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Automated Water Analyser Computer Supported System (AWACSS) Part II: Intelligent, remote-controlled, cost-effective, on-line, water-monitoring measurement system

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Abstract

A novel analytical system AWACSS (Automated Water Analyser Computer Supported System) based on immunochemical technology has been evaluated that can measure several organic pollutants at low nanogram per litre level in a single few-minutes analysis without any prior sample pre-concentration or pre-treatment steps.

Having in mind actual needs of water-sector managers related to the implementation of the Drinking Water Directive (DWD) [98/83/EC, 1998. Council Directive (98/83/EC) of 3 November 1998 relating to the quality of water intended for human consumption. Off. J. Eur. Commun. L330, 32–54] and Water Framework Directive (WFD) [2000/60/EC, 2000. 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. Off. J. Eur. Commun. L327, 1–72], drinking, ground, surface, and waste waters were major media used for the evaluation of the system performance.

The first part article gave the reader an overview of the aims and scope of the AWACSS project as well as details about basic technology, immunoassays, software, and networking developed and utilised within the research project. The second part reports on the system performance, first real sample measurements, and an international collaborative trial (inter-laboratory tests) to compare the biosensor with conventional anayltical methods. The systems' capability for analysing a wide range of environmental organic micro-pollutants, such as modern pesticides, endocrine disrupting compounds and pharmaceuticals in surface, ground, drinking and waste water is shown. In addition, a protocol using

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reconstitution of extracts of solid samples, developed and applied for analysis of river sediments and food samples, is presented. Finally, the overall performance of the AWACSS system in comparison to the conventional analytical techniques, which included liquid and gas chromatographic systems with diode-array UV and mass spectrometric detectors, was successfully tested in an inter-laboratory collaborative trial among six project partners.

Keywords: Automated Water Analyser Computer Supported System (AWACSS); Optical immunosensor; On-line biosensor monitoring; Emerging pollutants; Environmental analysis; Immunosensor network

1. Introduction

Industrial activities, extensive farming, sewage water, and many other human activities are polluting waterways throughout Europe. Rivers, channels, lakes, oceans, ground water, and even drinking water can be contaminated by a variety of organic pollutants that can have adverse effects on aquatic life and pose risks to human health (Barceló and Hennion, 1997a, 1997b). Examples include herbicides, insecticides, fungicides, antibiotics, drugs, detergents, oils, industrial waste or by-products, endocrine disrupting compounds, and carcinogens. Some man-made substances being released into environment are toxic to humans via food chain. The new water-related environmental legislation in Europe clearly defines a need for regular monitoring of wide, and ever growing, range of organic substances down to low nanogram per litre levels (2000/60/EC, 2000; 98/83/EC, 1998). Since many of the so-called priority substances (2000/60/EC, 2000) to be monitored in water are rather difficult to analyse European Union expert groups working at the implementation of the Water Framework Directive (WFD) (2000/60/EC, 2000) put a considerable effort to propose a range of techniques and methods for their monitoring. True enforcement demands more frequent monitoring of water catchment areas and also industrial plants need greater control of their waste water to meet the demands of increased regulation.

Currently, to analyse the different environmental pollutants, quantitative water analysis is performed with standard analytical methods such as high-performance liquid chromatography (HPLC), or gas chromatography coupled to mass spectroscopy (GC–MS) (Clement et al., 1997). The contaminants are invariably present in complex matrices and at low concentrations and, therefore, efficient sample concentration and clean-up methods followed by common analytical techniques equipped with sensitive and selective detectors are usually techniques of choice (Barceló, 2000). Usually, these methods need trained manpower and automation is limited, therefore, these methods are labour intensive, expensive, and time consuming. Despite the fast developments of these techniques over the past decades, water-sector managers and researchers still strive for a water-monitoring device, which would be robust, cost-effective, automated and able to measure several tens of organic pollutants at low nanogram per litre level in a short time, preferably without any time-consuming sample concentration step and prior sample pre-treatment. Another sought for feature is a possibility of instrument's remote control, automated data processing and generation of alarm signals when pollutant's concentration exceeds the pre-set threshold value. The use of such on-line sensors follows also new developments in the drinking water legislation and introduction of risk assessment/risk management approach (World Health Organization, 2003).

Immunochemically based techniques are a popular alternative to standard methods. Key features of immunoassays, such as their specificity, sensitivity and speed of analysis led to the development of numerous environmental applications (Barceló, 2000) and qualify them as suitable candidates to be used for environmental monitoring. Still, most of the commercially available techniques suffer from not fully described cross-reactivity of target analytes, matrix effects, limited availability of antibodies and not having capability of multi-analyte analysis (Oubina et al., 2000). Recently, the field of array-based technology for multianalyte detection has exploded. These techniques take advantage of the two-dimensional layout of recognition elements to allow simultaneous detection and quantification of multiple analytes (Rowe Taitt et al., 2002).

In the EU project AWACSS (Automated Water Analyser Computer Supported System; EVK1-CT-2000-00045), a multi-analyte immunoassay-based system has been constructed and successfully tested for analysis of aqueous samples. The first part article gave the reader an overview of the aims and scope of the AWACSS project as well as details about basic technology, immunoassays, software, and networking developed and utilised within the research project. This second part now reports on the system performance, first real sample measurements, and an international collaborative trial (inter-laboratory tests) to compare the biosensor with conventional anayltical methods.

2. Experimental

2.1. Materials

Common chemicals of analytical grade were purchased from Sigma–Aldrich, Deisenhofen, Germany, or Merck KGaA, Darmstadt, Germany. The pesticides atrazine, isoproturon, and propanil were purchased as PESTANAL®, the antibiotic sulphamethizole and the hormone estrone were purchased as VETRANAL® analytical standards from Riedl-de Haën Laborchemikalien GmbH & Co. KG, Seelze, Germany. The polymer plasticiser bisphenol A was also ordered from Sigma–Aldrich. The fluorescent dye CyDye™

Cy5.5 was purchased from Amersham Biosciences Europe GmbH, Fribourg, Germany. The aminodextrans Amdex™ with 40 and 170 kDa 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. UV–vis spectra were recorded using a Specord M500 spectrophotometer from Carl Zeiss Jena GmbH, Jena, Germany. The spatially resolved surface modification was performed using a parallel micro-dispensing system (TopSpot) with high-performance piezostack actuation system and integrated temperature adjustment from HSG-IMIT/IMTEK, Germany.

2.2. Instrumentation

The AWACSS instrument employs fluorescence-based detection of the binding of fluorophore-tagged biomolecules to the surface of an optical waveguide chip. 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/immunochemistry is used to sensitise 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 autosampler 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 15–18 min.

Key features of the instrument, chip fabrication, chip characterisation, hardware requirements, and further details of the instrumentation are described in the first part article published in a previous issue of the same journal.

2.3. Immunochemistry

The immunochemistry utilised in the project takes advantage of a binding inhibition test that requires antibodies directed against specific analytes and analyte derivatives that can be covalently bound to a transducer surface. The previously immobilised aminodextran layer is used to reduce non-specific binding to the surface. Dried (immobilised) aminodextran layers on a glass substrate showed a thickness between one and three nm. The thickness of welled aminodextran layers (aminodextran layers on a glass substrate in contact with buffer solution after a few minutes) were also verified by spectroscopic ellipsometry experiments and these experiments yielded values between 100 and 150 nm.

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 labelled with a fluorescent marker, they were developed into immunoassays and used in the project. The sample containing the analyte is incubated in solution with the labelled specific antibody. Therefore, 100 μL of the antibody stock solution are mixed with 900 μL of the sample by an autosampler 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 immobilise a huge excess of antigen derivatives. This was demonstrated by additional reflectometric interference spectroscopy (RIfS) measurements as already described in literature (Glaser, 1993). The surface evaluation was performed with covalently immobilised peptide nucleic acid (PNA) for the detection of different endocrine disruptors by the above mentioned label-free detection method. Within these experiments, a hybridisation capacity with DNA oligonucleotides of 180 fmol mm^{-2} on PNA surfaces has been reported (Kroger et al., 2002). Other experiments to evaluate a covalent strategy for immobilisation of DNA microspots suitable for microarrays with label-free and time-resolved optical detection of hybridisation resulted in hybridisation capacities of approximately 300 fmol mm^{-2} (Jung et al., 2001). We achieved linear correlations between the increasing fluorescence signal and the antibody concentration used within TIRF experiments. The linear behavior of the fluorescence signal shows that no saturation effects can be observed even with highest antibody concentrations. Therefore, the immobilised huge excess of antigen derivatives in comparison to the used amounts of antibodies could be verified.

2.4. Detection

The AWACSS instruments are based on evanescent field technology. Laser light is coupled into an optical transducer and guided down the integrated optical (IO) chip. 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), which upon binding to the transducer surface are

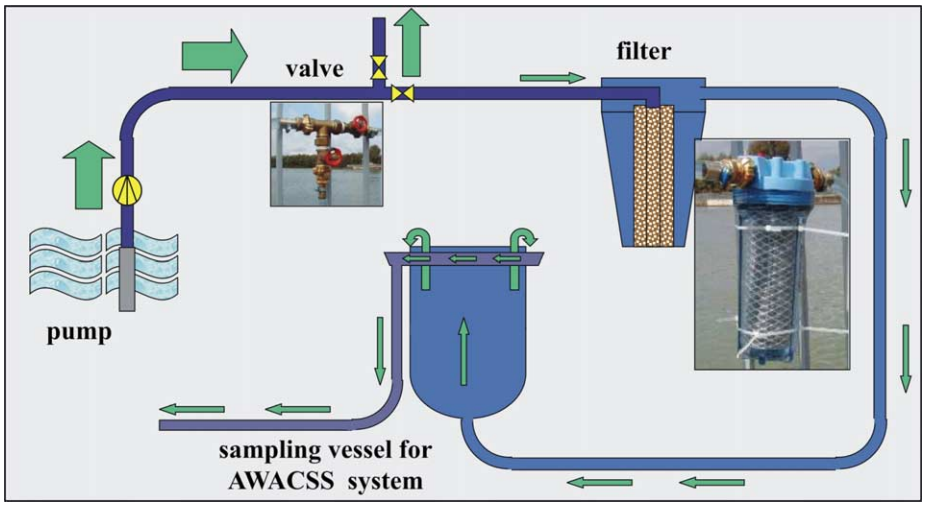


Fig. 1. Schematic set-up of the developed sampling system for on-line monitoring which allows direct intake of river water and its continuous transfer to the autosampler of the AWACSS system.

excited in the evanescent field. The emitted light is then collected for detection with 32 polymer fibres. The design allows for the simultaneous measurement of multi-analyte spots.

Analyte recognition is based on a binding inhibition assay. Analyte derivatives are immobilised onto the transducer surface prior to the assay. Next, analyte-specific antibodies labelled 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 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. Immobilisation

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 over night at room temperature. Finally, the solution was centrifuged (12,000 rpm) at approximately 4 °C and the supernatant was stored under refrigeration. 50 mg 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 over night at room temperature. A 10-fold volume excess of methanol precipitated the aminodextran conjugate. The supernatant was removed and the conjugate was freeze-dried. The IO 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 silanised 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 1.0–2.0 mg mL^{-1} and were immobilised by a parallel spotting device TopSpot from HSG-IMIT, Villingen-Schwenningen, Germany (http://www.hsg-imit.de) and IMTEK (Institute for Microsystem Technology), Fribourg, Germany (http://www.imtek.de).

2.6. Sampling

A sampling system for on-line monitoring has been developed which allows direct intake of river water and its continuous transfer to the autosampler of the AWACSS system. A schematic representation of the set-up is in Fig. 1.

2.7. Measurement

For the measurements, we used a polyclonal IgG antibody from sheep and a suitable analyte derivative. The entire sample volume was 1000 μL. For a calibration routine, 900 μL of spiked Milli-Q water was automatically mixed by the autosampler with 100 μL of an antibody stock solution containing the antibodies and ovalbumin from chicken eggs (OVA) in 10-fold phosphate buffered saline (PBS) (10-fold PBS: pH 6.8, 1500 mM sodium chloride, 100 mM potassium

phosphate monobasic). After a defined incubation time, this mixture was measured using the biosensor set-up. The experimental design for a calibration routine consists of nine independent blank (Milli-Q water) measurements and 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 (S.D.) for the replica was automatically calculated by the measurement control unit. 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 (Dudley et al., 1985) (parameters of a logistic function: A_1, A_2, x_0, and p) with three free parameters (A_2, x_0, and p) was used. A_1, as the upper asymptote was fixed to 100 % (relative signal for mean value of the blanks) 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) (Inczedy et al., 1998), the LOD is calculated as 3 times the S.D. of the blank measurements ($S.D._{blank}$) and the LOQ is calculated as 10 times the S.D. of the blank measurements ($S.D._{blank}$). All statistical procedures and further calculations are included within the AWACSS evaluation software package.

3. Results and discussion

3.1. Selection of AWACSS compounds

A set of new immunochemical reagents was developed for selected organic micro-pollutants to be analysed in environmental water samples. Major criteria for selection of target analytes were: (i) their presence in the environment; (ii) existing environmental legislation (98/83/EC, 1998; 2000/60/EC, 2000); (iii) technical possibilities to prepare sufficiently selective polyclonal antibodies and their corresponding analyte derivatives; and (iv) marketing considerations. More than 20 different polyclonal antibodies were isolated within the project and their corresponding analyte derivatives were synthesised. A priority was given to classes of pesticides, endocrine disrupting compounds, WFD Priority Substances, industrial pollutants and pharmaceuticals (for a list of AWACSS compounds, see Table 1).

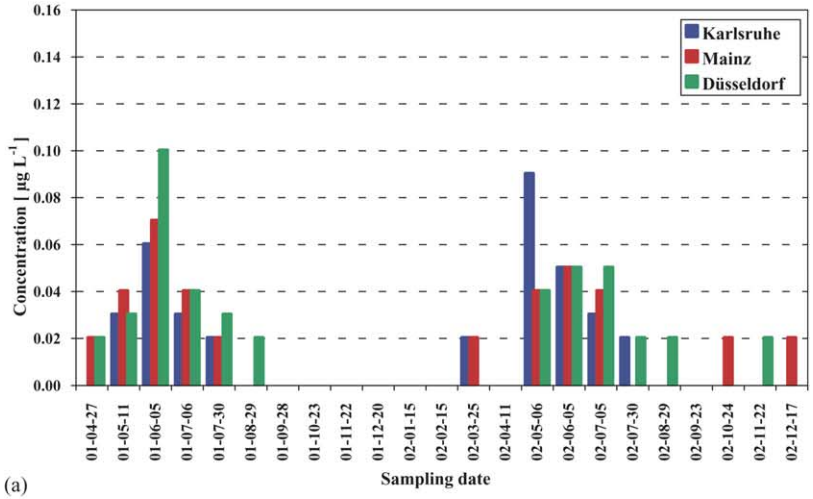
3.2. Regular monitoring of AWACSS compounds in European rivers

Sampling sites: Four project partners (IIQAB, Department of Environmental Chemistry, CID-CSIC, Barcelona, Spain; DVGW-Technologiezentrum Wasser, Karlsruhe, Germany; Environmental Institute, Kos, Slovak Republic; Water Research Institute, Bratislava, Slovak Republic) of the water-monitoring group have systematically investigated various

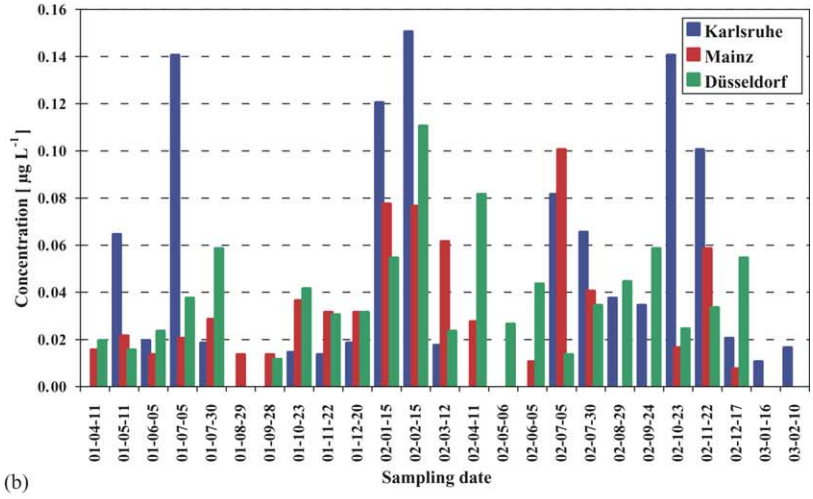
Table 1
A list of AWACSS compounds monitored in the surface water, ground water, municipal/industrial waste water and sediment samples within the years 2001–2003 with the number of positive findings and their concentration ranges

Compound[a]	No.[b]	Concentration[c]	Matrix[d]	Comment[e]
Alachlor	1	0.11	SED	SK, WFD
Pyrene	115	0.05–633	SED	SK
Benzo[a]pyrene	73	0.15–36.94	SED	SK, WFD
Fluorene	51	0.09–267	SED	SK
Fluoranthene	118	0.02–717	SED	SK, WFD
DEHP	116	1–2115	SED	SK, WFD
Bisphenol A	49	0.06–35.19	SW, GW, WW	SK
Nonylphenol	5	0.35–87.57	SW, GW, WW	SK, WFD
Benzene	11	0.6–177.5	SW, GW, WW	SK, WFD
Toluene	9	1.1–447.3	SW, GW, WW	SK
Xylene	7	1.0–31.9	SW, GW, WW	SK
Trichloroetylene	19	0.1–22852	SW, GW, WW	SK
Atrazine	21	0.2–4.46	SW, GW, WW	SK, WFD
Prometryn	4	0.13–1252	SW, GW, WW	SK
Ametryn	1	0.22	SW, GW, WW	SK
Terbuthylazine	1	0.14	SW, GW, WW	SK
Simazine	2	0.1–0.5	SW, GW, WW	SK, WFD
Benzenesulfonamide	3	0.42–4.86	SW, GW, WW	SK
Caffeine	15	1.3–112	SW, GW, WW	SK

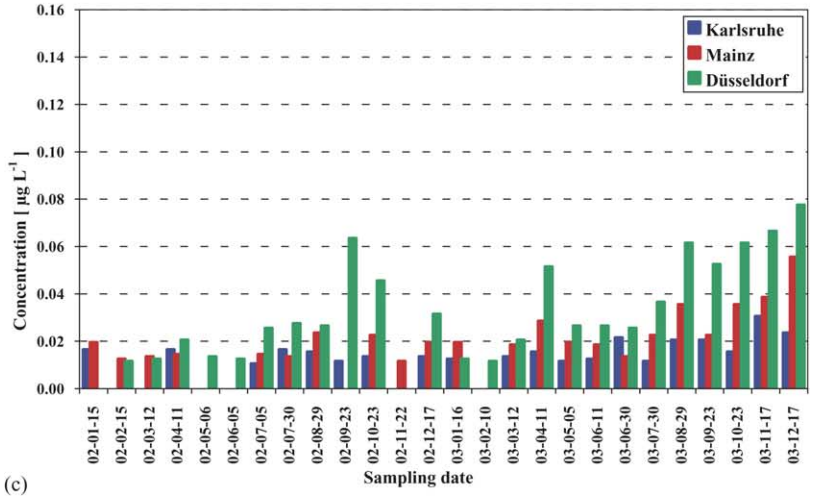
[a] Antibody and analyte derivative(s) developed or in the process of preparation.
[b] Number of positive findings.
[c] Concentration range in mg kg^{-1} for sediment samples (SED) and in $\mu g\,L^{-1}$ for water samples (SW, GW, and WW).
[d] SED: sediment; SW: surface water; GW: ground water; WW: waste water.
[e] SK: Slovak Republic; WFD: compound present on the list of Water Framework Directive Priority Substances.



(a)



(b)



(c)

Fig. 2. Characteristic pollution patterns by atrazine (a), bisphenol A (b), and sulphamethoxazole (c) at the River Rhine (Germany) by GC–MS. Sampling sites: Mainz, Karlsruhe, and Duesseldorf. Results were obtained in the period 2001–2003.

Table 2
Calculated relative signal values with standard deviations for all analytes and concentration steps

Concentration ($\mu g\,L^{-1}$)	Propanil	Atrazine	Isoproturon	Sulphamethizole	Bisphenol A	Estrone
0	100.00 ± 1.91	100.00 ± 2.37	100.00 ± 2.04	100.00 ± 2.79	100.00 ± 0.93	100.00 ± 0.56
0.009	96.43 ± 2.02	92.15 ± 2.19	96.45 ± 0.83	95.55 ± 2.23	98.01 ± 2.33	95.59 ± 2.04
0.027	92.87 ± 3.38	89.66 ± 3.27	92.05 ± 3.96	90.49 ± 2.24	95.84 ± 0.87	91.62 ± 2.48
0.09	75.69 ± 1.83	71.96 ± 3.49	70.47 ± 3.48	80.13 ± 1.80	86.75 ± 2.27	56.93 ± 2.48
0.27	49.85 ± 1.77	54.96 ± 3.69	40.62 ± 2.23	70.13 ± 1.32	81.76 ± 2.22	32.34 ± 1.58
0.9	26.95 ± 1.35	41.15 ± 1.53	18.24 ± 0.57	58.67 ± 0.10	70.34 ± 2.55	21.69 ± 0.41
2.7	17.44 ± 0.36	32.78 ± 1.86	12.52 ± 0.27	52.73 ± 1.06	58.67 ± 1.54	18.65 ± 0.46
9	12.41 ± 0.61	27.41 ± 0.98	9.85 ± 0.26	45.70 ± 1.14	49.31 ± 2.25	16.53 ± 1.00
27	9.39 ± 0.81	22.58 ± 0.91	8.24 ± 0.51	39.59 ± 0.88	40.25 ± 1.39	15.64 ± 1.56
90	7.87 ± 0.68	19.15 ± 0.63	7.10 ± 0.24	33.91 ± 0.92	34.22 ± 1.64	14.63 ± 0.23
A_1 (%)	100	100	100	100	100	100
A_2 (%)	9.34 ± 0.87	20.66 ± 1.65	8.39 ± 0.52	32.88 ± 2.41	28.10 ± 2.45	16.72 ± 1.05
x_0 ($\mu g\,L^{-1}$)	0.23 ± 0.01	0.22 ± 0.02	0.17 ± 0.01	0.47 ± 0.10	1.70 ± 0.29	0.09 ± 0.01
p	1.05 ± 0.04	0.74 ± 0.05	1.23 ± 0.04	0.57 ± 0.05	0.58 ± 0. 04	1.46 ± 0.12
LOD ($\mu g\,L^{-1}$)	0.019	0.010	0.020	0.018	0.008	0.007

Resulting parameters (with standard deviations) for the determined logistic fit functions and the validation parameter LOD for all analytes.

types of water around Europe in order to select the analyte panel applicable to real-world situations and future monitoring sites for the AWACSS system. Also, water matrix and cross-reactivity effects related to the AWACSS immunoassay chemistry were addressed.

In Spain, sampling sites at the Llobregat River, supplying drinking water to the city of Barcelona, two of its tributaries, the Ebro River and the Mediterranean Sea were regularly monitored by RIANA and LC–MS techniques. The occurrence of target analytes was monitored also in the ground water serving as a source for abstraction of drinking water. A removal efficiency of the detected pollutants was studied at the successive water treatment steps applied in the Sant Joan Despi waterworks.

The AWACSS monitoring network in Germany consisted of three sampling sites alongside the River Rhine. Among the techniques applied for monitoring were GC–MS and LC–DAD UV. Typical examples of pollution patterns by selected analytes over the years 2001–2003 are given in Fig. 2a–c. It is shown on the example of atrazine (Fig. 2a) that pesticide monitoring strongly depends on the seasonal variations, while pollution by industrial chemicals (bisphenol A, Fig. 2b) and pharmaceuticals (sulphamethoxazole, Fig. 2c) is relatively constant.

Slovak partners monitored four sampling sites on the Nitra River: Nedozery, a reference site upstream of major pollution sources; Prievidza, at the outlet of the municipal waste water treatment plant; Novaky, at the outlet of a discharge channel of large chemical industry and Chalmova, approximately 6 km downstream from the Novaky Chemical Plant. Both GC–MS screening and target analysis of AWACSS analytes using GC–MS, HPLC–DAD UV and ELISA were performed on a regular basis. Occurrence of AWACSS analytes was evaluated also within the National screening programmes of organic pollutants and pesticides in the Slovak Republic within the period 2001–2003. Concentrations ranges of AWACSS compounds detected in real-world samples are summarised in Table 1.

3.3. Development and testing of a sampling device

A sampling system was designed and realised that enables the continuous transfer of water from a river (or any other water source) to the autosampler of the AWACSS system (Fig. 1). The system included a pumping system, a valve for controlling the flow rates and the portion of water that is provided to the AWACSS system and a filter unit which allows the filtration of the water sample with a mesh size of one micrometer. By exchanging the (commercially available) filter cartridge, other filters with different pore sizes could be used.

In a sampling experiment at River Rhine, it could be shown that with this experimental set-up, a life-time of 3 months could be achieved before an exchange of the filter cartridge becomes necessary because a blocking of the filter occurs, assuming that the average turbidity of the river water is 15 FNU (for River Rhine most often it is lower) and that a flow rate of 1 L h^{-1} through the sampling vessel is applied. With this flow rate, the water in a 250 mL vessel is completely exchanged every 15 min.

3.4. Studies on the performance of the biosensor

3.4.1. Multi-analyte measurements

Feasibility studies on multi-analyte measurements were performed with the RIANA system for a mixture of atrazine, isoproturon and estrone. The results showed that sensitivity and selectivity of individual analytes remained unchanged compared to measurements of individual compounds. A good agreement between the results obtained by biosensor technique and the on-line SPE–LC–MS method were obtained for each analyte (Rodriguez-Mozaz and Reder, 2004). Follow-up

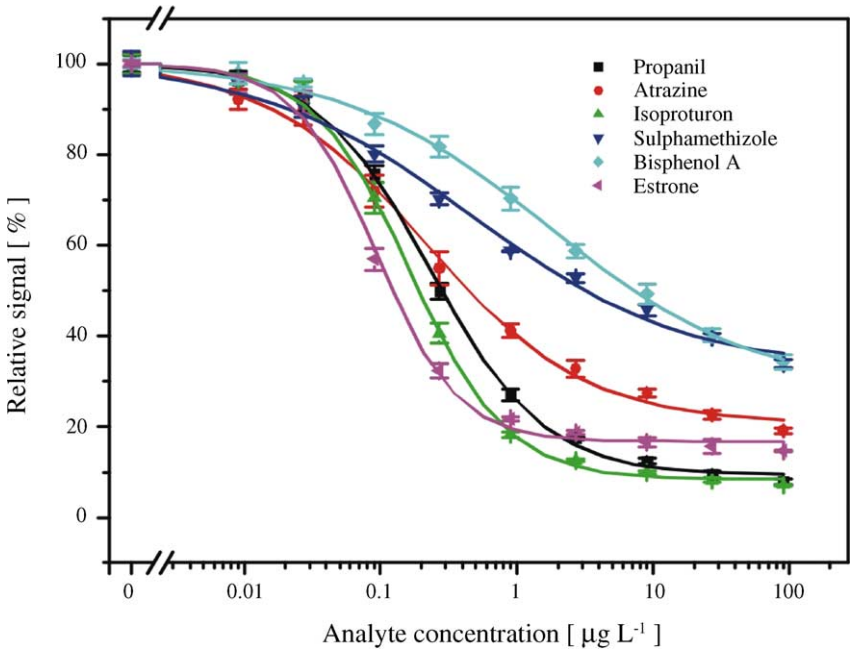


Fig. 3. The resulting set of calibration curves for atrazine, bisphenol A, estrone, isoproturon, sulphamethizole, and propanil which were measured in parallel on a multi-analyte transducer. For all compounds, the calculated LOD is below 0.020 $\mu g\,L^{-1}$.

determinations by HPLC–DAD UV method showed a small positive bias towards higher values obtained by the immunosensor for most of the tested analytes. In the final stage of the project, the multi-analyte determination capability of AWACSS was successfully tested with a mixture of six analytes. The AWACSS IO chip was modified with derivatives of atrazine, bisphenol A, estrone, isoproturon, sulphamethizole, and propanil according to the previously described immobilisation protocol. Then, a simultaneous calibration from 0 to 90 $\mu g\,L^{-1}$ analyte concentration in Milli-Q water with mixed analytes and an antibody stock solution containing the six corresponding polyclonal antibodies (anti-atrazine, anti-bisphenol A, anti-estrone, anti-isoproturon, anti-mixed sulphonamides, and anti-propanil) was performed. The resulting set of calibration curves is shown in Fig. 3. For all compounds, the calculated LOD is below 0.020 $\mu g\,L^{-1}$ and all validation parameters, calculated relative signals, and standard deviations are summarised in Table 2.

This multi-analyte calibration demonstrated the possibility to quantify pesticides from three different classes (triazines, phenylurea herbicides and anilides), endocrine disrupting compounds (bisphenol A), steroid hormones (estrone), and pharmaceuticals (sulphamethizole) within one single measurement cycle, which only takes approximately 18 min. No cross-reactivity effect was observed for any of the tested analytes.

3.4.2. Matrix effects

As expected from the mode of antibody action, matrix effects were observed for different types of samples. The effects were studied in various types of water matrices, including Milli-Q, ground, surface and mixed municipal/industrial waste water. Typical changes in profiles of calibration curves are shown for estrone by Rodriguez-Mozaz and Reder (2004), where the most pronounced effect was observed in the waste water sample. It has been concluded that at analysis of such complex samples a care should be taken to establish calibration curves in similar matrix. Several strategies were proposed and tested to avoid the matrix effects: (i) adjustment of ionic strength and pH of the sample; (ii) dilution of the sample; (iii) sample clean-up; and/or (iv) enrichment of the sample on SPE column and reconstitution of the extract.

One of the major advantages of biosensors compared to conventional techniques is their ability to analyse selectively target analytes in complex matrices. Therefore, a protocol was developed allowing for analysis of solid matrices such as sediments and food samples. In the procedure one gram of solid sample is extracted in ultrasonic bath with 10 mL acetonitrile or methanol, the upper layer of extract (5 mL) is diluted in Milli-Q water in a way to reduce concentration of organic solvent to 2% and further treated/analysed as a liquid sample. Feasibility of the approach was proven in the inter-laboratory study comparing results obtained by online SPE–LC–DAD UV system and ELISA for atrazine and alachlor spiked into the Nitra River sediments and baby food (strawberry) samples at 50 and 500 $ng\,g^{-1}$ levels. Recoveries rates of both analytes ranged from 89 to 108%.

A separate study on the effects of residual amounts of organic solvents acetonitrile and methanol on the immunoassay performance was conducted with ELISA for several antibodies by Abuknesha and Griffith (submitted for publication), Abuknesha et al. (submitted for publication) and Abuknesha and Luk (submitted for publication).

Table 3
Results obtained by AWACSS system within the collaborative trial

Compound	Assigned value ($\mu g\,L^{-1}$)[a]	Milli-Q water matrix			Assigned value ($ng\,g^{-1}$)[a,b]	Sediment matrix		
		Mean ($\mu g\,L^{-1}$)	Mean (%)	CV (%)[c]		Mean ($ng\,g^{-1}$)	Mean (%)	CV (%)[c]
Atrazine								
Level 1	0.095	0.11	116	9.6	45	49.0	109	5.0
Level 2	0.950	1.12	118	8.3	450	384	85	3.6
Bisphenol A								
Level 1	0.097	0.08	82	4.0	44.5	51.7	116	27.8
Level 2	0.974	1.25	128	16.9	445	423	95	7.5
Estrone								
Level 1	0.076	0.08	105	7.0	36	25.4	71	3.2
Level 2	0.763	1.04	136	4.4	360	333	93	10.7

Milli-Q water and sediment samples were spiked at 0.1 and 1.0 $\mu g\,L^{-1}$ (water samples) and 50 and 500 $ng\,g^{-1}$ (sediment samples), respectively.
[a] Corrected for recoveries of analytes on the SPE cartridges and blank measurements.
[b] Corrected for extraction recoveries from sediments.
[c] Calculated from three measurements.

3.5. Comparison of AWACSS performance to conventional analytical techniques

The overall performance of the AWACSS system in comparison to the conventional analytical and immunosensor techniques was tested in the inter-laboratory collaborative trial among the project partners. In total, eleven different analytical set-ups were used in six laboratories, among them four automated on-line SPE–LC–DAD UV systems (Slobodník et al., 1993), on-line SPE–LC–FLD, on-line SPE–LC–MS (Slobodník and Brinkman, 2000), off-line SPE/LVI–GC–MS (Korenková et al., 2001), two RIANA prototypes (Tschmelak et al., 2004a, 2004b, 2004c, 2004d, 2004e, 2004f), ELISA and AWACSS. The tested matrices were Milli-Q water and freeze-dried 63 μm fractions of river sediments from the Nitra River. Each of them was spiked with three analytes: (1) atrazine as a representative of pesticide class, being also on the list of WFD Priority Substances; (2) bisphenol A—industrial pollutant known as an endocrine disrupting compound; and (3) estrone—hormone with endocrine disrupting effects, often present in outlets of municipal waste water treatment plants. Spiking levels were 0.1 and 1.0 $\mu g\,L^{-1}$ in water matrices and 50 and 500 $ng\,g^{-1}$ in sediment. In order to prevent decomposition of analytes during transport, water samples were directly loaded onto small 10 mm × 2.0 mm i.d. SPE cartridges packed with polymeric sorbent, which fitted into the automated sample preparation device PROSPEKT available in all partner's laboratories. Sediment samples were first extracted by ultrasonic extraction into acetonitrile, extract diluted in Milli-Q water and concentrated on the same cartridges. Each sample has been prepared in triplicate and sets of cartridges, including blanks, were distributed among the partners. Cartridges were measured either directly by techniques using on-line SPE-LC set-ups (Brinkman et al., 1994) or eluted by 1 mL acetonitrile, which was then reconstituted into 20 mL volume by Milli-Q water (resulting in an acetonitrile concentration of 2%) for analysis by RIANA, ELISA and off-line SPE/GC–MS. For more details on the procedures, the reader is addressed to a reference (Slobodník et al., 2004).

The results showed that, in terms of accuracy, AWACSS performance in Milli-Q water and sediment samples is fully comparable to conventional chromatography-based techniques (see Tables 3 and 4). Recoveries of atrazine and bisphenol A ranged from 82 to 126% compared to the assigned values, while those for estrone were between 71 and 136%. In general, the AWACSS results were less biased towards higher values in comparison to ELISA and RIANA immunosensor techniques. An evaluation of the collaborative trial using the *Z*-score methodology as an expression of deviation of the measured from assigned value showed that none of the results obtained by AWACSS would be excluded from the evaluation. Here, it should be mentioned that analysis of reconstituted sediment extracts by on-line SPE–LC–DAD UV techniques was possible only by using time-consuming mathematical deconvolution and/or subtraction of the blank signals. As regards the reproducibility, all results by AWACSS had a coefficient of variation lower than 17% (Table 3), the only exception being bisphenol A at the lower spiking level in sediment (27.8%). The results were well within the range obtained by both chromatography-based and other

Table 4
Comparison of atrazine determinations in Milli-Q water obtained by conventional liquid chromatography-based analytical techniques, ELISA, the immunosensor RIANA, and the AWACSS instrument

	Mean ($\mu g\,L^{-1}$)	CV (%)
Conventional methods		
SPE–HPLC–DAD UV (Lab 1)	0.11	14.3
SPE–HPLC–DAD UV (Lab 2)	0.14	3.5
SPE–HPLC–DAD UV (Lab 3)	0.07	20.8
Immunochemistry methods		
RIANA	0.16	24.1
AWACSS	0.11	9.6
ELISA	0.14	3.6

Spiked concentration: 0.1 $\mu g\,L^{-1}$; number of measurements = 3.

immunosensor techniques (for an example of Milli-Q water, see Table 4).

4. Conclusions

Two prototypes of the AWACSS system were designed and constructed within the project and over 20 antibodies and their analyte derivatives were developed. Analytical performance of AWACSS systems was tested in comparison to a wide range of conventional chromatography-based analytical techniques, such as on-line SPE–LC–DAD UV, on-line SPE–LC–MS and GC–MS systems. Feasibility studies on the multi-analyte analysis and/or matrix effects were conducted with immunosensor techniques RIANA and ELISA. Among the tested matrices were surface, ground, drinking and waste water and, using special sample preparation protocol, sediment samples. The results showed that AWACSS is fully comparable to conventional analytical techniques in terms of accuracy, repeatability and reproducibility. Detection limits of all tested analytes were in the low nanogram per litre range, while selectivity allowed for trace analysis even in complex matrices such as sediment extracts. Time of a single analytical run was less than 18 min and during the system validation more than 70 analyses were performed within a day in a fully automated regime. Four water-monitoring groups from the project team were extensively assessing various fields of potential application of the system and measurement sites that could accommodate the final instruments for testing. The fact that monitoring of many of the AWACSS compounds is required by the present environmental EU legislation (98/83/EC, 1998; 2000/60/EC, 2000) and that most of the compounds are being frequently detected in real water samples all over Europe gives good perspectives to the system to be placed among the current state-of-the-art analytical instruments.

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References

98/83/EC, 1998. Council Directive (98/83/EC) of 3 November 1998 relating to the quality of water intended for human consumption. Off. J. Eur. Commun. L330, 32–54.

2000/60/EC, 2000. 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. Off. J. Eur. Commun. L327, 1–72.

Abuknesha, R.A., Griffith, H.M.T. A broad specificity antiserum to steroid estrogens generated with 17β-estradiol-17-*O*-hemiglutarate-KLH immunogen: assessment of binding properties and use to develop an immunoassay for total natural steroid estrogens and ethinyl estradiol in water. Anal. Bioanal. Chem., submitted for publication.

Abuknesha, R.A., Griffith, H.M.T., Ossowska, K. A highly sensitive and specific ELISA for estrone and use in the analysis of surface water samples. Anal. Bioanal. Chem., submitted for publication.

Abuknesha, R.A., Luk, C. Generation and assessment of a polyclonal antiserum to a mixture of six sulfonamides: potential use of a mixed-specificity antiserum in screening for pluralities of sulfonamide residues. Anal. Bioanal. Chem., submitted for publication.

Barceló, D., Hennion, M.C., 1997a. Trace Determination of Pesticides and Their Degradation Products in Water. Elsevier.

Barceló, D., Hennion, M.C., 1997b. Sampling of polar pesticides from water matrices. Anal. Chim. Acta 338 (1–3), 3–18.

Barceló, D. (Ed.), 2000. Environmental Analysis: Sample Handling and Trace Analysis of Pollutants—Techniques, Applications and Quality Assurance. Elsevier.

Brinkman, U.A.Th., Slobodník, J., Vreuls, J.J., 1994. Trace-level detection and identification of polar pesticides in surface water: the SAMOS approach. Trends Anal. Chem. 13 (9), 373–381.

Clement, R.E., Yang, P.W., Koester, C.J., 1997. Environmental analysis. Anal. Chem. 69 (12), 251–288.

Dudley, R.A., Edwards, P., Ekins, R.P., Finney, D.J., McKenzie, I.G., Raab, G.M., Rodbard, D., Rodgers, R.P., 1985. Guidelines for imunnoassay data processing. Clin. Chem. 31 (8), 1264–1271.

Glaser, R.W., 1993. Antigen-antibody binding and mass transport by convection and diffusion to a surface: a two-dimensional computer model of binding and dissociation kinetics. Anal. Biochem. 213 (1), 152–161.

Inczedy, J., Lengyel, T., Ure, A.M., 1998. Compendium of Analytical Nomenclature—The Orange Book, third ed. Blackwell Science.

Jung, A., Stemmler, I., Brecht, A., Gauglitz, G., 2001. Covalent strategy for immobilization of DNA-microspots suitable for microarrays with label-free and time-resolved optical detection of hybridization. Fresenius J. Anal. Chem. 371 (2), 128–136.

Korenková, E., Matisová, E., Slobodník, J., 2001. Large volume injection in capillary gas chromatography. Chemicke Listy 95 (9), 528–539.

Kroger, K., Jung, A., Reder, S., Gauglitz, G., 2002. Versatile biosensor surface based on peptide nucleic acid with label free and total internal reflection fluorescence detection for quantification of endocrine disruptors. Anal. Chim. Acta 469 (1), 37–48.

Oubina, A., Ballesteros, B., Carrasco, P.B., Galve, R., Gascon, J., Iglesias, F., Sanvincens, N., Marco, M.-P., 2000. Immunoassays for environmental analysis. In: Barceló, D. (Ed.), Environmental Analysis: Sample Handling and Trace Analysis of Pollutants—Techniques, Applications and Quality Assurance. Elsevier, pp. 287–339.

Rodriguez-Mozaz, S., Reder, S., López de Alda, M., Gauglitz, G., Barceló, D., 2004. Simultaneous multi-analyte determination of estrone, isoproturon and atrazine in natural waters by the RIver ANAlyser (RIANA), an optical immunosensor. Biosens. Bioelectron. 19 (7), 633–640.

Rowe Taitt, C., Anderson, G.P., Lingerfelt, B.M., Feldstein, M.J., Ligler, F.S., 2002. Nine-analyte detection using an array-based biosensor. Anal. Chem. 74 (23), 6114–6120.

Slobodník, J., Brinkman, U.A.Th., 2000. LC/MS interfacing systems in environmental analysis: applications to polar pesticides. In: Barceló, D. (Ed.), Environmental Analysis: Sample Handling and Trace Anal-

ysis of Pollutants—Techniques, Applications and Quality Assurance. Elsevier, pp. 935–1001.

Slobodník, J., Groenewegen, M.G.M., Brouwer, E.R., Lingeman, H., Brinkman, U.A.Th., 1993. Fully automated multi-residue method for trace-level monitoring of polar pesticides by liquid chromatography. J. Chromatogr. 642 (12), 359–370.

Slobodník, J., Oswald, P., Kozmenko, H., Korenková, E., Proll, G., Tschmelak, J., Sacher, F., Stien, J., Rodriguez-Mozaz, S., Tóthová, L., Krascsenits, T., 2004. Analysis of environmental pollutants in water and sediment samples by the multi-analyte biosensor AWACSS: comparison of performance to conventional analytical techniques. Manuscript in preparation.

Tschmelak, J., Kumpf, M., Proll, G., Gauglitz, G., 2004a. Biosensor for seven sulphonamides in drinking, ground, and surface water with difficult matrices. Anal. Lett. 37 (8), 1701–1718.

Tschmelak, J., Proll, G., Gauglitz, G., 2004b. Immunosensor for estrone with an equal limit of detection as common analytical methods. Anal. Bioanal. Chem. 378 (3), 744–745.

Tschmelak, J., Proll, G., Gauglitz, G., 2004c. Sub-nanogram per litre detection of the emerging contaminant progesterone with a fully automated immunosensor based on evanescent field techniques. Anal. Chim. Acta, doi:10.1016/j.aca.2004.06.031.

Tschmelak, J., Proll, G., Gauglitz, G., 2004d. Ultra-sensitive fully automated immunoassay for detection of propanil in aqueous samples: steps of progress toward sub-nanogram per liter detection. Anal. Bioanal. Chem., doi:10.1007/s00216-004-2695-x.

Tschmelak, J., Proll, G., Gauglitz, G., 2004e. Verification of performance with the automated direct optical TIRF immunosensor (River Analyser) in single and multi-analyte assays with real water samples. Biosens. Bioelectron. 20 (4), 743–752.

Tschmelak, J., Proll, G., Gauglitz, G., 2004f. Immunosensor for estrone with an equal limit of detection as common analytical methods. Anal. Bioanal. Chem. 378 (3), 744–745.

World Health Organization, 2003. Guidelines for Drinking Water Quality, third ed. World Health Organization, Geneva, Switzerland.