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Environmental Biosensors for Organochlorines, Cyanobacterial Toxins and Endocrine Disrupting Chemicals

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Abstract Environmental biosensors and related techniques for monitoring organochlorines, endocrine disrupting chemicals and cyanobacterial toxins are described. The practical requirements for an ideal environmental biosensor are analyzed. Specific case studies for environmental applications are reported for triazines, chlorinated phenols, PCBs, microcystins, and endocrine disrupting chemicals. A new promising approach is reported for microcystins and alkylphenols that utilize electrooptical detection.

Keywords: innovative technologies, rapid measurements, immunosensors, microcystins, multiarray sensors

INTRODUCTION

Very few analytical methods for environmental monitoring that are fast, low-cost and continuous are currently available. The monitoring of residue or contamination in soil, water and air can be classified into two main categories. These are: (i) screening or diagnostic techniques in which only a yes-or-no (qualitative) answer is required, and (ii) semi-quantitative or quantitative techniques in which the detection of unwanted chemicals, and the testing of whether or not the residues of the contaminants are within permissible levels are required. It is possible for the former methods to generate false positive or negative results if the sensitivities are insufficient for the detection of the threshold levels.

The successful coupling of suitable transducers (*e.g.* electrochemical, optical or mass) with biomolecular systems is of great importance in the search for novel sensing technologies that are inexpensive, highly selective and capable of generating early warning signals in the presence of toxic environmental chemicals. The emergence of on-site chemical and immunochemical biosensors for environmental pollutant monitoring has tremendous potentials due to their small size, low costs and the ease of analytical signal generation in real time [1-3]. These sensors represent a step forward over the conventional laboratory analytical methods.

In recent years, biosensors that can detect a range of analytes in environmental samples have been developed [4,5]. Basically, a sensor consists of a chemically selective layer, a transducer, and a signal processor. If the selective layer utilizes a biological or biochemical species, then it can be classified as a biosensor. Thus, an immunosensor is a subset of biosensor since it comprises of either an antibody or an antigen. Each sensor has a number of desirable characteristics depending on its applications. Essentially, a practical biosensor for the monitoring of environmental pollutants must be specific, reversible, able to provide fast response time, and capable of direct detection of an immunoreaction with minimal sequential addition of immunoreagents. Also, the sensor may be capable of continuous flow measurements and suitable for determining multiple analytes in complex samples with little or no need for sample preparation steps. Finally, the sensor must be able to process signals, or capable of being integrated into other devices that can exercise real-time feedback as required for pollution monitoring or surveillance studies. Although, a number of pollutant measurement techniques have been reported, only few possess these specific requirements. One of the major objectives of our research is to develop field-portable sensors that meet or exceed the above sensor requirements for use in the assessment of toxic chemical residues in various environmental media. This paper summarizes chemical and biosensors developed in our laboratory for pesticides, chlorinated organics, endocrine disruptors and toxins.

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MATERIALS AND METHODS

Reagents and Stock Solutions

Cystamine dihydrochloride (2,2'-diaminodiethyldisulfide), 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC), and HEPES (*N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid], microcystin-LR, cysteine, 4-methylumbelliferyl phosphate, 3-aminopropyltriethoxysilane (APTS), glutaraldehyde (GA, 25% solution in

water), 2-chlorophenol (2-CP), 2,4,6-trichlorophenol (2,4,6-TCP), bovine serum albumin (BSA) and okadaic acid were purchased from Sigma. Protein phosphatase PP1, purified from rabbit skeletal muscle diluted in 50% glycerol, 20 mM 4-morpholinepropanesulfonic acid (pH 7.5), 60 mM 2-mercaptoethanol, 0.15 M NaCl, 0.1 mM $MnCl_2$, 1 mM $MgCl_2$, 2 mM EGTA, and 0.1 mg/ml serum albumin as well as the phosphatase assay buffer (50 mM Tris-HCl, 0.1 mM $CaCl_2$, pH 7.0), were obtained from UBI (Upstate Biotechnology, Inc., New York, USA). Cyanazine hapten (Mw 310 g/mol) was a gift from Dr. James Fleeker, North Dakota State University. Potassium phosphate (dibasic and monobasic) were from Fisher Scientific Company. Potassium ferrocyanide, $K_4Fe(CN)_6$ was from ACE Scientific Supply Co. Anti-cyanazine antibody solutions were made in phosphate-buffered saline containing 0.05% Tween 20 (PBST), pH 7.2. Deionized water was used for the preparation of all aqueous solutions.

Instrumentation

The instrumentation used for electrochemical experiments consists of an EG&G PAR potentiostat/Galvanostat Model 263A equipped with an EG&G 270 software. Cyclic voltammetry measurements were carried out with a Ag/AgCl reference electrode, platinum wire counter electrode and gold (A = 0.2 cm^2) as working electrode. For fluorescence measurements, solution emission and excitation spectra were recorded at 293 K with an SLM 48000S lifetime fluorescence spectrophotometer equipped with a red sensitive Hamamatsu R928 photomultiplier tube. Emission maxima were reproducible to within 2 nm. A SF2000 Miniature Fiber Optic Spectrophotometer (Ocean Optics ™) was utilized for the optic fiber measurement. A light-emitting diode (LS-450 Blue LED) which produces either pulsed or continuous spectral output at 470 nm (the blue region) for fluorescence measurements is integrated with this spectrometer. Data was collected with Windows-based software (OIFOXY™). Aroma Scan Model A32S (AromaScan, Inc., NH, USA) was used for the multiarray electronic nose experiments.

Sensor Preparation and Characterization

Electrochemical Immobilization: The Au electrode was pretreated by boiling in 2 M KOH solution for two hours. Immediately before any hapten immobilization the electrode was thoroughly rinsed with water, soaked in concentrated nitric acid for 10 min and rinsed again. The electrode was then incubated in 0.02 M cystamine aqueous solution for 2 h in room temperature. The electrode was rinsed with water and incubated in a solution containing 3 mM cyanazine hapten and 10 mM EDC in 0.01 M HEPES buffer, pH 7.3 for 3 h at room temperature after which it was rinsed with water. The modified electrode was used in the electrochemical analysis, first without soaking in an antibody solution. Later the electrode was incubated in an anti-cyanazine antibody solution at 35°C using a thermostated water-bath. All cyclic voltammetry experiments were conducted at the same temperature. The other electrochemical immobilization procedures were as recently reported [6,7].

Antibody Production: The design and preparation of analyte analogs and immunogens are essential steps in the development of low molecular weight immunosensors. Triazine analogs were obtained from Dr. Fleeker's laboratory. These were prepared from active esters of the carboxylic acid analog of the triazine haptens using *N*-hydroxysuccinamide [8]. The triazines were coupled to a high molecular weight carrier, bovine serum albumin (BSA), or keyhole limpet hemocyanin (KLH) which were used for the production of the antibodies. The antibodies were purified by gel filtration and protein-A immunoaffinity columns, and were subsequently characterized using ELISA and nuclear magnetic resonance (NMR) techniques. By using these antibodies, sensors for *s*-triazine were developed based on the antibody inhibition of the current generated by the ferrocyanide mediator on antigen-immobilized gold electrodes.

RESULTS AND DISCUSSION

Pesticide Immunosensors

Pesticides and herbicides are routinely used to improve crop harvesting and pest-control. Due to the growing concern about health effects, several investigations have been conducted in order to understand how pesticides and herbicides degrade in the environment [9, 10]. Current methods of monitoring pesticides include liquid chromatography and gas chromatography with mass spectrometry. The high costs and labor involved in the chromatographic methods have led to the search for low-cost alternatives capable of providing rapid analysis. We report on the development of immunosensors for atrazine, cyanazine, simazine and their metabolites. The sensor chemistries are shown in Fig. 1.

The hapten monolayer electrode was used in the detection of cyanazine using cyclic voltammetry technique. The interaction of the electrode with different antibody concentrations resulted in the formation of an antibody-antigen (*Ab-Ag*) complex which insulated the electrode towards the $[Fe(CN)_6]^{4-}/Fe(CN)_6]^{3-}$ redox probe, hence resulting in less or no charge transfer.

The extent of the insulation depended on the antibody concentration and the time of exposure to the antibody solution. Typical voltammetric responses obtained for the cyanazine hapten monolayer electrode before and after exposure to 5 μL/mL antibody solution are shown in Fig. 2(a) and (b), respectively. The decrease in the amperometric response of the antigenic monolayer to the corresponding antibody solution for a fixed time produced a quantitative measurement of the antibody concentration (Fig. 3). The lowest detection limit achieved for the cyanazine sensor was 4.0 μg/mL with a response time of a few minutes and a less-than 2%

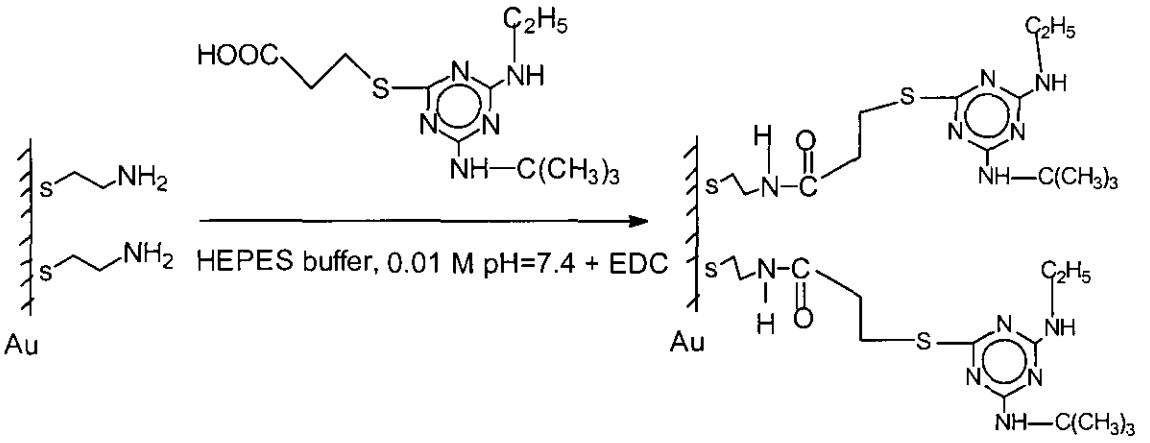

Fig. 1. The assembly of a cyanazine hapten monolayer on gold electrode.

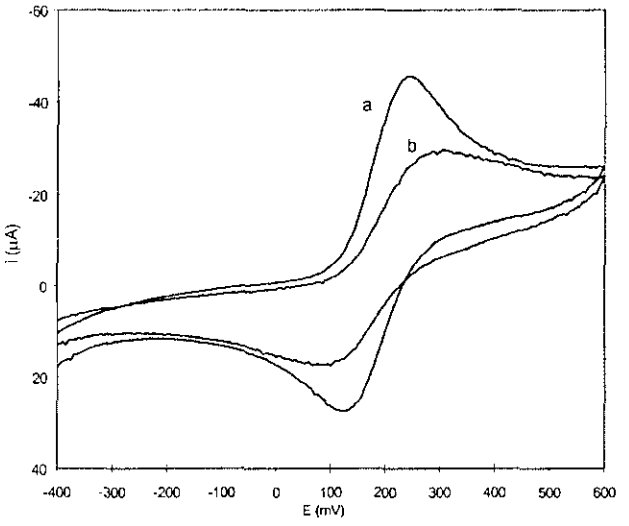

Fig. 2. Cyclic voltammograms of 1.1 mM $K_4Fe(CN)_6$ in phosphate buffer using Au-cyanazine hapten monolayer electrode a) before and b) after exposure to 5 μL/mL anti-cyanazine for 5 min.

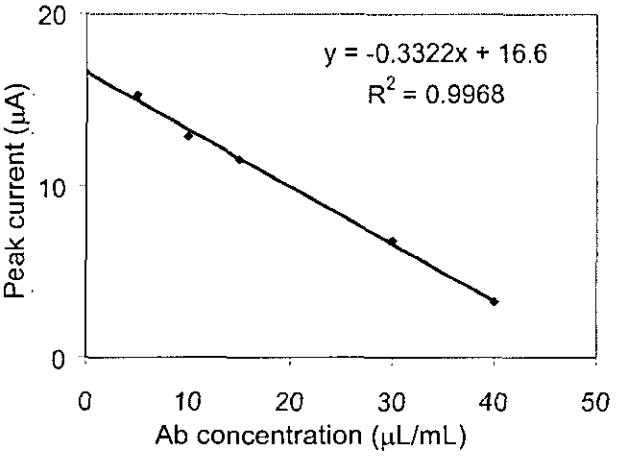

Fig. 3. Plot of cathodic peak current as a function of anti-cyanazine concentration. Peak current was measured for 1.1 mM $K_4Fe(CN)_6$ in phosphate buffer at 35°C using Au-cyanazine hapten monolayer electrode exposed to different concentrations of antibodies (5, 10, 15, 30 and 40 μL/mL) for 5 min each.

cross-reactivity to atrazine, simazine and other metabolites.

Multiarray Sensors for Organochlorines

The presence of halogenated organic compounds in the environment is a great concern due to their persistent toxicity and the ability to bioaccumulate. A 32-array conducting polymer sensor was used for the rapid measurement of volatile and semivolatile halogenated organic compounds of environmental interest. The mathematical expressions for the microscopic polymer network model and the analytical technique employed are fully described in our previous publication [7].

These sensor arrays were found to recognize small molecules on the basis of their chemical structures that were related to the nature of the chemical class, the number, the type and the position of the functional groups. Each sensor responded in varying degrees to chlorinated organic molecules with standard deviation of less than 0.05. The time averages for the sensor response databases, datamaps, response patterns, and the intensity profiles were obtained for 2,4,6-trichlorophenol and 2-CP. The limit of detection obtained for 2,4,6-trichlorophenol and 2-chlorophenol using the conducting polymer sensor array were 0.1 and 0.25 ng/mL respectively. These results demonstrated the viability of conducting polymer sensor arrays for the identification and quantitation of chlorinated organic phenols based on the differences in their Euclidean distances. The qualitative differences as defined by the Euclidean distance measurements were most clearly

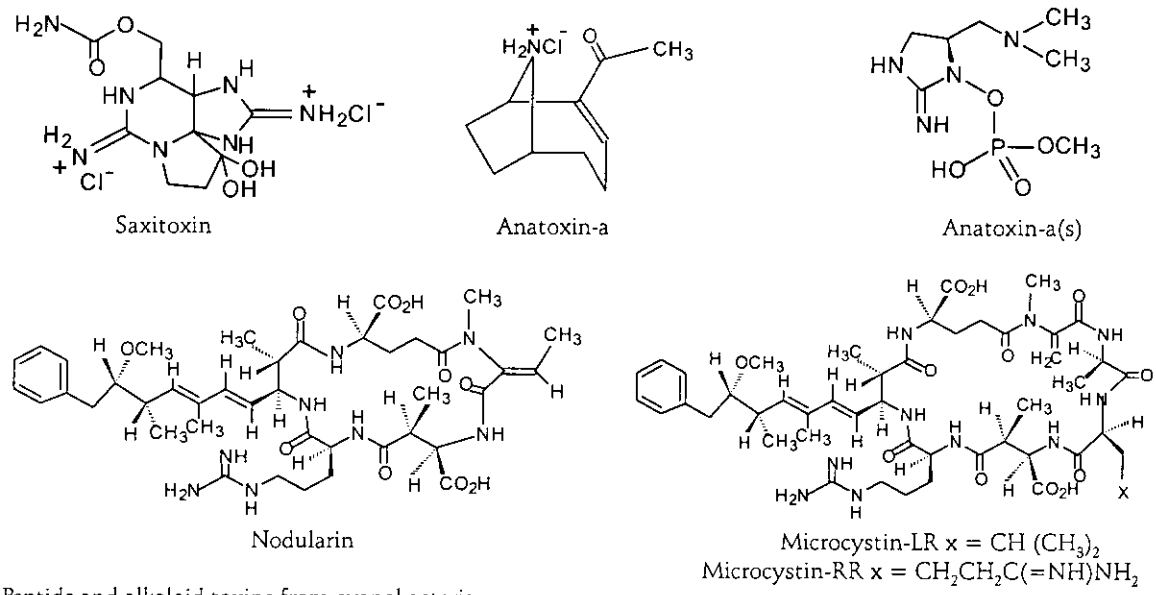

Fig. 4. Peptide and alkaloid toxins from cyanobacteria.

visible when the nature and the type of the functional groups were considered.

Direct Electrochemical Sensors for Polychlorinated Biphenyls (PCBs)

A direct electrochemical immunosensor has been developed for the determination of PCBs in water. The assay was based on the measurement of the current due to the specific binding between PCB and anti-PCB antibody-immobilized conducting polymer matrix. The linear dynamic range of the immunosensor was between 0.3-100 ng/mL with a correlation coefficient of 0.997 for Aroclor 1242 [6]. Well-defined responses were recorded for all Aroclors. The method detection limits for Aroclors 1242, 1248, 1254 and 1016 were 3.3, 1.56, 0.39, and 1.66 ng/mL respectively, and a signal-to-noise (S/N) ratio of 3. The immunosensor exhibited high selectivity for PCBs in the presence of potential interference such as chlorinated anisoles, benzenes and phenols. The highest cross-reactivity measured for chlorinated phenolic compounds relative to Aroclor 1248 was less than 3%. The recoveries of spiked Aroclors 1242 and 1254 from industrial effluent water, rolling mill and seafood plant pretreated water at 0.5 and 50 ng/mL ranged from 103-106%. The effect of ionic compounds on the detection indicated that no significant change in immunosensor signal was observed within the uncertainty of the assay procedure. The detection method can be used for continuous monitoring of effluent such as waste streams and ground water.

Optical Sensors for Cyanobacterial Toxins

Water blooms of toxic cyanobacteria are commonly encountered in freshwater and represent an increasing environmental hazard [11]. Several genera of fresh and brackish water cyanobacteria, *Microcystis, Oscillatoria, Anabaena, Aphanizomenon* and *Nodularia,* produce lethal toxins. Toxins from these genera include cyclic hepatotoxic hepta- or pentapeptides and neurotoxic alkaloids (Fig. 4) [12].

One major group of cyclic heptapeptides is called microcystins (MCs). More than 50 different microcystins have been isolated and characterized so far [13,14]. Of the toxic cyclic heptapeptides known, the most prevalent is microcystin-LR [15]. Cyanobacterial toxins present a significant analytical challenge due to their complex structures and frequent occurrence as mixtures of structural congeners. Requirements for the analysis of cyanobacterial toxins are increasing due to recognition of the hazards that they present to human and animal health [16-20]. The advantages and disadvantages of the different chemical, biochemical and biological analytical methods for microcystins as well as the overall analytical strategy have recently been reviewed [18].

We have developed a competitive optical sensing approach for the determination of microcystin-LR using a novel microcystin bioconjugate that was synthesized by coupling microcystin with fluorescein. Fluorescence experiments verify the formation of new fluorescently-active analog of MC-LR, the emission wavelength shifted to the lower position as compared with the free fluorophore reagent (FITC) (Fig. 5). The emission wavelength for FITC is 528 nm. The new fluorescently-active compound exhibits emission wavelength at 514 nm. The linear response for fluorescently labeled MC-LR in solution was between 0.5 and 15 pM or higher. Okadaic acid was used for the selectivity study [21], our experimental data demonstrated that coated PP1 layer are resistant to okadaic acid up to concentrations of 48 nM. As MC-LR and other inhibitors prevent each other

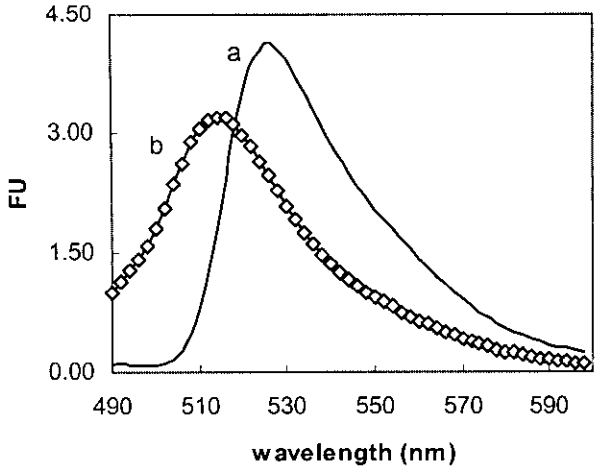

Fig. 5. Emission spectra of FITC (a) and MC-Cys-FITC (b). Emission spectra were collected at the excitation wavelength 480 nm.

from binding to PP1 or PP2A [22], we are currently testing other toxins such as nodularin, taunomycin, calyculin.

Endocrine Disrupting Chemicals-Alkylphenols

Bisphenol-A (4,4'-isopropylidenediphenol), nonylphenol and diethylstilbestrol (DES) are among the chemicals currently being studied as potential endocrine disruptors. These chemicals commonly known as endocrine disrupting chemicals (EDCs) disrupt the normal functioning of the endocrine system and their effects are observed as carcinogenic responses at hormonally sensitive organs, as well as defects in reproductive organs, body development and neural and immune systems [23-26]. DES was used in the past to prevent miscarriages. Its use has since been abolished due to observed increase in cancer and abnormalities in the reproductive organs of the offspring. Bisphenol-A is used in the manufacture of polycarbonates as well as epoxy and polyester-styrene resins. Nonylphenol is used in the preparation of lubricating oil additives, resins plasticizers and surface agents, it is also one of the major persistent metabolite of nonylphenol polyethoxylates surfactants [27,28]. These chemicals have shown to elicit estrogenic effects on both laboratory animals as well as in wild animals. Current methods for the analysis of EDCs are immunoassays and chromatographic techniques, both of which have shown some limitations [23,24].

Electrochemical analysis of four alkylphenols (bisphenol-A, nonylphenol, estradiol and DES) using cyclic voltammetry method gave irreversible oxidation peaks at 0.9 V, 1 V, 1 V and 0.85 V versus Ag/AgCl, respectively (Fig. 6(a)-(d)). The formation of an insulating film was observed for bisphenol-A and estradiol, a characteristic generally observed with phenolic compounds [29,30]. The limit of detection using this technique was < 0.5, < 0.8, < 1 and < 0.8 mM for bisphenol-A, nonylphenol, estradiol and DES, respectively. These values are however expected to improve with further optimi-

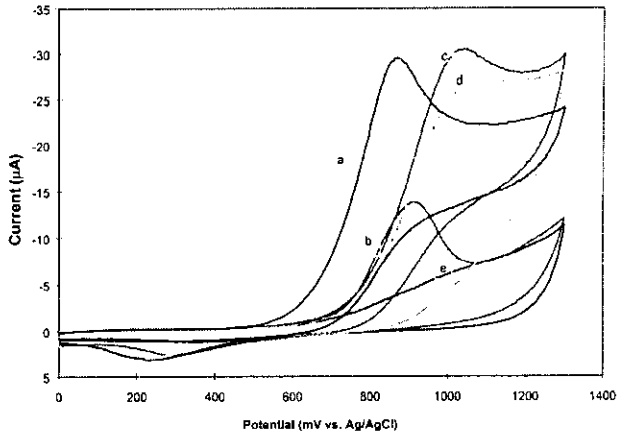

Fig. 6. Cyclic voltammograms for (a) 4 mM DES (b) bisphenol-A, (c) 4 mM nonylphenol, (d) 4 mM estradiol and (e) 0.05 M tetrabutylammonium perchlorate (TBAP) in acetonitrile/water (50:50). All CVs for the alkylphenols were recorded with TBAP as supporting electrolyte in acetonitrile/water solvent (50:50) using platinum working electrode, Ag/AgCl (3 M NaCl) reference electrode and platinum wire as counter electrode.

Table 1. Summary of environmental sensors reported

Analyte	Sensing method	Limit of detection
Cyanazine	Electrochemical immunosensor	4.0 μg/mL
2,4,6-TCP	Multiarray sensor	0.1 ng/mL
2-CP	Multiarray sensors	0.25 ng/mL
Aroclor 1242	Direct electrochemical immunosensor	3.3 ng/mL
Aroclor 1248	Direct electrochemical immunosensor	1.56 ng/mL
Aroclor 1254	Direct electrochemical immunosensor	0.39 ng/mL
Aroclor 1016	Direct electrochemical immunosensor	1.66 ng/mL
Bisphenol A	Cyclic voltammetry	0.5 mM
Nonylphenol	Cyclic voltammetry	0.8 mM
Estradiol	Cyclic voltammetry	1.0 mM
DES	Cyclic voltammetry	0.8 mM
Microcystin-LR	Optical sensor	0.5 mM
PCBs	Direct electrochemical immunosensor	0.3 ng/mL

zation of the technique and especially by eliminating the high non-faradaic currents.

CONCLUSION

We have developed various sensors for the detection of pesticides, organochlorines, PCBs, microcystins and alkylphenols (Table 1). Low detection limits for cyanazines, PCBs, toxins and alkylphenols were realized, with values as low as 4.0 μg/mL, 0.39-3.3 ng/mL, 0.5

pM and 1mM respectively. The sensors developed for the analysis of these compounds gave rapid measurements with response time of several minutes. The combination of gas sensor arrays and pattern recogni-tion techniques resulted in a fast and objective method for the simultaneous measurement of a wide range of volatile and semi-volatile organics.

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