass transducers and integrated optical (IO) waveguide chips). As already mentioned, immunoassays depending on the binding inhibition assay have been developed to detect bio-active organic pollutants (chiefly organic pollutants) in various aqueous samples. High-affinity recognition molecules were labeled with an organic dye (usually CyDye Cy5.5^{TM} from Amersham Biosciences Europe GmbH, Fribourg, Germany). Specific locations on the transducers were modified with immobilized aminodextran conjugates of the target molecules. Upon binding of a fluorescent recognition element, the light intensity of fluorescent spots was detected using photo diodes. Fully automated and manual data analysis (depending on the used biosensor system) was used to determine calibration curves for several analytes. Bio-active organic pollutants were measured in laboratory water (Milli-Q), tap water, and different environmental samples without sample pre-treatment nor sample pre-concentration steps. Results are shown for rapid analyses of progesterone, testosterone, estrone, propanil, and isoproturon as examples for several other possible analytes. Most of the bio-active organic pollutants were detected at levels as low as 1.0 pg mL^{-1} or even below.

2. Experimental

2.1 *Materials*

Common chemicals of analytical grade were purchased from Sigma-Aldrich, Deisenhofen, Germany, or Merck KGaA, Darmstadt, Germany. The hormones progesterone, testosterone and estrone were purchased as VETRANAL® and the pesticides propanil and isoproturon were purchased as PESTANAL®, analytical standards from Riedl-de Haën Laborchemikalien GmbH & Co. KG, Seelze, Germany. Two monoclonal IgG1 antibodies from mouse (anti-testosterone BM2076 and anti-progesterone BM2068) were purchased from Acris Antibodies GmbH, Hiddenhausen, Germany. The polyclonal antibodies IgG from sheep (anti-estrone,

anti-total-estrogen, anti-propanil, and anti-progesterone) were delivered by Ram Abuknesha, King's College London, UK within the AWACSS project. The monoclonal anti-isoproturon (GSF) was kindly provided by Petra Kraemer from the GSF–National Research Center for Environment and Health, Institute of Ecological Chemistry, Neuherberg, Germany. The fluorescent dye CyDye^{TM} Cy5.5 was purchased from Amersham Biosciences Europe GmbH, Fribourg, Germany. The aminodextrans Amdex^{TM} with 40 K and 170 K Dalton molecular weight were purchased from Helix Research Company, Springfield, OR, USA. Labelling and purification of antibody were carried out as described in the product information sheet supplied with the labelling kit from Amersham Biosciences Europe GmbH, Fribourg, Germany. UV-Vis spectra were recorded using a Specord M500 spectrophotometer from Carl Zeiss Jena GmbH, Jena, Germany. The spatially resolved surface modification on AWACSS chips was performed using a parallel micro-dispensing system (TopSpot) with high-performance piezostack actuation system and integrated temperature adjustment from HSG-IMIT/IMTEK, Germany. The immobilization in spatially distinct loci on RIANA chips required another micro dispensing device. The MicroDrop® (Microdrop GmbH, Norderstedt, Germany) micro dispenser system consists of a glass capillary surrounded by a piezo actuator. Applying a voltage pulse to the piezo causes it to contract, creating a pressure pulse in the liquid inside the capillary. At the nozzle, the pressure is transferred into a highly accelerated motion that expels a small droplet. This droplet flies with a velocity of 1.5 up to 3.0 m s^{-1}. Typical drop diameters are 30–100 μm, depending on the nozzle diameter, and the volume variation is less than 1%.

2.2 *Biosensors*

The AWACSS instrument employs fluorescence-based detection of the binding of fluorophore-tagged biomolecules to the surface of an integrated optical waveguide chip (IO-transducer). The fibre-pigtailed chip, driven by a semiconductor laser, consists of a waveguide circuit, which distributes excitation light to 32 separate sensing patches on the chip surface. Bio/immuno-chemistry is used to sensitize each of the 32 patches to a specific analyte and a micro fluidic system is used to automatically handle the sample injection over the sensor surface, enabling rapid, simultaneous and high-sensitivity fluorescence detection of up to 32 pollutants. A fibre-coupled detection array is used to monitor the 32 separate fluorescence signals, and software has been developed for control of the optics and fluidics and data acquisition and processing for the fluorescence signals, laser power, and ambient and chip temperature. An HTC PAL auto sampler with cycle composer software (CTC Analytics AG, Zwingen, Switzerland) was used for dilutions, sample preparations (transferring 100 μL of the antibody stock solution to 900 μL of the sample followed by one or two mixing strokes) and the sample transfer to the AWACSS instrument. Liquid handling and data acquisition are fully automated and computer controlled. One measurement cycle with washing steps, injection of the sample and regeneration of the surface takes about 15 to 18 min. Key features of the instrument, chip fabrication, chip characterization, hardware requirements, and further details of the instrumentation are described in the literature [3, 4].

The set-up of RIANA consists of the following components as described by Barzen *et al*. [5]: Light from a laser diode (Coherent Deutschland GmbH, Dieburg, Germany)

with 15 mW operating at 635 nm is coupled into the bevelled edge of a bulk optical glass slide (Schott Spezialglas GmbH, Gruenenplan, Germany). The laser beam is guided by total internal reflection along the sensitive area of the glass transducer. Fluorescent dyes near to the chip surface are excited by the evanescent field of the reflection spots. The emitted light is collected by polymer optical fibers, filtered by absorption filters with cut-on at 690 nm (Omega® Optical Inc., Brattleboro, VT, USA) and detected by photodiodes using lock-in detection. An HTS PAL auto sampler with cycle composer software is used for dilutions, sample preparations and the sample transfer to the RIANA. Liquid handling and data acquisition are fully automated and computer controlled. One measurement cycle with washing steps, injection of the sample and regeneration of the surface takes about 12 min. The regeneration protocol consists of a flow cell flushing with 400 to 800 µL of a diluted sodium dodecyl sulfate (SDS) solution (0.5% SDS in Milli-Q water at pH 2) followed by a rinsing step with buffer. Such a regeneration cycle can be repeated up to 1000 times depending on the stability of the immobilized analyte derivatives on the transducer surface. Afterwards, transducers can be recycled by starting the surface chemistry (see below) from the beginning.

2.3 *Immunochemistry*

The immunochemistry utilized in this research takes advantage of a binding inhibition assay in heterogeneous phase that requires antibodies directed against specific analytes and analyte derivatives that can be covalently bound to a sensor surface (IO-transducer or bulk optical glass slide). The immobilized aminodextran layer is used to reduce non-specific binding to the surface. A huge number of polyclonal antibodies and their corresponding analyte derivatives have been produced for a variety of organic micro-pollutants. After being purified and labeled with a dye (CyDye™ Cy5.5), they were developed into immunoassays and used in both biosensors (AWACSS and RIANA). The sample containing the analyte is incubated in solution with the labeled specific antibody. Therefore, 100 µL of the antibody stock solution are mixed with 900 µL of the sample by an auto sampler and are incubated for approximately 5 min. The antibody binds the analyte during the incubation step until a well-defined condition of the reaction is reached. When the sample is pumped over the sensor surface, only the antibodies with free binding sites can bind to the surface. For the binding inhibition assay to be quantitative, the binding of the antibody to the surface must be mass transport-limited. This allows the signal to be a function of the diffusion rate to the surface and not of the kinetics of the surface binding. The number of high affinity binding sites on the surface has to be much higher than the number of antibodies used for one measurement. To be sure, that the binding is mass transport-limited, we use small amounts of antibodies and on the sensor surface we immobilize a huge excess of antigen derivatives. This was demonstrated by additional reflectometric interference spectroscopy (RIfS) measurements as already described in the literature [6] and surface evaluation experiments [7, 8]. We achieved linear correlations between the increasing fluorescence signal and the antibody concentration used within TIRF experiments. The linear behaviour of the fluorescence signal shows that no saturation effects can be observed even with highest antibody concentrations. Therefore, the immobilized huge excess of antigen derivatives in comparison to the used amounts of antibodies could be verified.

2.4 *Detection*

AWACSS and RIANA are based on evanescent field technology. Laser light is coupled into an optical transducer and guided down the integrated optical (IO)-chip of the AWACSS system or via total internal reflection along the sensitive area of the bulk optical glass transducer of the RIANA instrument. The transducer surface is chemically modified in spatially distinct loci with analyte derivatives. Analyte-specific antibodies are labelled with a fluorescent marker (CyDye Cy5.5^{TM}), which upon binding to the transducer surface are excited in the evanescent field. The emitted light is then collected for detection in the polymer fibers. The design allows for the simultaneous measurement of multi-analyte spots. Analyte recognition is based on a binding inhibition assay. Analyte derivatives are immobilized onto the transducer surface prior to the assay. Next, analyte-specific antibodies labeled with fluorescent markers are incubated with the analyte samples. After the short incubation period, the analyte solution flows over the transducer. Only analyte-specific antibodies with free binding sites will bind to the transducer surface whereas, at the same time, antibodies that have two analyte molecules bound to each epitope will not bind to the surface. The surface bound labelled antibodies are excited in the evanescent field and the fluorescence light is detected. As a result, an inverse analyte signal is measured, with samples having low analyte concentrations giving rise to high fluorescence signals and samples with high analyte concentrations resulting in low fluorescence.

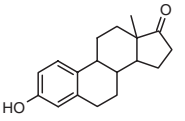
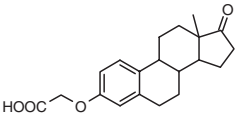
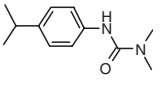
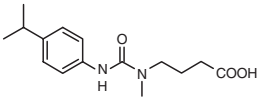
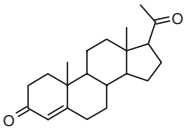
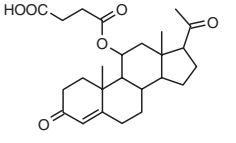
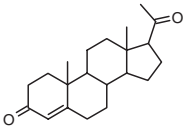
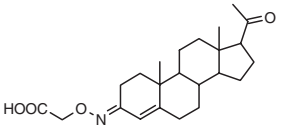
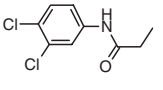
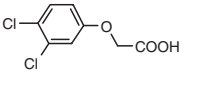
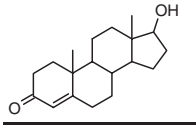
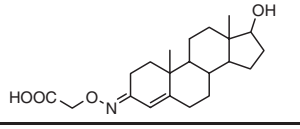
2.5 *Immobilization*

Active esters were prepared with the derivatives, which are analyte molecules modified with a spacer containing a carboxyl group. Approximately 5.0 mg of the derivative were dissolved in 100 μL of dry N,N-dimethylformamide (DMF). N-Hydroxysuccinimide (NHS) and N,N′-dicyclohexylcarbodiimide (DCC), each in 1.1-fold molar excess (referring to the amount of analyte derivative) were added to the solution. After stirring for several minutes, the solution was kept overnight at room temperature. Finally, the solution was centrifuged at approximately four degrees centigrade and the supernatant was stored under refrigeration. Fifty milligrams aminodextran were dissolved in a mixture of 500 μL Milli-Q water and 500 μL DMF. The active ester solution was added, mixed thoroughly and kept overnight at room temperature. A ten-fold volume excess of methanol precipitated the aminodextran conjugate. The supernatant was removed and the conjugate was freeze-dried. The chips were cleaned in a freshly prepared mixture (ratio 2 : 3) of hydrogen peroxide (30% H_2O_2) and concentrated sulphuric acid (65% H_2SO_4) for approximately 10 min and rinsed with Milli-Q water. After drying under a nitrogen flow, 25 μL of (3-glycidyloxypropyl)trimethoxysilane (GOPTS) were applied to the surface and reacted for up to 60 min. The silanized surface was rinsed with dry acetone and dried under a flow of nitrogen. Subsequently, the aminodextran conjugates were dissolved in Milli-Q water at a concentration of 1.0 up to 2.0 mg mL^{-1} and were immobilized by a parallel spotting device TopSpot from HSG-IMIT, Villingen-Schwenningen, Germany (www.hsg-imit.de) and IMTEK (Institute for Microsystem Technology), Fribourg, Germany (www.imtek.de) or by the piezoelectric ink-jet system from Microdrop GmbH, Norderstedt, Germany (www.microdrop.de).

2.6 *Measurements*

For the measurements, we used polyclonal IgG antibodies from sheep, monoclonal IgG1 antibodies from mice and suitable analyte derivatives (the haptens or similar substances – for details see table 1). The entire sample volume was 1000 µL. For a calibration routine, 900 µL of spiked Milli-Q water was automatically mixed by the auto sampler with 100 µL of an antibody stock solution containing the antibodies and ovalbumin from chicken eggs (OVA) in ten-fold phosphate buffered saline (PBS) (ten-fold PBS: pH 6.8, 1500 mM sodium chloride, 100 mM potassium phosphate monobasic). After a defined incubation time of approximately 5 min this mixture was measured using the biosensor setups. The experimental design for a calibration routine consists of nine independent blank (Milli-Q water) measurements and eight

Table 1. Summary of the structures of the used targets (analytes) and used derivatives for surface modification.

Analyte	Derivative
Estrone	Estrone-3-carboxymethylether ($E_1$3CME)
	
Isoproturon	3-(4-Isopropylphenyl)-1-carboxypropyl-1-methyl urea (IP30)
	
Progesterone	Progesterone-11-hemisuccinate (P11HS)
	
Progesterone	Progesterone-3-O-carboxymethyloxime (P3CMO)
	
Propanil	3,4-Dichlorophenoxyacetic acid (PRN-H4)
	
Testosterone	Testosterone-3-O-carboxymethyloxime (T3CMO)
	

or nine concentration steps (each measured as three replica) of the analyte (spiked Milli-Q water). For all concentration steps and the blank measurements (nine replica) the mean value and the standard deviation (SD) for the replica was automatically calculated by the AWACSS measurement control unit or manually for the RIANA system. The measured signal for the mean value of the blanks was set to 100% and all spiked samples could be obtained as a relative signal below this blank value. To fit the data set a logistic fit function [9] (parameters of a logistic function: A_1, A_2, x_0, and p) was used. A_1, as the upper asymptote and A_2 is the lower asymptote. The range between A_1 and A_2 is the dynamic signal range. The inflection point is given by the variable x_0 and represents the analyte concentration, which corresponds to a decrease of 50% of the dynamic signal range (IC_{50}). The slope of the tangent in this point is given by the parameter p. In compliance with the IUPAC rules (The Orange Book) [10], the limit of detection (LOD) is calculated as three times the SD of the blank measurements (SD_{blanks}). The use of LOQ for logistic calibration curves is sceptical, because with its non-linear behaviour the results for immunoassays are often worse than they need to be. A real alternative is the use of the 95% confidence belt and the related minimum detectable concentration (MDC) and reliable detection limit (RDL) [11]. O'Connell *et al.* reported on calibration and assay development using the four-parameter logistic model and assay quality control procedures. We used Origin 7G SR4 (OriginLab Corporation, Northampton, MA, USA) to fit the data set and to calculate the 95% confidence belt. The received data was used to calculate the validation parameters LOD, LOQ, MDC, and RDL. All important statistical procedures and further calculations are included within the AWACSS evaluation software package. For the RIANA the data evaluation has to be done in a semi-automated procedure.

3. Results

3.1 *Testosterone*

Testosterone, an aromatizable androgen, masculinizes resulting masculine endocrinology or behaviour. In females or neonatally castrated males, female features develop in the absence of aromatizable androgen. Testosterone is the most important sex hormone that men have. It is responsible for the typical male characteristics and helps maintain sex drive, sperm production, pubic and body hair, muscle, and bone. The brain controls the production of testosterone by the testes. The occurrence of this hormone in surface waters has become a topic of concern because of potential adverse effects including disruption of the endocrine system of aquatic organisms. Sources of hormones to natural waters include disposal of effluents from municipal sewage-treatment plants and animal feeding operations. Detecting testosterone in the lower nanogram per litre range with a cost-effective, robust and quasi-continuous monitoring system can be very important for the future safety of our drinking water. We used testosterone, for example, to adapt the first commercially available assay on our biosensors. The derivative for surface modification used was testosterone-3-O-carboxymethyloxime (T8390) from Sigma-Aldrich (Schelldorf, Germany). The monoclonal IgG1 antibody anti-testosterone (BM2076) from mice with a proposed affinity constant (K) of 5×10^{10} was supplied by Acris Antibodies (Hiddenhausen, Germany). According to the data-sheet of the antibody, the immunogen was

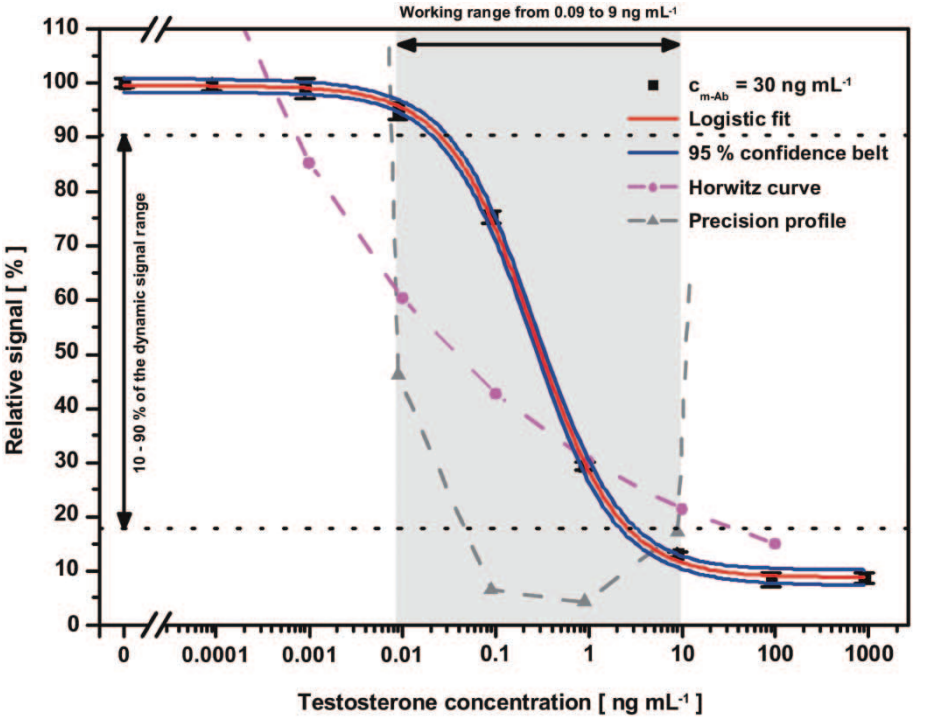


Figure 1. Calibration curve and 95% confidence belt for testosterone measured on a testosterone-3-O-carboxymethyloxime (derivative) modified transducer from 0.09 pg mL^{-1} to 900 ng mL^{-1} (eight steps). The antibody (BM2076: monoclonal anti-testosterone) concentration (c_{m-Ab}) was 30 ng mL^{-1} in each sample. The working range from 0.09 to 9 ng mL^{-1} is shaded in gray.

'Testosterone-3-CMO-BSA' and applications like ELISA and RIA were tested with this antibody. In a first step, a freshly prepared transducer with the derivative testosterone-3-O-carboxymethyloxime (see table 1) immobilized on the complete sensitive area of the chip was calibrated with testosterone from 0.09 pg mL^{-1} to 900 ng mL^{-1} in eight steps by using 30 ng labeled anti-testosterone per sample (sample volume: 1.0 mL). The calibration resulted in a limit of detection (LOD) of 6.0 pg mL^{-1}, a limit of quantification (LOQ) of 23.2 pg mL^{-1}, a minimum detectable concentration (MDC) of 2.7 pg mL^{-1}, and reliable detection limit (RDL) of only 5.3 pg mL^{-1}. The calibration curve is shown in figure 1 and all parameters of the logistic fit function and calculated results for the validation parameters are summarized in table 2. Relative signal values of the calibration curve are collected in table 3. To ensure high-precision testosterone quantification using our immunosensors, we spiked river water at six different testosterone levels from 0.9 pg mL^{-1} to 90 ng mL^{-1}. The obtained data for triplicate measurements has been evaluated using the corresponding calibration curve (see figure 1) and parameters of the corresponding logistic fit (see table 2) respectively. Out of these evaluated results, we calculated mean recovery rates as percentage of testosterone contamination. The recovery rates could be obtained between 57.6 and 127.7% and the results are shown in figure 4. If you leave out the lowest spiking level of only 0.9 pg mL^{-1}, which is below the LOD, and the highest spiking level of 90 ng mL^{-1}, which is beyond the working range of 0.09–9 ng mL^{-1}, all recovery rates (accurately

Table 2. Summary of all parameters of the logistic fit functions (A_1, A_2, x_0, and p) and all validation parameters (LOD, LOQ, MDC, and RDL) for the calibration curves.The parameter x_0 of the logistic fit function is equivalent to the test mid-point IC_{50}. Calibrations were performed for estrone, isoproturon, progesterone (with monoclonal and polyclonal antibody on different derivatives), propanil and testosterone.

Analyte surface	Estrone $E_1$3CME	Isoproturon IP30	Progesterone P11HS	Progesterone P3CMO	Propanil PRN-H4	Testosterone T3CMO
A_1 (%)	100.00	100.79	100.00	99.97	100.00	99.53
A_2 (%)	17.2	15.6	3.4	19.5	19.4	8.8
x_0 (ng mL^{-1})	0.06	0.17	0.71	0.53	0.05	0.25
p (–)	0.6	0.6	0.5	0.6	0.7	0.9
c_{ab} (ng mL^{-1})	3	50	25	30	3	30
LOD (pg mL^{-1})	0.2	0.8	0.4	1.7	0.6	6.0
LOQ (pg mL^{-1})	1.4	8.9	4.9	16.6	4.5	23.2
MDC (pg mL^{-1})	n.c.	0.2	n.c.	1.7	n.c.	2.7
RDL (pg mL^{-1})	n.c.	0.4	n.c.	5.3	n.c.	5.3

n.c.: not calculated.

Table 3. Summary of the calculated relative signal values with corresponding errors (replica measurements) as percentage. Results for calibrations with isoproturon, progesterone and testosterone are shown and all others can be found in the mentioned references.

Concentration (ng mL^{-1})	Isoproturon[a] (%)	Progesterone[b] (%)	Testosterone[c] (%)
0	100.00 ± 1.38	100.00 ± 1.00	100.00 ± 0.86
0.00009	100.96 ± 1.74	98.91 ± 0.72	99.68 ± 0.97
0.0009	95.68 ± 0.50	96.93 ± 0.49	98.99 ± 1.78
0.009	87.09 ± 0.49	94.06 ± 0.39	94.90 ± 1.55
0.09	65.52 ± 0.83	79.08 ± 1.80	75.30 ± 1.10
0.9	38.82 ± 1.12	51.36 ± 1.53	29.38 ± 0.64
9	24.34 ± 0.72	35.12 ± 1.46	13.03 ± 0.48
90	18.15 ± 0.58	24.54 ± 1.40	8.39 ± 1.31
900	15.83 ± 0.65	19.05 ± 1.88	8.77 ± 0.90

[a]Surface: 3-(4-isopropylphenyl)-1-carboxypropyl-1-methyl urea (IP30); antibody: monoclonal anti-isoproturon (GSF).
[b]Surface: progesterone-3-O-carboxymethyloxime (P3CMO); antibody: monoclonal anti-progesterone (BM2068).
[c]Surface: testosterone-3-O-carboxymethyloxime (T3CMO); antibody: monoclonal anti-testosterone (BM2076).

between 80.5 and 114.7%) were between 70 and 120% as recommended by the AOAC International chiefly for water analysis. This first achievement motivated us to plan further improvements and to start work on assay optimization to reach lower detection and quantification limits for testosterone in different water matrices (drinking, ground, and surface waters). Furthermore, the assay must be adjusted to the needs of measuring biological fluids. A complete study on our fully automated immunoassay for testosterone in aqueous samples (including biological fluids) will be published soon.

3.2 *Estrone*

Our polyclonal antibodies (anti-estrone and anti-total-estrogen) can detect not only estrone but also several other estrogenic compounds such as ethinylestradiol, estradiol, and estriol. Throughout the scientific world, the hypothesis has been put forward that humans and wildlife species suffer adverse health effects after exposure to endocrine disrupting compounds (EDCs). Reported adverse effects include declines

in populations, increases in cancers, and reduced reproductive function [12]. A huge amount of natural hormones and endocrine disrupting chemicals are reaching surface waters [13]. The main sources of this pollution are wastewater treatment plants and intensive stock rearing.

We have optimized our fully automated immunoassay for estrone and this procedure resulted in a LOD of only 0.2 pg mL^{-1} and a LOQ of 1.4 pg mL^{-1}. These extremely low detection and quantification limits for the hormone estrone are the result of the continuous optimization of the assay [14]. The basis of such sensitive assays is the antibody with a highly affinity constant towards the target analyte and a high affinity towards the immobilized derivative (in this case estrone-13-carboxymethylether, see table 1 for the structure) used for the transducer modification. During the optimization process, we reduced the amount of antibody per sample and improved the chip surface modification. Finally, this proceeding led to a calibration routine with an antibody amount of only 3 ng per sample. The reduction of the amount of antibody per sample results in better validation parameters, but this reduction led to the current device-related limitation of the RIANA. The achieved parameters of the logistic fit function and calculated results for the validation parameters are shown in table 2. For some endocrine disrupting compounds, no effect levels (NOELs) in the low nanogram per litre range are reported. This defines the challenge, which analytical methods have to compete with and our RIANA instrument with its improved sensitivity for the detection of a single hormone in the very low nanogram per litre range is a powerful tool in aquatic analytics in addition to the common analytical methods. Further details about this work on estrone can be found in the literature [14].

3.3 *Progesterone*

Progesterone is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals and it can be found in various surface waters which are partly used as drinking water resources [12, 15]. Therefore, immunoanalytical methods at a very low LOD and a low LOQ are becoming more and more important for environmental analysis and especially for monitoring drinking water quality. Biosensors have suitable characteristics such as efficiency in allowing fast, sensitive, and cost-effective detection in aqueous samples. We have developed a sophisticated fully automated immunoassay for progesterone with a LOD in the sub-nanogram per liter range and a LOQ in the lower nanogram per liter range [16]. The assay resulted in an LOD of only 0.4 pg mL^{-1} and a LOQ of 4.9 pg mL^{-1} by using 25 ng of the antibody (polyclonal anti-progesterone) per sample. Calculated results for the validation parameters and achieved parameters of the logistic fit function are shown in table 2. The basis of our ultra-sensitive assay is the antibody with a high affinity constant towards progesterone and the robust biosensor set-up used. These results have been achieved with the polyclonal antibody anti-progesterone together with immobilized progesterone-11-hemisuccinate (see table 1) which have been kindly supplied by Ram Abuknesha during the AWACSS project. To compare this amazing fully automated immunoassay for progesterone detection to a commercial available pair of antibody and derivative, a monoclonal IgG1 antibody was purchased anti-progesterone (BM2068) from mouse with a proposed affinity constant (K) of 1.2×10^{10} from Acris Antibodies (Hiddenhausen, Germany) and a new derivative for surface modification progesterone-3-O-carboxymethyloxime (P3277) was purchased

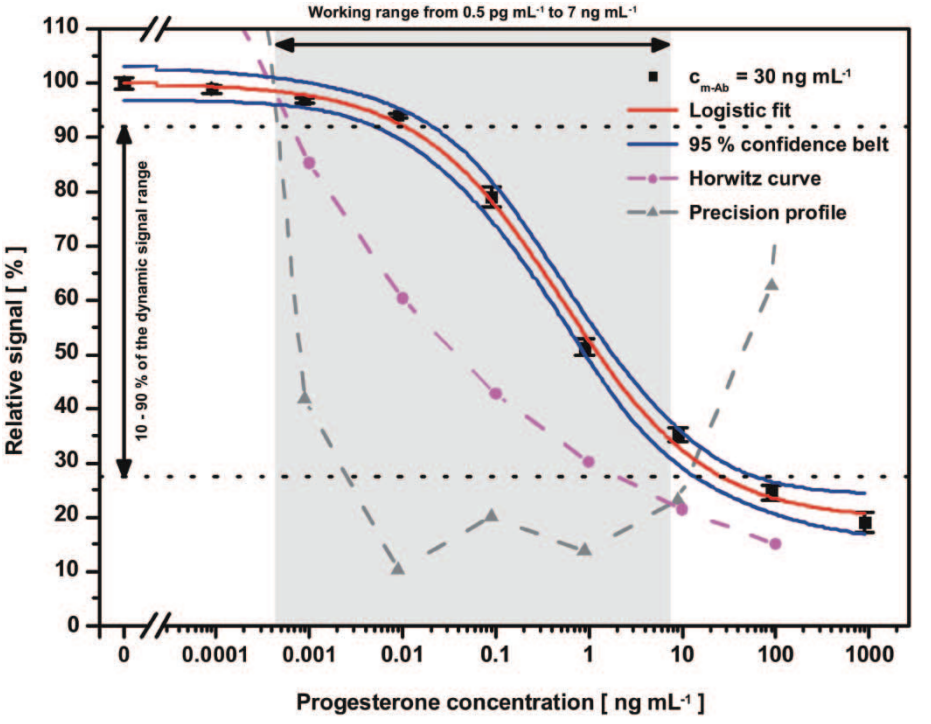


Figure 2. Calibration curve and 95% confidence belt for progesterone measured on a progesterone-3-O-carboxymethyloxime (derivative) modified transducer from 0.09 pg mL^{-1} to 900 ng mL^{-1} (eight steps). The antibody (BM2068: monoclonal anti-progesterone) concentration (c_{m-Ab}) was 30 ng mL^{-1} in each sample. The working range from 0.5 pg mL^{-1} to 7 ng mL^{-1} is shaded in gray.

from Sigma-Aldrich (Schelldorf, Germany). According to the data-sheet of the antibody, the immunogen was 'Progesterone-3-CMO-BSA' and applications like ELISA and RIA were tested with this antibody. For comparison, a freshly prepared transducer with the derivative progesterone-3-O-carboxymethyloxime (see table 1) immobilized on the complete sensitive area of the chip was calibrated with progesterone from 0.09 pg mL^{-1} to 900 ng mL^{-1} in eight steps by using 30 ng labeled anti-progesterone per sample (sample volume: 1.0 mL). The calibration resulted in a LOD of 1.7 pg mL^{-1}, a LOQ of 16.6 pg mL^{-1}, a MDC of 1.7 pg mL^{-1}, and RDL of only 5.3 pg mL^{-1}. All parameters of the logistic fit function and calculated results for the validation parameters are summarized in table 2 and the calibration curve is shown in figure 2. Relative signal values of the calibration curve are collected in table 3. Having in mind the better results of the progesterone assay using the polyclonal antibody and the immobilized progesterone-11-hemisuccinate, the results with the commercially available pair of antibody and derivative were disappointing but this assay has not been optimized so far. To verify the assay performance for quantifying progesterone in real-world samples using our immunosensors, we spiked river water at six different progesterone levels from 0.9 pg mL^{-1} to 90 ng mL^{-1}. The resulting data for triplicate measurements has been evaluated using the corresponding calibration curve (see figure 2) and parameters of the corresponding logistic fit (see table 2), respectively. Out of these evaluated results, we calculated mean recovery rates as percentage of

progesterone contamination. The recovery rates were between 71.1 and 155.8% and the results are shown in figure 4. If you leave out the lowest spiking level of only $0.9\,pg\,mL^{-1}$, which is below the LOD all recovery rates (accurately between 71.1 and 107.8%) were between 70 and 120% as recommended by the AOAC International chiefly for water analysis. Furthermore, good results with commercial products on our biosensors are incredibly important since the number of commercial available antibodies rises daily. Therefore, more important analytes will be detectable on our ultra-sensitive biosensors for environmental monitoring in future.

3.4 *Propanil*

The widely used pesticide propanil is a selective post-emergent general-use acetanilide herbicide registered for control of broadleaf and grass weeds in rice, small grain, and turf. Because broad application and quite heavy use of this herbicide lead to contaminated sites and, consequently, contaminated water, immunoanalytical methods with very low LOD are becoming increasingly important for environmental analysis and, especially, for monitoring drinking-water quality. We described the steps of progress toward sub-nanogram per litre detection of propanil with a fully automated immunoassay [17]. In contrast with common analytical methods such as GC- or HPLC-MS the biosensor used requires no sample pre-treatment and pre-concentration. The basis of our very sensitive assay is an antibody with a high affinity constant toward propanil. During the optimization process, we compared four different derivatives for surface modification, checked scores of real world samples and reduced the amount of antibody per sample. In fact, optimization of the assay resulted in an LOD of only $0.6\,pg\,mL^{-1}$ and a LOQ of $4.5\,pg\,mL^{-1}$ by using only three nanogram antibody per sample without any sample pre-treatment nor pre-concentration. The acquired parameters of the logistic fit function and calculated results for the validation parameters are shown in table 1. The results for propanil with our biosensors, and the improved sensitivity for detection of a single pesticide at the low nanogram per litre range, show that our biosensors can compete with common analytical methods in the field of water analysis.

3.5 *Isoproturon*

Pesticides like isoproturon are well known for their ecotoxic potential. Therefore, the European Union (EU) has adopted directives (e.g. EU Authorisation Directive 91/414/EEC) for maximum concentrations in drinking water ($0.1\,ng\,mL^{-1}$ for single substances and $0.5\,ng\,mL^{-1}$ for the sum concentration) [18]. The phenyl urea herbicide isoproturon is used against annual grasses and broad-leaved weeds in cereal production across Europe, resulting in both surface water and ground water pollution [19]. In Germany, isoproturon can be found in several rivers and waterworks close to those rivers may run into problems if they supply polluted water. Krämer *et al.* described enzyme-linked immunosorbent assays (ELISAs) based on rabbit polyclonal and rat monoclonal antibodies against isoproturon [20]. They described the production and characterization of rabbit polyclonal antisera and rat monoclonal antibodies against isoproturon and they conducted intensive measurements in Milli-Q water, real water samples and organic solvent containing water samples. The reported LOD is between 0.003 and $0.01\,ng\,mL^{-1}$ and in fact, the reported results are very good for ELISAs.

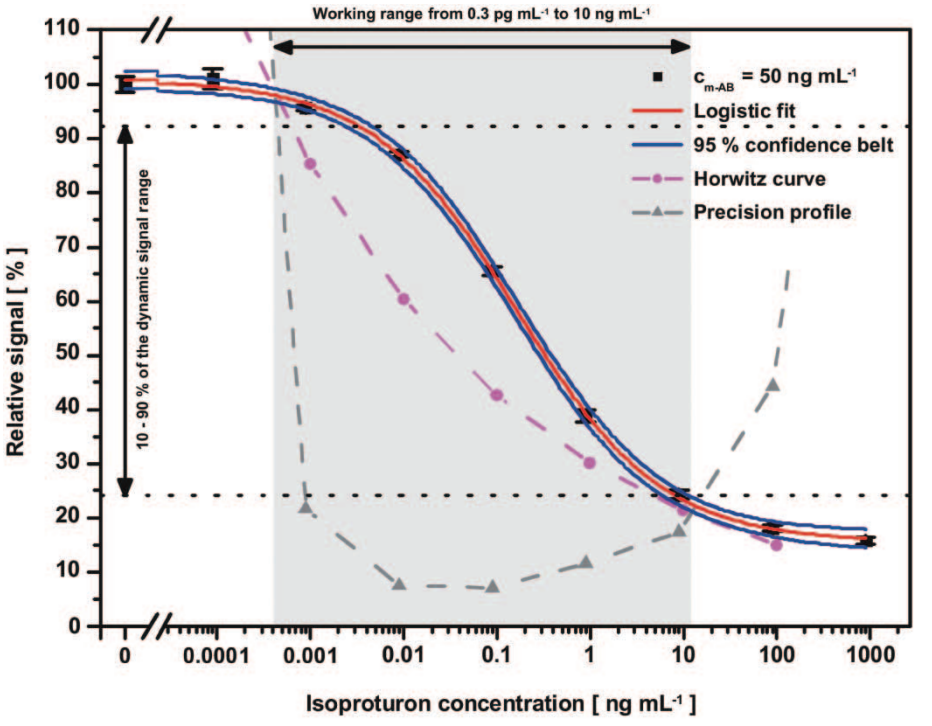


Figure 3. Calibration curve and 95% confidence belt for isoproturon measured on a 3-(4-isopropylphenyl)-1-carboxypropyl-1-methyl urea (derivative) modified transducer from 0.09 pg mL^{-1} to 900 ng mL^{-1} (eight steps). The antibody (GSF: monoclonal anti-isoproturon) concentration (c_{m-Ab}) was 50 ng mL^{-1} in each sample. The working range from 0.3 pg mL^{-1} to 10 ng mL^{-1} is shaded in grey.

Therefore, we contacted Petra Kraemer from the GSF–National Research Center for Environment and Health, Institute of Ecological Chemistry, Neuherberg, Germany and received a cup of monoclonal anti-isoproturon as a present for research. After the standard labeling procedure and the preparation of our sensor surface with 3-(4-isopropylphenyl)-1-carboxypropyl-1-methyl urea (see table 1) from the AWACSS project the new pair of antibody and surface has been developed into a new immunoassay. The system was calibrated with isoproturon from 0.09 pg mL^{-1} to 900 ng mL^{-1} in eight steps by using 50 ng labeled anti-isoproturon per sample (sample volume: 1.0 mL). The calibration resulted in a LOD of 0.8 pg mL^{-1}, a LOQ of 8.9 pg mL^{-1}, a MDC of 0.2 pg mL^{-1}, and RDL of only 0.4 pg mL^{-1}. These outstanding values for LOD, MDC, and RDL are the result of the tight 95% confidence belt, the high accuracy, and the reproducibility of the isoproturon assay. The calibration curve is shown in figure 3 and all parameters of the logistic fit function and calculated results for the validation parameters are summarized in table 2. Relative signal values of the calibration curve are collected in table 3. To guarantee high-precision isoproturon quantification in drinking water using our immunosensors, we spiked drinking water at six different isoproturon levels from 0.9 pg mL^{-1} to 90 ng mL^{-1}. The obtained data for triplicate measurements has been evaluated using the corresponding calibration curve (see figure 3) and parameters of the corresponding logistic fit (see table 2), respectively. Out of these evaluated results, we calculated mean recovery rates as

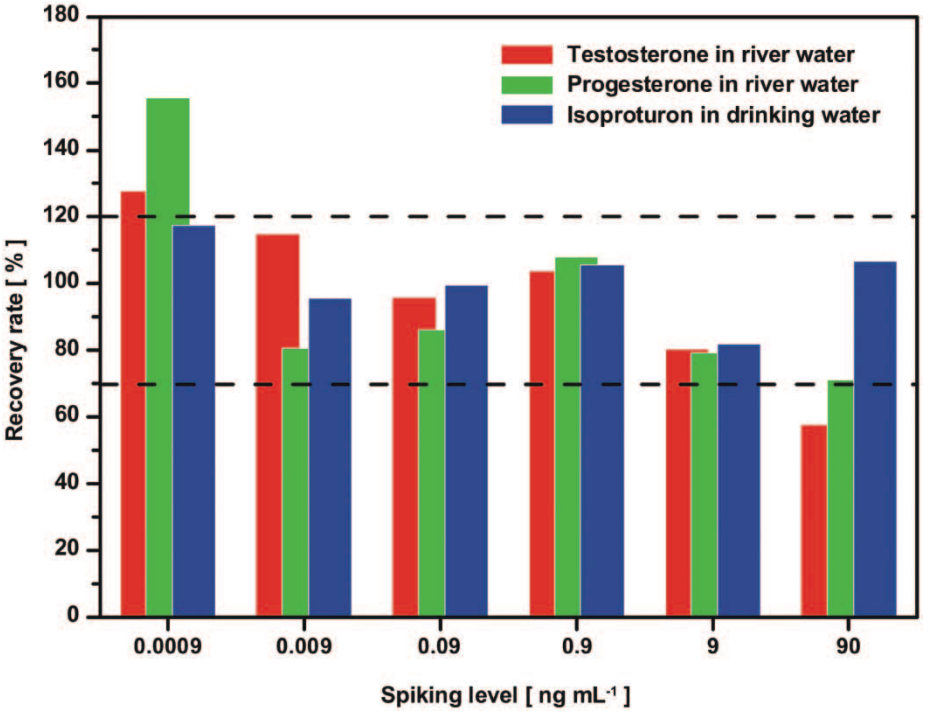


Figure 4. Recovery rates (mean value) for testosterone and progesterone detection in spiked river water and isoproturon detection in spiked drinking water. All spiked real-world samples were evaluated with the corresponding calibration curve (see figures 1–3).

percentage of isoproturon contamination. The recovery rates were between 82.1 and 117.6% and the results are shown in figure 4. Once again, all recovery rates were between 70 and 120% as the AOAC International recommends it chiefly for water analysis. The working range lasts from 0.3 pg mL^{-1} to 10 ng mL^{-1}, which covers over four orders of magnitude. This achievement motivated us to plan further improvements and to start a collaboration to compare the mentioned ELISAs to our biosensors RIANA and AWACSS.

Acknowledgements

This work was funded by the 'Automated Water Analyser Computer Supported System' (AWACSS) (EVK1-CT-2000-00045) research project supported by the European Commission under the Fifth Framework Programme and contributing to the implementation of the Key Action 'Sustainable Management and Quality of Water' within the Energy, Environment and Sustainable Development. The development of the former biosensor used was funded by the European Commission under the Environment and Climate Program (Fourth Framework Programme) River Analyser (RIANA) (ENV4-CT95-0066) project. The monoclonal antibody anti-isoproturon was a gift for research from Petra Kraemer, GSF–National Research Centre for Environment and Health, Institute for Ecological Chemistry,

Neuherberg, Germany. All other non-commercial antibodies and derivatives were kindly supplied by Ram Abuknesha, King's College London, London, UK. Jens Tschmelak is a scholarship holder and Guenther Proll is participant of the research training group 'Quantitative Analysis and Characterization of Pharmaceutically and Biochemically relevant Substances' funded by the Deutsche Forschungsgemeinschaft (DFG) at the Eberhard-Karls-University of Tuebingen, Tuebingen, Germany.

References

[1] 98/83/EC: COUNCIL DIRECTIVE (98/83/EC) of 3 November 1998 relating to the quality of water intended for human consumption. Official Journal of the European Community, **L330**, 32–54 (1998).
[2] 2000/60/EC: DIRECTIVE 2000/60/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 23 October 2000 establishing a framework for Community action in the field of water policy. Official Journal of the European Community, **L327**, 1–72 (2000).
[3] J. Tschmelak, G. Proll, J. Riedt, J. Kaiser, P. Kraemmer, L. Bárzaga, J.S. Wilkinson, P. Hua, J.P. Hole, R. Nudd, M. Jackson, R. Abuknesha, D. Barceló, S. Rodriguez-Mozaz, M.J. López de Alda, F. Sacher, J. Stien, J. Slobodník, P. Oswald, H. Kozmenko, E. Korenková, L. Tóthová, Z. Krascsenits, G. Gauglitz. *Biosensors Bioelectron*., **20**, 1499 (2005).
[4] J. Tschmelak, G. Proll, J. Riedt, J. Kaiser, P. Kraemmer, L. Bárzaga, J.S. Wilkinson, P. Hua, J.P. Hole, R. Nudd, M. Jackson, R. Abuknesha, D. Barceló, S. Rodriguez-Mozaz, M.J. López de Alda, F. Sacher, J. Stien, J. Slobodník, P. Oswald, H. Kozmenko, E. Korenková, L. Tóthová, Z. Krascsenits, G. Gauglitz. *Biosensors Bioelectron*., **20**, 1509 (2005).
[5] C. Barzen, A. Brecht, G. Gauglitz. *Biosensors Bioelectron*., **17**, 289 (2002).
[6] R.W. Glaser. *Anal. Biochem*., **213**, 152 (1993).
[7] K. Kroeger, A. Jung, S. Reder, G. Gauglitz. *Anal. Chim. Acta*, **469**, 37 (2002).
[8] A. Jung, I. Stemmler, A. Brecht, G. Gauglitz. *Fresenius' J. Anal. Chem*., **371**, 128 (2001)
[9] R.A. Dudley, P. Edwards, R.P. Ekins, D.J. Finney, I.G. McKenzie, G.M. Raab, D. Rodbard, R.P. Rodgers. *Clinical Chem*., **31**, 1264 (1985).
[10] J. Inczedy, T. Lengyel, A.M. Ure. *Compendium of Analytical Nomenclature – The Orange Book*, 3rd Edn, Blackwell Science, Oxford (1998).
[11] M.A. O'Connell, B.A. Belanger, P.D. Haaland. *Chemometrics and Intelligent Laboratory Systems*, **20**, 97 (1993).
[12] R.J. Kavlock, G.P. Daston, C. DeRosa, P. Fenner-Crisp, L.E. Gray, S. Kaattari, G. Lucier, M. Luster, M.J. Mac, C. Maczka, R. Miller, J. Moore, R. Rolland, G. Scott, D.M. Sheehan, T. Sinks, H.A. Tilson. *Environ. Health Persp*., **104**, 715 (1996).
[13] D.G.J. Larsson, M. Adolfsson-Erici, J. Parkkonen, M. Pettersson, A.H. Berg, P.-E. Olsson, L. Forlin. *Aquatic Toxicol*., **45**, 91 (1999).
[14] J. Tschmelak, G. Proll, G. Gauglitz. *Talanta*, **65**, 311 (2005).
[15] L.S. Shore, M. Gurevitz, M. Shemesh. *Bull. Environ. Contamin. Toxicol*., **51**, 361 (1993).
[16] J. Tschmelak, G. Proll, G. Gauglitz. *Anal. Chim. Acta*, **519**, 143 (2004).
[17] J. Tschmelak, G. Proll, G. Gauglitz. *Anal. Bioanal. Chem*., **379**, 1004 (2004). Erratum: *Anal. Bioanal. Chem*., **381**, 526 (2005).
[18] A. Craven. Pesticides in air, plant, soil & water system. In *Proceedings of the 12th Symposium on Pesticide Chemistry*, pp. 417–421, Piacenza, Italy, June 4–6 (2003).
[19] S.R. Sorensen, Z. Ronen, J. Aamand. *Appl. Environ. Microbiol*., **67**, 5403 (2001).
[20] P.M. Krämer, M.H. Goodrow, E. Kremmer. *J. Agric. Food Chem*., **52**, 2462 (2004).