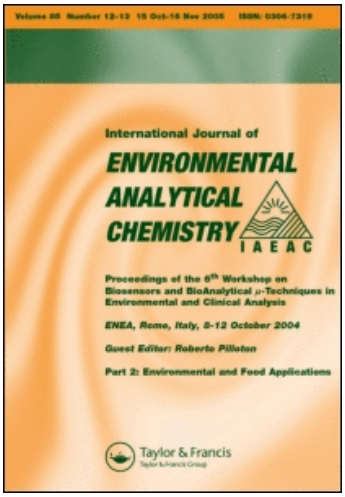

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Detection of Pesticides in the Environment Using Biosensors Based on Cholinesterases

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To cite this Article Skládal, P. , Fiala, M. and Krejcí, J.(1996) 'Detection of Pesticides in the Environment Using Biosensors Based on Cholinesterases', International Journal of Environmental Analytical Chemistry, 65: 1, 139 — 148
To link to this Article: DOI: 10.1080/03067319608045549
URL: http://dx.doi.org/10.1080/03067319608045549

Intern. J. Environ. Anal. Chem., Vol. 65, pp. 139–148
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DETECTION OF PESTICIDES IN THE ENVIRONMENT USING BIOSENSORS BASED ON CHOLINESTERASES

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(Received, 9 September 1995; in final form, 28 March 1996)

Amperometric biosensors based on acetylcholinesterase or butyrylcholinesterase were used for the kinetic determination of organophosphate and carbamate pesticides. The current of the biosensor I_{ss} was measured continuously with substrate; the addition of samples with pesticides resulted in the time decrease of the current dI/dt. The relative inhibition $RI = (dI/dt)/I_{ss}$ was used as the signal for evaluations. For several pesticides, different calibration curves (dependencies of RI on concentration c) were obtained depending on the affinity of the individual pesticide to the cholinesterase used. This affinity was described using the bimolecular inhibition constant k_i. The single calibration curve independent on the type of pesticide was obtained as the dependence of RI on the product $k_i c$, thus indicating the effects of both concentration and inhibiting properties on the response of the biosensor. The relative inhibition was used to characterise anticholinesterase toxicity of the sediments collected from the Morava River and its tributary streams. The influence of both point (large cities) and nonpoint (agriculture) sources of pollution was identified.

KEY WORDS: Amperometric biosensor, cholinesterase, organophosphates, carbamates, river sediments.

INTRODUCTION

Organophosphate and carbamate pesticides are widely used in agriculture. These compounds are preferred for relatively low persistency, on the other hand, some of them exhibit rather high acute toxicity[1]; consequently, the monitoring in water, soil and food products is highly desired to protect human health and living organisms. The bioanalytical detection of organophosphates and carbamates in the environment has been for a long time conveniently carried out using their inhibition effects on cholinesterases[2,3], a commercial test kit is available from Boehringer (Cholinesterase Inhibition Test, Cat. No. 1293 460). Also systems based on immobilised cholinesterase appeared soon[4], the application for cholinesterases in biosensors followed[5]. Small portable biosensor systems are generally considered as very promising tools for the analysis of toxic substances directly in the field conditions[6]. The biosensor measurements are economically effective and fast and are thus suitable for pre-screening of large number of samples.

The substantial part of the enzyme-based biosensors for environmental analysis employs either acetycholinesterase (AChE, EC 3.1.1.7) or butyrylcholinesterase (BChE, EC 3.1.1.8) as biorecognition elements in combination with a variety of physical

transducers, pH sensitive glass electrodes[7] and ISFETs[8], potentiometric[4], amperometric[9,10] and conductometric[11] electrodes, fibre optic systems[12]. The results obtained from biosensor analysis have been successfully validated using standard chromatographic techniques[13]. In most approaches, a decrease of the signal (original, S_o, final, S_i) after preincubation with the sample is used to calculate the percentual inhibition[2]:

$$I\% = 100\,(1 - S_o/S_i).$$

The length of the incubation interval is quite important (longer incubation improves sensitivity) as short incubation could result in the undesired negative identification of toxic samples.

The recently described kinetic measurement allows much faster analysis with very good sensitivity[14]. The current of the amperometric biosensor with substrate I_{ss} is monitored continuously; in the presence of pesticides, a time decrease of the signal dI/dt occurs which is used to calculate the relative inhibition:

$$RI = (dI/dt)/I_{ss}$$

RI serves as a parameter characterising the toxicity of analysed samples. In this work, the effects of the concentration and inhibiting properties of pesticides on the signal of the biosensor will be studied with respect to the practical use of the biosensor for analysis of real samples—river sediments.

EXPERIMENTAL

Chemicals Acetylcholinesterase from electric eel (15 μkat/mg), butyrylcholinesterase from horse serum (16 μkat/mg) and paraoxon were obtained from Sigma, all other pesticides were kindly provided by Dr. B. Šafář (Research Institute MVCHO, Brno). Cobalt phthalocyanine was from Aldrich, graphite powder was supplied by Merck. All other reagents of the highest purity available were from Lachema (Brno).

Construction of biosensors[15] The biosensors (Figure 1) were based on the screen-printed electrode system (a ceramics strip 25 × 7 mm) which is commercially available from Krejčí Engineering (Tišnov, Czech Rep.). The original platinum-based working electrode (Ø 0.8 mm) was covered by the graphite/acetylcellulose (92 and 4%) composite layer containing cobalt phthalocyanine (4%) as a modifier, the resulting active area was 1.5 mm^2. The original silver electrode was coated by a layer of AgI in the presence of 1 mM iodide and it than served as a Ag/AgI/0.5 mM iodide reference electrode.

The enzyme layer was coated over the working electrode only. A mixture (1 μl) of cholinesterase (either AChE or BChE, 600 nkat/ml), bovine serum albumin and glutaraldehyde (0.35 and 0.08%, respectively) was applied on the surface and allowed to dry in a closed vessel. The resulting biosensors were stored dry in refrigerator.

Electrochemical measurements The reference and working electrodes of the biosensor were connected to the amperometric detector ADLC 2 (Laboratory Instruments, Prague). The operating potential was +400 mV vs. the internal reference electrode (i.e. +250 mV vs. the common Ag/AgCl/3 M KCl reference). The output signal was transferred through

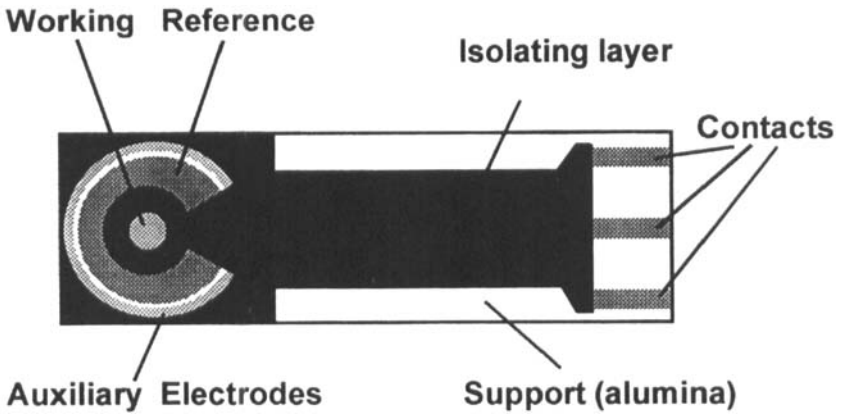

Figure 1 The design of the biosensor. The screen-printed electrode system (Krejčí Engineering, Tišnov) was used as the transducer. The working electrode was covered by the graphite/acetylcellulose/cobalt phthalocyanine composite layer, which was further coated with the enzyme layer composed of AChE (BChE)/ albumin/glutaraldehyde. The dimensions of the sensor are 25 × 7 mm.

the 12 bit A/D converter (Datik, Brno) to the PC AT/386 computer for storage and evaluations using an own software.

The kinetic measuring procedure is schematically shown in Figure 2. The biosensor was placed in a vessel thermostated to 30°C with 3 ml of 50 mM phosphate buffer (pH 7.0). The background current was allowed to stabilise, and the substrate (acetylthiocholine or butyrylthiocholine iodide for AChE or BChE biosensors, respectively) was added to 0.5 mM final concentration. The increase of current I_{ss} was recorded and immediately the addition of sample (10 µl, methanolic solution) followed. The time decrease of current dI/dt was determined. After washing, the same biosensor was used again for new measurements providing that the next signal I_{ss} was higher than 50% of the value obtained for a fresh biosensor (typically close to 100 nA).

Analysis of real samples The sampling sites (46) in the Morava River watershed covered both the main watercourse and the most important tributaries. Sediment samples were obtained as a mixture from 3 cores deep 10 cm under the sediment surface (stainless steel core, inner Ø 2 cm). In laboratory, samples were dried (25°C) and sieved on 50 µm mesh. A portion of 0.40 g was placed in a small plastic test tube, 1 ml of methanol was added and the mixture was vigorously agitated for 10 min. The supernatants obtained after centrifugation (10000 r.p.m., 10 min) were analysed using biosensor as described above. The values of anticholinesterase toxicity were calculated as the relative inhibition per 1 g of the dry sediment.

RESULTS AND DISCUSSIONS

Response of biosensors to individual pesticides

The calibration curves (*RI* vs. concentration *c*) for several pesticides using the BChE based sensors are shown in Figure 3. All the curves exhibit biphasic character. For the

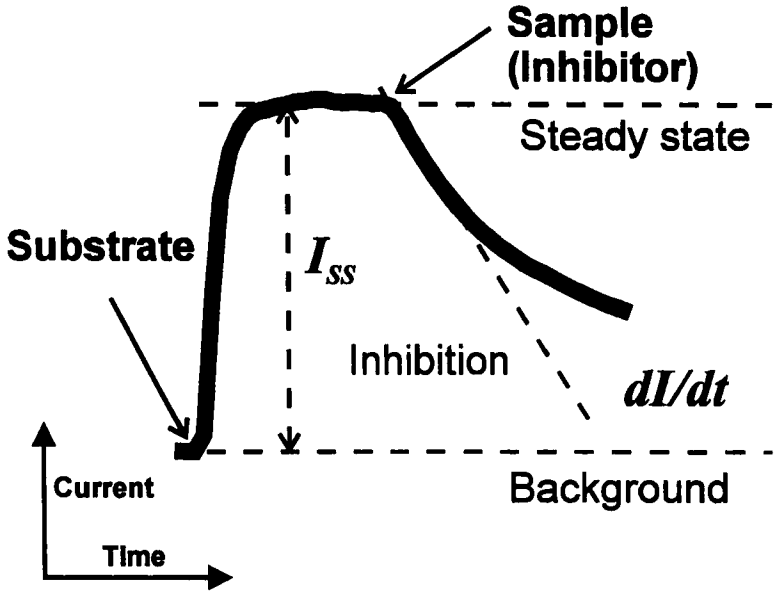

Figure 2 Kinetic determination of the relative inhibition using the biosensor.

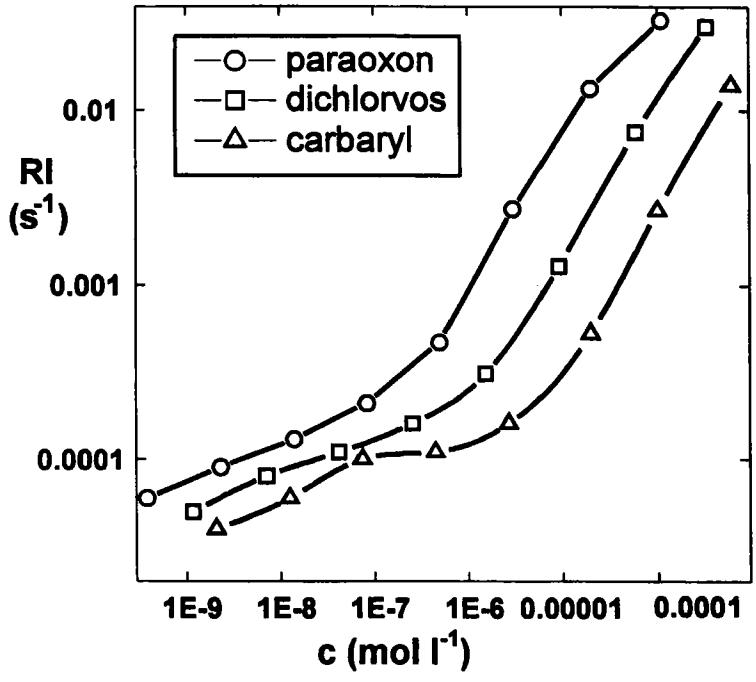

Figure 3 Examples of calibration curves for organophosphate (paraoxon and dichlorvos) and carbamate (carbaryl) pesticides obtained using the BChE-based biosensor.

higher concentrations of inhibitors, the dependencies are linear in the double logarithmic plots with values of slopes very close to 1, thus indicating the existence of linear behaviour also for the dependencies of *RI* on *c*. The parameters for both log *RI* vs. log *c* and *RI* vs. *c* dependencies are summarised in Table I. For the construction of calibration curves, several biosensors were used. The reproducibility of measurements was always better than 5%, it was mostly due to the differences among individual biosensors resulting from manual manufacturing.

The horizontal position of the individual calibration curve is determined by the inhibiting effect of the pesticide on cholinesterase. This explains variable detection limits for individual inhibiting compounds. The detection limit is given by the lowest reliably detectable decrease of current. In the employed arrangement, this level is given by the consumption of substrate by the biosensor inside the measuring vessel[16], which sets the smallest measurable relative inhibition at 5×10^{-5} s^{-1}. The corresponding lowest detectable concentrations range from 0.3 to 11 nM for paraoxon and carbaryl, respectively. This is equal to 23 nmol/g (60 ng/g) of paraoxon in the original dry sediment. The detection limit could possibly be further improved by increasing the ratio between the volume of the sample (10 µl) and the reaction mixture (3 ml). For methanolic extracts analysed here, this small ratio is necessary to minimise effects resulting from the addition of pure methanol (electrochemical oxidation); nevertheless, these disturbances are negligible for 0.33% contents. Methanol is an excellent solvent for the extraction of pesticides from soil samples, it could be used also for the elution of pesticides from water samples extracted on C18 modified matrices[17]. The steps involving the extraction of pesticides to organic solvents improve also the specificity of the measurement. In fact, cholinesterase could be inhibited also by fluoride[9] and especially by heavy metals[18]. The effect of some heavy metals on our biosensor system is summarised in Table II. Due to the kinetic measuring procedure, the metals could not influence only cholinesterase, but also the electrochemical system. Using methanol for extraction, these concurrent inhibition effects are completely removed and the observed inhibition is only due to the presence of organic substances in the original samples. For

Table I Parameters for linear regressions on parts of the curves from Figure 3, $\log RI = A1 + B1 + {}^*\log c$, as well as for the original dependencies $RI = A2 + B2 * c$. In both cases, the units were s^{-1} for *RI* and mol l^{-1} for *c*. Only the last four points for the highest concentration of pesticides were considered for calculations.

Pesticide	*A1*	*B1*	*regr. coeff.*	*A2*	*B2*	*regr. coeff.*
Carbaryl	0.810	0.83	0.994	0.000150	23.5	0.9998
Dichlorvos	1.50	0.864	0.9993	0.00082	90.9	0.998
Paraoxon	2.41	9.908	0.991	0.00305	282	0.976

Table 2 The effect of some heavy metals on the BChE-based biosensor.

Metal ion	*Inhibition (detection limit)*
Fe^{3+}, PB^{2+}, Zn^{2+}	not observed up to 5 mM
Cd^{2+}	30 µM
Cu^{2+}	10 µM
Hg^{2+}	40 µM (+ electrochemical disturbance)

future applications, the operation of cholinesterase based sensors in pure organic solvents should be considered. It was shown that AChE biosensors are compatible with hydrophobic solvents[19] and we have successfully tested this approach with the BChE biosensor described here[20]. The combination of solid phase extraction with direct biosensor measurements in the organic phase extracts could be useful for achievement of lower detection limits.

The biosensor could be re-used many times provided that no inhibition is detected in the analysed sample. Here, up to 20 samples were analysed with a single sensor. The operational stability of the biosensor is very good. After 8 hours of continuous operation in stirred solution (no inhibition), the decrease of signal due to the long-term use was below 10% both for AChE and BChE-based sensors. On the other hand, the low price of the biosensor (less than 1 ECU at present) makes this sensor easily disposable. The shelf live of biosensors is satisfactory, the activity decreased with the half-time $\tau_{1/2}$ of 340 days during storage in dry state at 4°C. Using the concept of relative inhibition, the gradual decrease of activity during storage is efficiently compensated.

The influence of the bimolecular inhibition constants k_i on response of the biosensor is evident from Figure 4, where the dependence of slopes d*RI*/d*c* of calibration curves (the parameters *B2* in Table I and other data not shown here) is plotted vs. the values of k_i obtained from ref.[21]. The values for strong cholinesterase inhibitors, sarin and soman, were added for comparison (data kindly provided by Dr. B. Šafář). The results of the

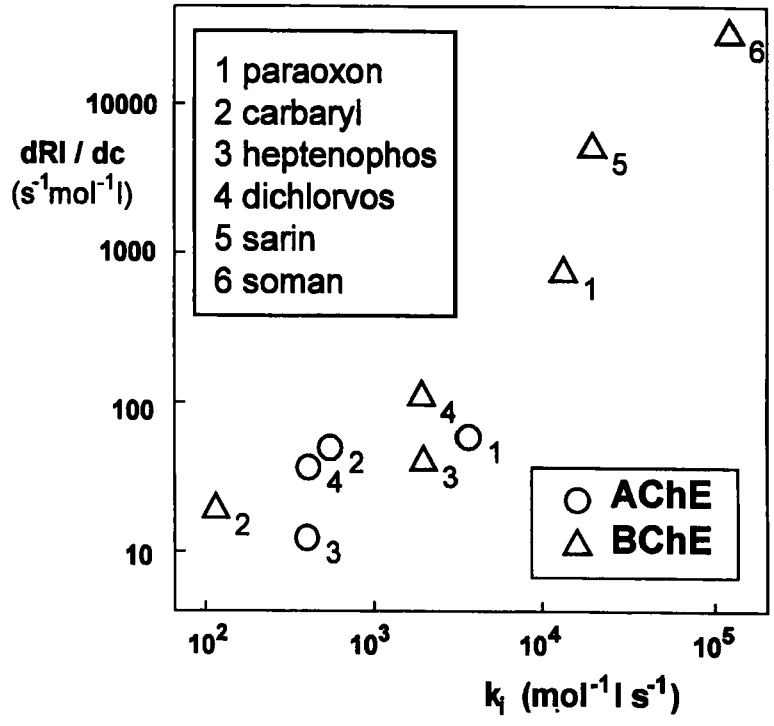

Figure 4 Dependence of the slopes d*RI*/d*c* of the linear regions of calibration curves (higher concentrations, Figure 3) on bimolecular inhibition constants k_i (values from ref.[21]). The data for sarin and soman were provided by B. Šafář.

biosensor measurements depend both on concentrations and on inhibiting properties of pesticides. For analysis of real samples, when the type of the toxic compound is unknown, the result could hardly be interpreted as a concentration, perhaps the apparent content of some reference compound (paraoxon) could only be calculated[22]. Here, we prefer to utilise the resulting relative inhibition as a toxicity parameter. We tried to combine the two variables which determine the measured biosensor signal, the concentration c and the inhibition constant k_i. In Figure 5, all the measured RI values for several pesticides and for both AChE and BChE sensors were plotted against the corresponding products $k_i c$. As can be seen, a single dependence was obtained, the shape of which resembles the calibration curves presented in Figure 3.

In the double logarithmic plot in Figure 5, two linear regions could be identified. For the linear equation:

$$\log RI = A + B * \log (k_i c),$$

the following parameters were obtained (r represents the regression coefficient, n is the number of experimental points):

$k_i c > 0.1\ s^{-1}$: $A = -2.855 \pm 0.053$ $B = 0.724 \pm 0.056$ $r = 0.94$ $(n = 25)$

$k_i c < 0.1\ s^{-1}$: $A = -3.602 \pm 0.055$ $B = 0.178 \pm 0.018$ $r = 0.88$ $(n = 35)$

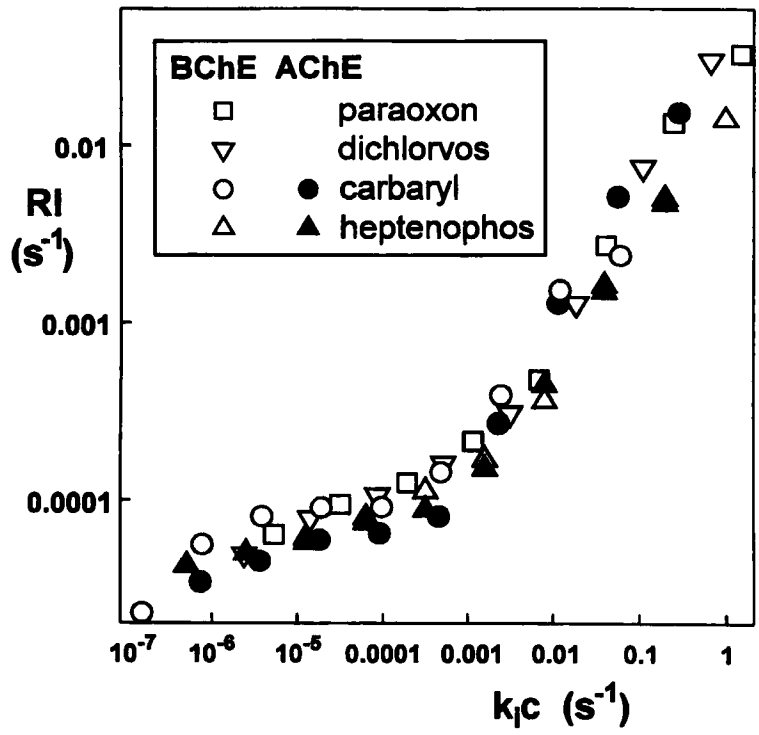

Figure 5 Dependence of the relative inhibition RI on the corresponding $k_i c$ values calculated from the experimental concentrations c and bimolecular inhibition constants k_i.

The normalised single calibration curve was obtained which now does not depend on the type of pesticide. Thus, for evaluation of the cholinesterase inhibitions, the concept of toxicity seems to be much more useful than calculation of concentrations of some reference pesticides. For several pesticides present together, the overall toxicity will be proportional to the sum of their individual ($k_i c$) values. The question appears if to use as the toxicity the measured *RI* values directly, or to calculate the corresponding $k_i c$ values. Here, the *RI* values were used as the toxicity parameter.

The proposed approach seems to be better than the usually published percentual inhibition of cholinesterase, which depends on the incubation interval. As the incubation interval is not standardised, the results from various laboratories could not be compared easily. This type of biosensor could detect toxicity only and it does not indicate what substances are responsible for the inhibition of the biorecognition element. This approach could conveniently be used for screening purposes; the positive samples should be further analysed and confirmed using conventional chromatographic methods. Another possibility could be the combination of chromatographic separation of substances from the unknown sample and the use of cholinesterase-based biosensor as a specific detector[23]. On the other hand, the advantage of biosensors—fast, low-cost and easy operation will be lost.

Analysis of river sediments

The biosensor was applied to the analysis of the pollution caused by organophosphate and carbamate pesticides in the Morava River watershed. In this area, agriculture is the most important factor responsible for nonpoint pollution[24]. Sampling sites were chosen to cover both the main course of the river as well as the most important tributary streams and the expected point sources of pollution. The obtained anticholinesterase (anti-BChE) toxicities are summarised in Figure 6. The vertical axis represents the location on the Morava River course. The source is located in the upper part (above Hanušovice), 0 km corresponds to the crossing of the border of Czech Republic. The names of important cities are included directly in Figure 6, the tributary streams are identified by numbers explained in the legend.

The trend of increasing toxicity values from source along the course of the Morava corresponds with the increasing intensity of agriculture in the same direction. Similar values of toxicity were found for sites on the Morava stream and for tributary streams in the same location. The application of organophosphate and carbamate pesticides reached up to 3.9 and 7.8 kg/km^2 in the middle and lower parts of the watershed, respectively. The unexpectedly increased toxicity found near the source in Hanušovice (the mountain area with less intensive agriculture) is most probably caused by a local point pollution. The effects of point pollution can be seen also for several other cities, the value of toxicity sharply increases below the town in comparison with the value measured above. This happens for Olomouc, Napajedla, Uherské Hradiště and especially for Vnorovy.

The biosensor measurements are capable to identify point sources of pollution, thus enabling to arrange some measures to improve the danger situation. Moreover, this analysis could help to evaluate the nonpoint load of pesticides resulting from agriculture. On the other hand, the threshold toxicity value has to be established, it is supposed that it should be below $10^{-3}s^{-1}g^{-1}$. Further investigations are needed to clarify the relations between the concentration of pesticides in water and their contents in sediments. It is expected that the stability of pesticides is higher in sediments than in water, and some accumulation in sediments could occur, too.

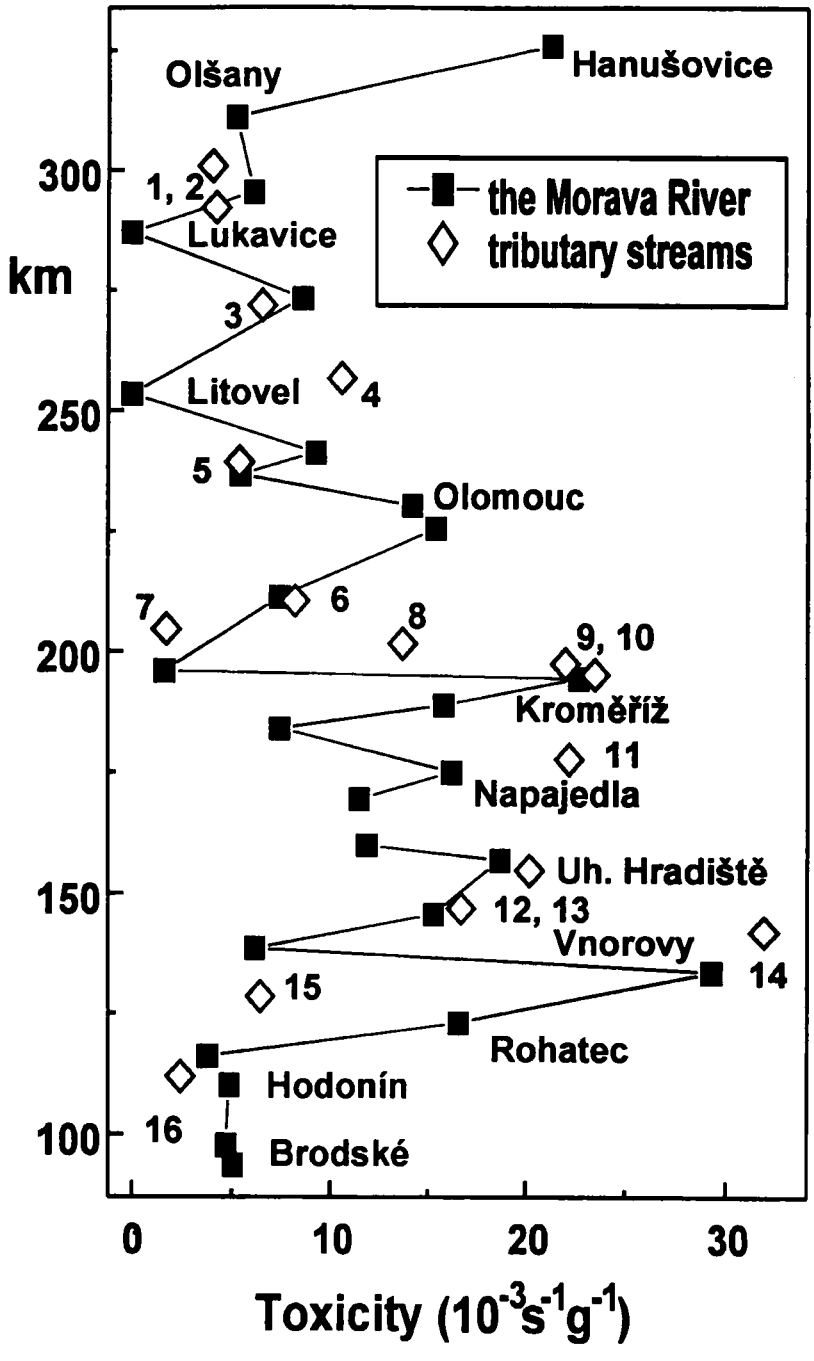

Figure 6 Toxicities (BChE-based biosensor) measured on the Morava River and its tributary streams (1 Desná, 2 Moravská Sázava, 3 Třebůvka, 4 Třídvorka, 5 Oskava, 6 Bečva, 7 Valová, 8 Blata, 9 Haná, 10 Moštěnka, 11 Dřevnice, 12 Olšava, 13 Dlouhá řeka, 14 Okluky, 15 Velička, 16 Salajka). The important cities are indicated directly in picture. The 0 km for the vertical axis represents the border of Czech Republic, the source of the Morava River is located above Hanušovice.

From the practical point of view, real samples did not exhibit any problems to the performance of the biosensor. No electrode fouling occurred and no interference from oxidisable substances was observed. The latter was probably prevented by the relatively low operating potential of the graphite-based electrodes.

The biosensor analysis of anticholinesterase toxicity in the river sediments exhibits a high potential for the effective environmental monitoring of the use of organophosphate and carbamate pesticides.

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