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EDITH VIIRLAID

Biosensing Pesticides in Water Samples

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Biosensing Pesticides in Water Samples

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Institute of Chemistry, Faculty of Science and Technology, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

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III. **Viirlaid, E.**, Riiberg, R., Mäeorg, U., Rinken, T. Glyphosate attachment on aminoactivated carriers for sample stabilization and concentration. *Agronomy Research*, 2015, 13(4), 1152–1159.

Author's contribution

Paper I: Performed all experimental work and data analyses, and was involved in the interpretation of results and the preparation of the manuscript.

Paper II: Performed the experimental work and calculations related to biosensor optimization and measurements, responsible for the interpretation of biosensor measurement results and was involved in the preparation of the manuscript.

Paper III: Performed partially the experimental work and calculations, responsible for the interpretation of results and was involved in the preparation of the manuscript.

ABBREVIATIONS AND SYMBOLS

A	whole signal change parameter
AFeNP	aminoactivated magnetic iron oxide nanoparticles
ASB	aminoactivated silica beads
B	kinetic parameter
BIA	bead injection analysis
DOC	dissolved oxygen concentration
EDC	N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride
ELISA	enzyme-linked immunosorbent assay
EPSPS	enzyme 5-enolpyruvylshikimate-3-phosphate synthase
FIA	flow injection analysis
GC	gas chromatography
HPLC	high-performance liquid chromatography
I	signal intensity
K_d	dissociation constant
$K_{1/2}$	half-limiting value constant
LOD	limit of detection
LOQ	limit of quantification
NHS	N-hydroxysuccinimide
PBS	phosphate buffer saline
5-TAMRA	5-carboxytetramethylrhodamine
5-T-g	5-TAMRA-glyphosate
UV-Vis	Ultraviolet-visible

INTRODUCTION

Pesticides are natural or synthetic compounds mainly employed in the agricultural, but also other sectors to control all kinds of pests [1,2]. The global pesticide use is increasing steadily and the pesticides sales number was approximately 3.6 million tons in 2017 [2,3]. Majority of the applied pesticides potentially contaminate water and soil, and cause unfavourable effects on biota [4]. To eliminate risks to living organisms, allowed values for drinking and surface water have been set for most pesticides. EU provided Maximum Concentration Levels for pesticides in drinking water are quite conservative, being 0.1 μg/l for each individual pesticide [5]. The detection of pesticides is commonly carried out with chromatographic techniques integrated with UV-Vis, fluorescence and/or mass-spectrometric detectors allowing to achieve limit of detection (LOD) values under the maximum tolerated levels for all pesticides in drinking water. However, these methods are time consuming due to the need of sample pre-treatment and sophisticated equipment, which make them unsuitable for out-of-lab analyses [6]. In order to achieve capability for on-site specific testing of pesticides, biosensors are regarded as a prospective option.

Different biosensors have been proposed for the determination of pesticides, but there are few suitable technologies available that allow sensitive and quick out-of lab detection of pesticides without pre-treatment.

The main goal of the present work was to develop biosensing platforms for the detection of two pesticides – carbaryl and glyphosate, which can be potentially used for quick on-site monitoring of these compounds in water. These two pesticides are among the most problematic ones: glyphosate is the most extensively used herbicide (and also pesticide) globally and carbaryl was a widely used insecticide until recently. Although carbaryl is banned in EU now, it is still one of the most popular substances used against insects in the United States and Asian countries.

A major issue in pesticide laboratory-based analyses is the delay due to the transportation of collected samples. So, one of the aims of the current study was to study sample stabilization and preconcentration options to increase the sensitivity and reliability of pesticide analyses.

1. LITERATURE OVERVIEW

1.1. What are pesticides? The use of pesticides

Pesticides are substances to control all kinds of pests, that is any animal or plant detrimental to humans or human concerns [1]. Pesticides are classified according to their chemical composition or target organisms. Based on composition, pesticides are grouped into four main families: (a) organochlorides, including chlorinated aromatics and alicyclics; (b) organophosphates; (c) esters of carbamic acid or carbamates; and (d) pyrethrins – naturally occurring biodegradable terpenoids, which synthetic analogues are called pyrethroids [7]. However, pesticides are commonly known by their trade names and not by their systematic names. Based on targeted organism, the term pesticide comprises herbicides, insecticides, rodenticides, fungicides etc., whereas herbicides are accounting for about 50% of all pesticides used [2].

Pesticides are mainly used in the agricultural, but also other sectors like industrial, commercial and governmental; and in home & gardening [2]. The global volume of pesticide use is increasing steadily. Based on the estimations of the US Environmental Protection Agency (EPA) and global pesticides sales, in 2017 the total amount of pesticides used was approximately 3.6 million tons [2,3], making 500 g/year per every single person. In Estonia, the use of pesticides has increased over 4 times within last two decades and reached to more than 800 t in 2016 [8] (> 600 g/year per person).

Considering individual compounds, the use of some pesticides has decreased due to their harmful effects discovered. For example, in 2002 carbaryl (1-naphthyl *N*-methylcarbamate) was classified as a potential human carcinogen [9] and since then its agricultural use in United States has dropped considerably: from more than 1,360 t in 1997 to less than 363 t in 2016 [10]. In EU, carbaryl has been totally banned since 2007 [11].

1.2. Fate of pesticides in the environment

Majority of the applied pesticides potentially contaminate water and soil, and cause unfavourable effects on biota [4]. The fate of a pesticide in the environment, its persistence and mobility are affected by its chemical and physical properties along with site characteristics like soil and groundwater features, climate, local weather conditions, biological population, and last but not least – handling practices of the pesticide user [12]. The persistence of a pesticide is characterised in terms of its half-life, which can vary from several weeks (e.g. 2,4-Dichlorophenoxyacetic acid in soil) to tens of years (e.g. 30 years in soil or even longer in water for DDT (dichlorodiphenyltrichloro-ethane)) [13–15].

Pesticides with longer half-lives have higher potential for mobility and movement in the environment. Pesticides with high sorption potential (e.g. glyphosate) can bind to soil particles almost covalently, so being inaccessible to

microorganisms and affecting also non-target organisms or soil after leaching [16,17]. To eliminate any potential risks to human health, maximum residue limits in environment and advised daily intake (ADI) levels in drinking water and food have been set for most pesticides – these are discussed hereinafter.

1.2.1. Accumulation and metabolism in living organisms

Whether a toxic compound can enter into a living organism from the surrounding environment depends on its physico-chemical properties, mainly lipophilicity. Lipophilic pesticides (and their metabolites) can get absorbed in fatty tissues of higher organisms hence resulting in persistence of pesticides for extended periods [18]. For example, DDT bioaccumulates in living organisms and although its use was banned in many countries almost 50 years ago it is still detected in bottom sediments and food [19]. Accumulated pesticides can enter from fat into metabolic pathways, which are generally aiming to reduce and eliminate the toxic impact of xenobiotics. However, metabolites can sometimes be even more persistent and/or toxic than the original compound [20,21]. For example, the major metabolite of DDT DDE (dichlorodiphenyldichloroethylene) is considered to be the most persistant contaminant in the environment [22].

The metabolic degradation pathways of pesticides can be extremely complicated and are depending on organism. For example, at least 9 different metabolic products have been identified for insecticide aldrin (1,2,3,4,10,10-hexachloro1,4,4α,5,8,8α-hexahydro-1,4-endo,exo-5,8-dimethanonaphthalene) (Fig. 1) [23–25].

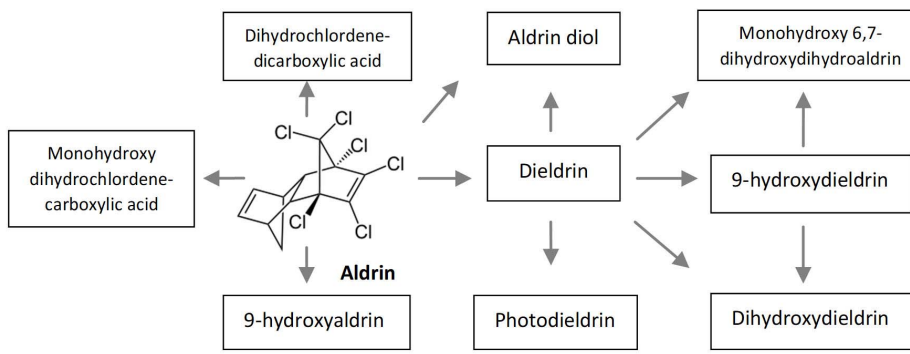


Figure 1. The degradation metabolites of aldrin

In addition, to increase the overall effect, commercial pesticide preparations often contain several additives (solvents, surfactants, preservatives and/or other pesticides) beside the main ingredient, leading to even more complicated degradation processes and enhanced toxicities [26,27].

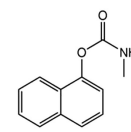
1.3. Some most problematic pesticides

The impact of a pesticide on environment is determined by its physico-chemical properties and amount applied. The most problematic pesticides are organochlorides as they remain intact for years and bioaccumulate in living organisms [28]. DDT and aldrin are still among the most widely used pesticides in developing countries of Asia although they have been banned in developed countries [11,28,29].

Currently, carbamates and organophosphates are preferred due to their relatively low persistence and potential bioaccumulation [2]. In addition, the use of pyrethroids and other new chemical compounds is increasing due to their lower toxicity to birds and mammals [1,2,28].

The following review will mainly focus on carbaryl (belonging to carbamates), and an organophosphorus compound glyphosate, as the development of biosensors for the detection of these two particular pesticides was the aim of the present study.

1.3.1. Carbaryl

Carbaryl (1-naphthyl *N*-methylcarbamate, 

) is an insecticide introduced in 1956 [30]. It is interesting to mention that besides agri- and horticulture, it has been used in veterinary practice [31,32]. The most common trade name of carbaryl is Sevin®, but it is also known as Carbamine, Denapon, Dicarbam etc. [30,33]. Carbaryl-containing pesticide products are available in both solid and liquid forms [34,35]. Carbaryl is a crystalline solid with very low solubility in water (0.01 g/l at 20 ºC) [33], so in order to enhance it's solubility other ingredients are added. Powder products of carbaryl contain crystalline silica and carbonates [34].

Although carbaryl is banned in EU, it is still one of the most popular carbamate insecticides in the United States [2,10,11] where its agricultural use in 2016 was approximately 363 t [10].

Carbaryl's low vapour pressure (0.041 mPa at 23.5 ºC) and low Henry's law constant (2.74×10^{-9} atm m^3/mol at 25 ºC) indicate that it does not volatilize easily [30]. Although carbaryl has poor solubility in water both the experimental and estimated soil sorption coefficient (log $K_{OC} \approx 2.3$) values indicate moderate soil mobility and leaching to ground water may occur [36]. At soil

and water surfaces carbaryl is degraded quickly by photodecomposition, hydrolysis and microbial activity. Carbaryl degradation is mainly initiated by hydroxyl radical attack and results in the formation of 1-naphtol, carbon dioxide, and methylamine [30]. 1-naphthol degrades rapidly to other compounds [30,36,37]. Average half-life of carbaryl is 4 days in water and 15 days on soil surfaces [30,37,38]. In anaerobic sediments carbaryl breaks down more slowly with half-life of 125 days [38].

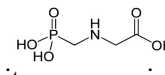
Carbaryl acts via inhibition of enzyme acetylcholinesterase (AChE), which is responsible for the degradation of the neurotransmitter acetylcholine in insects [30]. For humans it is categorized as moderately toxic and moderately irritating based on oral LD_{50} values (233–840 mg/kg) for rats [39,40]. The acceptable daily intake level (ADI) in EU for carbaryl is set to 0.0075 mg per kilogram of body weight per day (mg/kg/day) [29], World Health Organization (WHO) has set ADI at a similar level – 0.008 mg/kg/day [31], the corresponding value in US is slightly different – 0.01 mg/kg/day [41]. The Health Advisory Program in US serves the informal technical guidance for carbaryl in drinking water, set as high as 400 µg/l [41], which is contrary to daily consumption. In Canada the allowed level of carbaryl for livestock water is as high as 1100 µg/l, which is 12 times higher than the allowed level for human drinking water [42,43]. In many countries, there are no formal guideline values for carbaryl. The allowed levels for carbaryl in drinking and surface waters in different countries are shown in Table 1.

Table 1. Allowed levels for carbaryl residues.

	Drinking water, µg/l	**Freshwater short / long-term exposure, µg/l**	**Marine water short / long-term exposure, µg/l**	**Ref.**
EU	0.1*			[5]
US	400**	110 / 6***		[41,44]
Canada	90	3.3 / 0.2	5.7 / 0.29	[42,43]
Australia	5			[45]

* Though carbaryl is forbidden in the EU, the maximum level for each pesticide (0.1 µg/l) can be applied.
** Drinking Water Equivalent Level (DWEL) – the informal technical guidance for unregulated drinking water contaminants.
*** US EPA's Office of Pesticide Programs Aquatic Life Benchmarks for fish.

1.3.2. Glyphosate

Glyphosate (N-(phosphonomethyl)glycine, ) has become the most widely used herbicide in the world after its commercialization in 1974 [46]. Its use in agriculture has increased considerably after the introduction of genetically-modified glyphosate-tolerant crops in 1997 [47]. The global consumption of glyphosate reached almost 700,000 tons in 2016 [47]. In the same year the quantities of glyphosate marketed in Estonia were approximately 400 tons whereas glyphosate was accounting for about 50% of all pesticides marketed [8]. The best-known trade names of glyphosate include Roundup®, Glypro®, Rodeo®, Aquamaster® etc. [48]. Glyphosate is an acid (pK_a=0.8, pK_a1=2.3, pK_a2=6.0, pK_a3=11.0). For handling, packing and improved solubility in water (10.1 g/l at 20 ºC) commercial glyphosate formulations contain also various excipients (solvents, stabilizers, surfactants, pH-regulators) [49,50]. Glyphosate is usually formulated as a salt with isopropylamine, diammonium, monoammonium or potassium as the counterion [26,47,49,50]. Formulation with the isopropylamine salt increases the solubility of glyphosate 36 times [50]. Some ingredients may be active biologically, physically and chemically theirselves and thus increase the negative impact of glyphosate containing products [26,47]. For example, surfactant polyoxyethyleneamine (POEA) has been found to be significantly more toxic than glyphosate [51–54].

Glyphosate's low vapour pressure (0.013 mPa at 25 °C) and low Henry's law constant (1.44×10^{-12} atm m^3/mol at 25 ºC) indicate that it does not spread by volatilization [55,56]. Several studies indicate that glyphosate binds tightly to soil particles (log K_{OC} up to 4.3) and its leaching to groundwater is unlikely [16,50,57]. However, adsorption of glyphosate depends on soil characteristics (soil composition, pH, phosphate content) and any changes may cause desorption and leaching [16]. Glyphosate is relatively stable to chemical and photodegradation and decays mostly through soil microbial action [58]. There are two known pathways of glyphosate decomposition. One leads to the formation of inorganic phosphorous and sarcosine, the latter fully metabolizes to CO_2 and NH_3 [16,59]. The other yields in glyoxylate and aminomethylphosphonic acid (AMPA), which metabolize until complete mineralization [16,59]. The half-life of glyphosate can vary from 2 to 197 days [60]. It may be even longer as the degradation of glyphosate and AMPA is very dependent on soil characteristic and climate conditions [16,59,61]. For example an autumn application of glyphosate in Finland has resulted in half-life values as long as 240 days [61]. It has been demonstrated that soils enriched with aluminium silicates and iron oxides are effective glyphosate sorbents resulting in its longer half-life [16]. AMPA may adsorb onto soil particles more strongly than glyphosate, be less accessible to soil microorganism and thus it is more persistent in the environment and accumulates in soils [16,56,59]. The half-life of AMPA is ranging from 76 to 240 days [60]. In addition, the toxicity of AMPA is comparable to that of glyphosate [60]. Rain and erosion increase the reach of glyphosate and

AMPA into surface water [17,62]. The half-life of glyphosate in water varies from a few days to 91 days [60].

Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), participating in the production of phenylalanine, tyrosine, and tryptophan in plants [63]; but this enzyme is not found in mammals, birds, reptiles, amphibians, and insects [64]. Therefore, for years, glyphosate was considered to be safe for human health. According to the oral LD50 for rat (5600 mg/kg), it is categorized as practically nontoxic [63]. However, recent studies have indicated its potential genotoxity on living organisms due to DNA damaging, cytotoxic, and endocrine disruption effects along with inhibitory effects on enzymes involved in biosynthesis [47]. International Agency of Research on Cancer (IARC) has classified glyphosate as "probably carcinogenic to humans" [65]. The current acceptable daily intake level in EU is 0.5 mg glyphosate per kilogram; WHO's ADI level is 1 mg/kg/day; the corresponding level in US is 2 mg/kg/day, indicating diverging opinions of authorities [41,66,67]. In US the drinking water maximum concentration level (MCL) set for glyphosate is 700 µg/l [41]. The Health Advisory Program in US serves the informal technical guidance for glyphosate [41] which is 100 times higher than MCL and it does not match daily consumption. US EPA's Office of Pesticide Programs present guidlines for aquatic life (Tabel 2). Interestingly long-term exposure limit for fish is higher than short-term [44]. There is also an allowed glyphosate level for lifestock water in Canada, which is similar to the one set for human consumption [42,43]. Allowed levels set for glyphosate in drinking and surface waters in different countries are shown in Table 2.

Table 2. Allowed levels for glyphosate residues.

	Drinking water, µg/l	**Freshwater short / long-term exposure, µg/l**	**Ref.**
EU	0.1		[5]
Netherland	0.1	77	[5,68]
US	700	21,500 / 25,700*	[41,44]
Canada	280	27,000 / 800	[42,43]
Australia	10		[45]

* US EPA's Office of Pesticide Programs Aquatic Life Benchmarks for fish.

1.4. Methods of detection of pesticide residues in water

1.4.1. Chromatographic methods

Detection of pesticide residues and their metabolites is commonly carried out with chromatographic techniques – gas chromatography (GC), liquid chromatography (LC) or high-performance liquid chromatography (HPLC) integrated with a UV-Vis, fluorescence and/or mass-spectrometric detector [69–74]. For the detection of carbaryl and glyphosate LC and HPLC methods are acknowledged as standard methods by the International Organization for Standardization (ISO) and United States Environmental Protection Agency (EPA) [75–79]. The LOD values of some standard methods for the detection of carbaryl and glyphosate are given in Table 3.

Table 3. LOD values of some standard methods for the detection of carbaryl and glyphosate in water

	Standard	Method	LOD (µg/l)	Ref.
Carbaryl	EPA 632	HPLC/UV	0.02	[75]
Carbaryl	EPA ECM MRID: 45116202	LC/MS/MS	0.03	[76]
Glyphosate	ISO 16308:2014	HPLC/MS/MS	0.03	[77]
Glyphosate	ISO 21458:2008	HPLC/fluorimetry	0.05	[78]
Glyphosate	EPA ECM MRID: 40881601	HPLC/fluorimetry	0.05	[79]

In scientific studies, even lower LOD values have been achieved – 0.01 µg/l for carbaryl with LC/UV and 0.025 µg/l for glyphosate with GC/MS/MS [69,80].

Although these techniques allow trace analysis with excellent sensitivity, reliability and high reproducibility, they are time consuming due to the need of sample pre-treatment and sophisticated equipment making these methods unsuitable for on-site or in-field analyses [6].

1.4.2. Other lab-based methods

In addition to chromatographic methods spectrophotometry, capillary electrophoresis coupled with amperometry or electrochemiluminescence detection, and enzyme-linked immunosorbent assays (ELISA) have been proposed for pesticide detection [70]. The first two methods mentioned have similar limitations as chromatographic methods like the need for sample pre-treatment and sophisticated equipment. In addition, compared to chromatography these methods have quite high LOD values: 9.9–2000 and 60–7600 µg/l, respectively (Table 4) [81–88].

Several ELISA methods involving specific antigen/antibody interaction have been proposed for majority of pesticides with low LOD [89–95]. For the

detection of carbaryl, a LOD value as low as 0.01 μg/l has been achieved with ELISA [91]. For the detection of glyphosate, ELISA combined with a sample pre-treatment step enabled to achieve LOD value 0.1 μg/l [95]. The commercially available ELISA Plate Kit for glyphosate has LOD as low as 0.05 μg/l with detection time of 2 hours [96]. Compared to chromatography ELISA methods allow similar LOD values, but the quite long analyses time (1−4 h) prevents the implementation of ELISA for on-site applications [70,91–95].

1.4.3. Sample pre-treatment

The application of laboratory requires the transportation of samples to a laboratory for analysis. Sampling over an extended period of time can result in losses of easily degradable pesticides. For example glyphosate may degrade rapidly and bind differently to metals and colloids at different pH [97]. In that case sampling requires also fixing and storage procedures [97]. Refrigeration (4 ºC) and acidification (pH=2) has been recommended by Kylin to prevent glyphosate losses [97], but these condition are not suitable for all analytical methods.

Depending on the method of analysis extraction of pesticides from sample and extract clean-up may be implemented. Methods like liquid-liquid extraction, solid phase extraction and cation/anion exchange have been regularly employed for the extraction and pre-concentration of analytes before final analysis with chromatographic methods [49,92,98,99]. After separation small molecules which are highly ionic, poorly volatile and lack a chromophore require either pre- or post-column derivatization [49]. Detection of glyphosate at trace levels in environmental samples is difficult due to its zwitterionic nature and its complexation with metal ions [98]. Fluorenylmethylchloroformate (FMOC-Cl) is the most common pre-column derivatization reagent used in combination with chromatography and its reported reaction times with glyphosate range from 30 min to overnight [95,98–100].

Hsu et al. used quick extraction/preconcentration method prior to glyphosate detection with capillary electrophoresis and electrochemical detection with LODs 0.3 μg/l [101]. Due to the high specificity of alumina towards the phosphate groups of glyphosate, alumina-coated magnetic iron oxide nanoparticles as the affinity adsorbent have been used for micro-scale solid phase extraction [101]. This procedure took 5 min and magnetic nanoparticles were isolated from sample solution by employing an external magnet [101].

1.4.4. Biosensors for the detection of pesticides

In order to achieve capability for on-site specific testing of pesticides, biosensors are regarded as a prospective option. Considering the wide selection of potential natural & synthetic biorecognition elements for biosensors and signal transduction technologies (electrochemical, optical, piezoelectric and thermal)

which can be combined, numerous different biosensors have been proposed for the detection of pesticides [67,102,103].

1.4.4.1. Immunosensors

Immunosensors are analytical devices integrating immunoactive specific material (antibody, aptamer) with some transducer [103,104]. Depending on if labels are used or not, immunosensors are divided into two categories: labelled and label-free type sensors [104]. Widely used labels are enzymes, fluorescent markers, chemiluminescent dyes and nanoparticles [103,104]. In labelled immunosensors two different assay formats are employed: sandwich and competitive assay [103,104]. The most widely employed signal transduction method in immunosensors is optical [6,103,104], providing a facile, rapid, low-cost and sensitive detection of pesticides [6].

A portable label-free immunosensor based on surface plasmon resonance (SPR) technology has been accomplished for carbaryl analysis by Mauriz et al. This biosensor had LOD 1.38 μg/l and a complete assay cycle, including regeneration, was accomplished in 20 min [105]. A bismuth-based piezoelectric transducer immunosensor enabled to achieve LOD of carbaryl as low as 0.11 μg/l [106]. García et al. performed the immunodetection of carbaryl with two different acoustic wave label-free biosensors and obtained LODs 0.09 μg/l and 0.14 μg/l [107]. All these label-free biosensors for carbaryl detection were based on competitive assays and used monoclonal antibodies as specific immunoreagent.

Competitive assays have been also used for the detection of glyphosate. Lee et al. proposed two different immunosensors in which glyphosate coupled either with gold or Co-B/SiO_2/dye nanoparticles competed with free glyphosate for the available IgG type anti-glyphosate antibody binding sites [108,109]. In both cases the measurements were carried out in two different ways: by detection of fluorescence intensity and detection of single-probe DNA with an UV spectrophotometer with LODs respectively 10 and 0.046 μg/l. The analyses time with above biosensors was 2 h [108,109]. Glyphosate detection with carbon dot labeled antibody and antigen-magnetic beads complexes had LOD 8 μg/l with analysis time 1 h [110]. Ding et al. replaced anti-glyphosate antibodies with oligopeptides in surface plasmon resonance (SPR) assay, which showed high specificity, but a relatively high LOD value (98 μg/l) [111]. In this biosensor glyphosate was covalently immobilized on amino-activated silica beads with the help of some carbodiimide. Silica beads served as a solid support to prevent the loss of glyphosate during screening procedures with oligopeptides used for glyphosate biorecognition [111]. González-Martínez et al. applied glyphosate derivatization prior to the assay by acetylation [93]. This immunosensor system was designed to carry out automatically all the steps in flow manifold. Sample pretreatment step enabled to achieve low LOD value (0.021 μg/l) with analysis time less than 1 h [93].

1.4.4.2. Inhibition-based biosensors

In addition to immunosensors, inhibition-based biosensors have been developed for the detection of pesticides, although these are less specific than immunosensors [6].

Enzymes used in this kind biosensors include cholinesterases (AChE, BChE), tyrosinase (TYR), horseradish peroxidase (HRP), urease etc. [112]. The inhibition-based biosensors are not selective to a particular pesticide, but to several compounds. For example, the activity of AChE is inhibited by carbamates, but also by organophosphorus compounds [6].

The biggest number of inhibition-based biosensors has been developed for the detection of carbaryl employing inhibition of AChE. These biosensors are mainly based on electrochemical detection with LODs ≥ 0.15 μg/l [113–119]. Inhibition-based biosensors developed for the detection of glyphosate mostly employ inhibition of peroxidases and are based on electrochemical detection with LODs ≥ 0.16 μg/l [120–122]. The analysis time depends on the incubation time of the analyte and enzyme and remains mostly within 30 min [113–115,120–122].

A condensed summary of the major analytical methods for carbaryl and glyphosate detection is in Table 4. Although there are several methods with their pros and cons available for the detection of pesticides, there are still no methods for real-time *in situ* detection of pesticides, allowing automated monitoring and control of these compounds.

Table 4. A short comparison of methods used for the detection of glyphosate and carbaryl.

Glyphosate detection	**Derivatization, pretreatment**	**Analysis time, h**	**LOD, μg/l**	**Ref.**
GC/MS/MS, HPLC/Fluorimetry, HPLC/MS/MS	+	> 6	≥0.025	[69,77–79]
ELISA	+/−	> 1	≥ 0.05	[96]
Spectrophotometrical	+	> 1.5	≥ 9.9	[81–83]
Immunosensor	+/−	< 2	≥ 0.021	[93,108–111]
Inhibition based biosensor	−	~ 0.5	≥ 0.16	[120–122]
Carbaryl detection				
LC/UV, HPLC/MS/MS, HPLC/UV	+	> 1	≥ 0.01	[31,75,76]
ELISA	−	< 3	≥ 0.01	[89,91]
Spectrophotometrical	+	~0.5	2000	[87]
Immunosensor	−	~1	≥ 0.09	[105–107]
Inhibition based biosensor	−	~ 0.5	≥ 0.15	[113–119]

2. AIMS OF THE STUDY

The main goal of the present study was the development of biosensing platforms for the detection of different pesticides – carbaryl and glyphosate, potentially allowing quick and easy on-site monitoring of these compounds in natural water. The more detailed sub-points were as follows:

- Design and preparation of biosensing systems for the detection of carbaryl and glyphosate.
- Studies on the mechanisms of reactions going on in biosensing systems.
- Establishing optimal protocols for the detection of these pesticides.
- Examination of sample pre-concentration and stabilization options for biosensor-based analyses to increase sensitivity and reliability of results.
- Comparison of the results obtained with biosensor and a standard method (HPLC/MS).
- Testing of biosensor applicability in real or spiked samples.

3. EXPERIMENTAL

3.1. Design and set up of biosensing systems for the detection of carbaryl and glyphosate

The studied biosensors for carbaryl and glyphosate were based on different bio-recognition and signal detection principles. The biosensing system for carbaryl was based on inhibition of tyrosinase by carbaryl. It comprised soluble tyrosinase (EC 1.14.18.1) and a cylindrical membrane-covered Clark-type amperometric oxygen sensor (Elke Sensor LLC), following the decrease of dissolved oxygen concentration (DOC) in samples which was caused by tyrosinase-catalyzed oxidation of tyrosine [I] (Fig. 1).

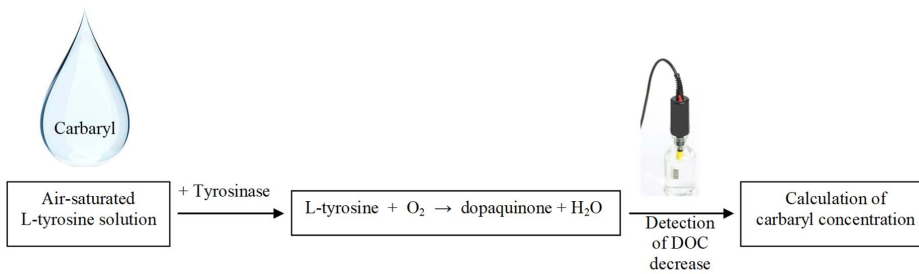


Figure 1. Scheme of carbaryl detection system based on inhibition of tyrosinase by carbaryl.

The biosensing system for glyphosate was based on the detection of fluorescence signal generated by quantitative replacement of glyphosate by 5-carboxytetramethylrhodamine (5-TAMRA) conjugated glyphosate in glyphosate/anti-glyphosate antibody immunocomplexes, which were attached onto bioactivated microbeads (Fig. 2).

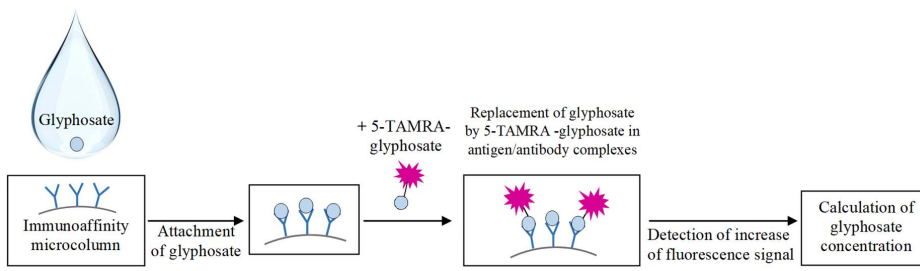
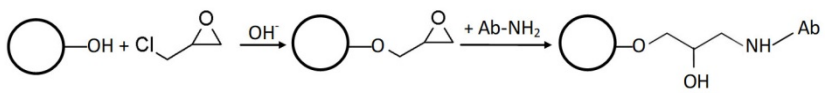


Figure 2. Scheme of glyphosate immunosensing system based on bead injection analysis.

These complexes were formed on microbeads, decorated with covalently bound anti-glyphosate antibodies, and forming single-use renewable micro-columns for the attachment of glyphosate from flows. The measurements were carried out on FIAlab 3500B system (FIAlab Instruments) and the fluorescence intensity of bound 5-TAMRA-glyphosate (5-T-g) was detected with an OceanOptics 2000+ spectrometer (with advanced electronics and extended 200 µm wide slit). For light transmission, fibres with core diameter of 400 µm were used. The anti-glyphosate IgY-type antibodies (Agrisera AB, AS132739) were immobilized onto microbeads (Sephadex G50 Medium, d=50–150 µm) with the help of epichlorohydrin [II]:



(1)

3.1.1. Synthesis of 5-TAMRA-glyphosate

5-T-g was synthesized from glyphosate tetrabutylammonium salt according to the following scheme [II] (Fig. 3). The product was purified directly with reverse phase flash-chromatography and analyzed with HPLC-MS. The synthesized 5-T-g was stored at -18 °C and dissolved in Dulbecco's phosphate-buffer on the day of the experiment [II].

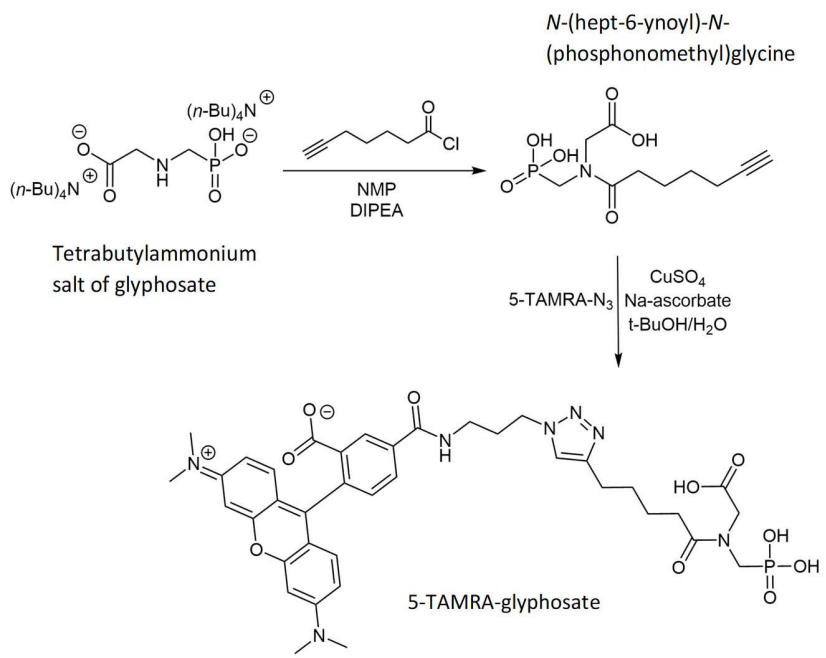


Figure 3. Synthesis of 5-TAMRA-glyphosate.

3.1.2. Characterization of anti-glyphosate antibody affinity towards 5-TAMRA-glyphosate

The anti-glyphosate antibody affinity towards 5-TAMRA-glyphosate was characterized using fluorescence anisotropy (FA) method. FA measurements were carried out in the kinetic mode with PHERAstar (BMG Labtech, Germany) microplate reader, using an optical module with 540 nm (excitation) and 590 nm (emission) Filters. Black 96-well flat bottom polystyrene nonbinding (NBS) surface microplates (Corning, Product No.3993) were used in all experiments [II].

The specific binding of 5-T-g to anti-glyphosate antibodies was measured in the absence or presence of an excess of non-labelled glyphosate, and the difference between these values was defined as specific binding [II]. For the measurement of dissociation, the excess of non-labelled glyphosate was added to the reaction medium after the equilibrium was reached. The binding affinity of 5-T-g to antibody was calculated from binding experiments, where the probe concentration was kept fixed and the antibody concentration was varied [II]. The FA signal at time moment *t* was calculated according to equation (2):

$$FA(t) = \frac{I(t)_{II} - I(t)_{\perp}}{I(t)_{II} + 2 \cdot I(t)_{\perp}} \quad (2)$$

where $I(t)_{\parallel}$ and $I(t)_{\perp}$ correspond to fluorescence intensities of parallel and perpendicular components of emitted light [II].

The K_D value, characterizing the affinity of 5-T-g binding was calculated with GraphPad Prism™ 5.04 (GraphPad Software, Inc., San Diego, CA, USA) according to a scheme proposed earlier [123] [II].

3.2. Pesticide assessment protocols

All kinetic measurements for the detection of carbaryl were carried out in air-saturated solutions under continuous stirring in an airtight thermostated glass cell (V=35 ml) at 25 ^{o}C. When the oxygen sensor output had stabilized after its immersement into the reaction medium containing L-tyrosine (final concentration 0.15 mM) and carbaryl in 0.1 M phosphate buffer solution (PBS) (pH=6.50), the reaction was started by injection of 100 µl of soluble tyrosinase (final concentration 7 µg/ml). Changes of dissolved oxygen concentration, dependent on carbaryl concentration were recorded with the interval of 1 s; the collected data was normalized (I_t/I_0) and debugged. The tyrosine oxidation reaction was characterized with the kinetic (coefficient *B*) or the whole signal change parameter (coefficient *A*), both calculated according to the dynamic biosensor model [124]:

$$\frac{I(t)}{I_o} = A\exp(-Bt) - 2A\sum_{n=1}^{\infty}(-1)^n \frac{\tau_s}{n^2/B - \tau_s}\left[\exp(-Bt) - \exp\left(-n^2\frac{t}{\tau_s}\right)\right] + C \quad (3)$$

We used SigmaPlot®5.0 (SPSS Software, USA) and GraphPad Prism®3.0 (GraphPad Software, San Diego, CA, USA) for data analysis and calculation of biosensor calibration parameters [I].

For the detection of glyphosate, an earlier immunoassay protocol in a similar BIA-based biosensor set-up [125,126] was modified, and optimal experimental conditions according to the specific characteristics of glyphosate measurements found. To obtain a detectable change in the biosensor signal, the amount of 5-T-g for the replacement of bound glyphosate was found by varying the concentration of 5-T-g from 0.1 to 1.9 μM. In addition, we modified the incubation times for the attachment of glyphosate to anti-glyphosate antibodies on microbeads and for the replacement of glyphosate by 5-T-g from 10 to 600 sec. This step was of major importance as glyphosate and 5-T-g have different affinities towards anti-glyphosate antibodies. Finally, the optimal amount of phosphate buffer saline (PBS; 0.1 M, 0.15 M NaCl; pH 7.2) and its flow rate for removing the unbound 5-T-g were found. All measurements were carried out at room temperature [II].

3.3. Validation and testing of biosensor results

3.3.1. Carbaryl biosensor

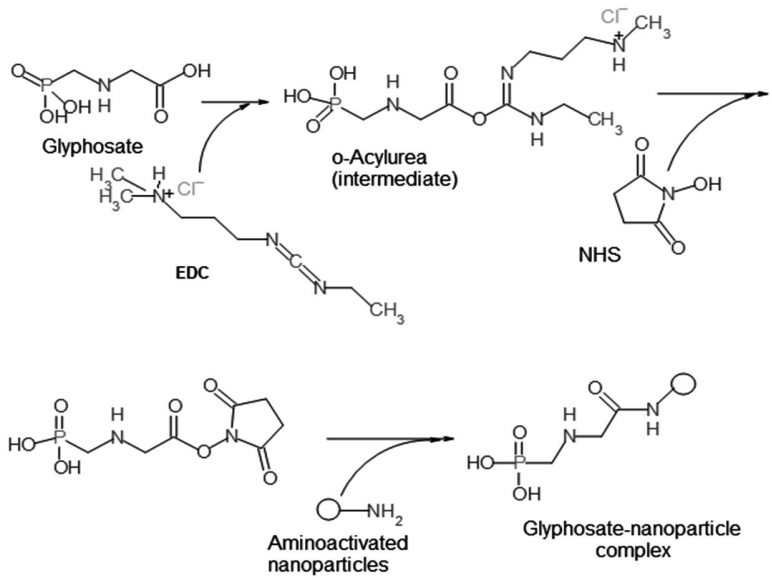
The carbaryl biosensor was tested with the potato peel removed from the washed potato by grating as thinly as possible. Grated wet mass of potato was added into the reaction medium and transferred to a measuring cell with PBS (0.1 M, pH 6.5). Dry matter of the grated potato crust was 16.44% (dried at 60 °C in an oven) [127].

3.3.2. Glyphosate biosensor

The results obtained with glyphosate immunoassay were validated with high-performance liquid chromatography (Infinity 1290, Agilent Technology), using tandem mass spectrometer with Agilent Jet stream electrospray ionization source (Agilent 6490) in spiked surface water samples from the river Valgejõgi collected according to ISO standard 5667-6 [II].

3.4. Concentration and stabilization of glyphosate in water samples with aminoactivated nanoparticles

In order to increase the sensitivity and reliability of glyphosate analysis, amino-activated nanoparticles were used for preconcentration and stabilization of glyphosate-containing samples. Glyphosate was attached onto two types of solid particles – aminoactivated silica beads (ASB, d=30 μm) and amino-activated magnetic iron oxide nanoparticles (AFeNP, d=14 nm). For the activation of glyphosate carboxyl groups, EDC (N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride) and NHS (N-Hydroxysuccinimide) chemistry was used:



(4)

Glyphosate attachment was carried out at room temperature within 1–120 minutes. Unbound glyphosate was removed with consecutive washing with 0.1% SDS solution and DI water [III]. The efficiency of glyphosate binding was determined with a Shimadzu UV-1800 spectrometer using the ascorbic acid method [128] [III].

4. RESULTS AND DISCUSSION

4.1. Carbaryl biosensor

The carbaryl biosensor was based on detection of the inhibiting effect of carbaryl on the catalytic activity of soluble tyrosinase. As the biosensor dynamic response was used instead of the steady-state signal for the detection of carbaryl, the inhibition kinetics was carefully characterized [I].

The decrease of DOC in time due to the tyrosinase-catalyzed oxidation of tyrosine was a typical S-shaped curve, which dynamics at constant initial tyrosine (0.15 mM was optimal for this study) and oxygen (0.26 mM) concentrations was dependent on the concentration of carbaryl (Fig. 4) [I].

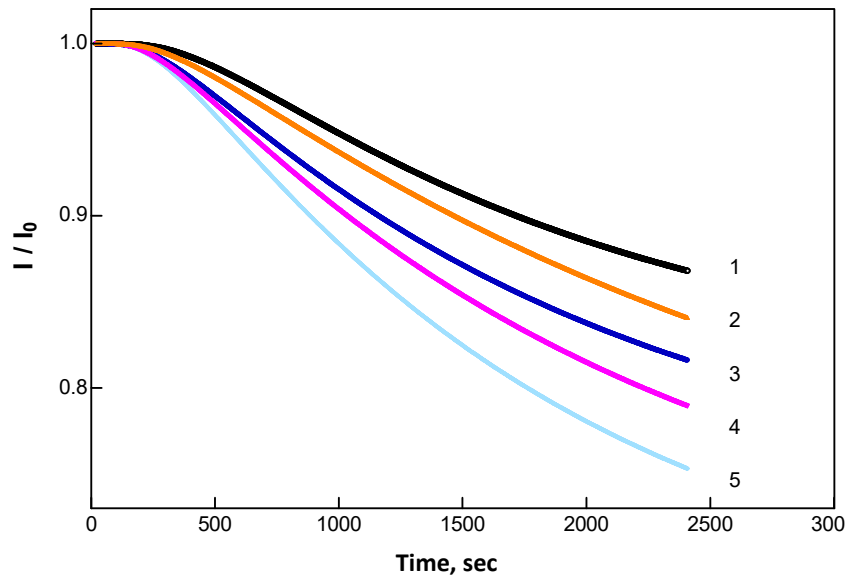


Figure 4. Normalized biosensor output current at different concentrations of carbaryl: (1) 0 mg/l; (2) 0.3 mg/l; (3) 1 mg/l; (4) 0.5 mg/l; (5) 0.7 mg/l). Experiments were carried out under constant stirring at 25 ^{o}C at tyrosinase concentratsion of 7 μg/ml and tyrosine concentratsion of 0.15 mM in PBS (0.1 M, pH 6.5).

These curves comprised of the initial "lag" period and the decrease of oxygen concentration due to the tyrosinase-catalyzed oxidation of tyrosine. The biosensor output curves obtained were characterized using a dynamic biosensor model (Eq. 3) [124]. The initial delay was caused both by the inertia of the oxygen sensor and the formation of the active form of tyrosinase, described in detail by Ramsden et al. [129]. The length of this "lag" period, calculated as the factor τ_s was in the range 180–300 s and its value did not depend on carbaryl

concentration. The reaction was characterized with two different process parameters – total signal change (parameter *A*) and the initial maximum slope of signal decrease (kinetic parameter *B*), which were calculated with high correlation coefficient (r>0.97) and irrelevant deviations of experimental data from the model (P>>0.05) [I]. Both parameters *A* and *B* were used to study the effect of the carbaryl concentration on the reaction dynamics. The curve of the kinetic parameter *B* as a function of carbaryl concentration at tyrosine concentration 0.15 mM is shown in Figure 5. This curve is linear up to carbaryl concentration 1.5 mg/l and has a flat, irregularly shaped maximum in the carbaryl concentration range between 3–6 mg/l [I].

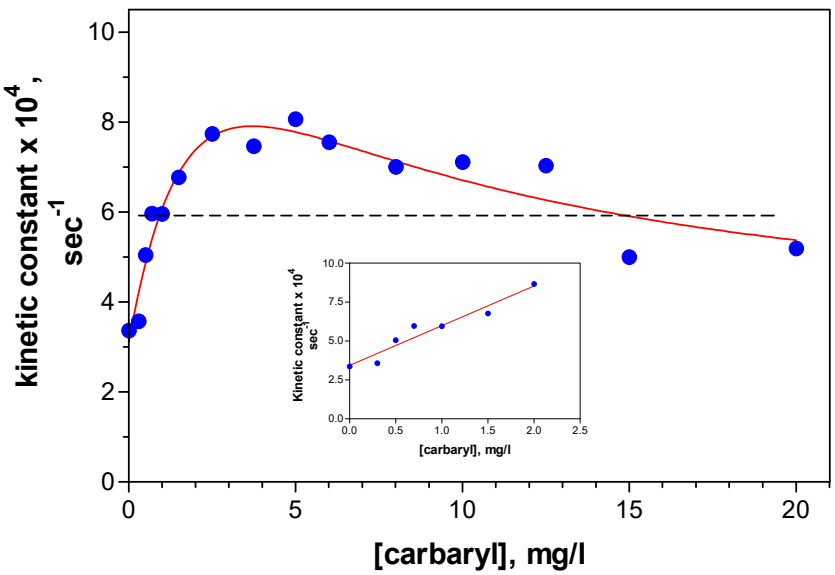


Figure 5. Calculated kinetic parameter at different carbaryl concentrations. Experiments were carried out under constant stirring at 25 °C at tyrosinase concentratsion of 7 µg/ml and tyrosine concentratsion of 0.15 mM in PBS (0.1 M, pH 6.5).

At all measured concentrations of carbaryl the calculated kinetic parameter *B* was greater than in the absence of carbaryl in the reaction mixture, indicating that carbaryl behaved not as an inhibitor against tyrosinase but as an inhibitory substrate. The reaction mechanism of carbaryl inhibition and the corresponding rate equation are analogous to these usually considered for uncompetitive inhibition:

$$B = \frac{V' c_{carbaryl}}{K'_M + c_{carbaryl} + {(c_{carbaryl})^2}/{K_i}} \quad (5)$$

where V' and K'_M correspond, but are not equivalent to Michaelis-Menten parameters, B is the kinetic parameter [I]. Based on the data received the dependence $1/B$ vs $1/c_{\text{carbaryl}}$ was also constructed. The obtained hyperbola indicates that carbaryl acts as an inhibiting substrate on tyrosinase and this may be the reason why in some cases it has not been possible to determine low carbaryl concentrations with biosensors using kinetic measurements [117] [I].

Carbaryl concentration cannot be directly determined from the kinetic parameter because to each value of the kinetic parameter correspond two different carbaryl concentrations (Fig. 5). Therefore additional criteria need to be used. The total signal change (parameter A) calculated from output signal of a biosensor along with parameter B suits best for this purpose. The dependence of total signal change A on carbaryl concentration is shown in Figure 6. Parameter A depends on the carbaryl concentration hyperbolically, with the apparent values for the half-limiting value constant $K_{1/2}$=7.8±4.4 mg/l and limiting value A_{max}=0.21±0.04 [I].

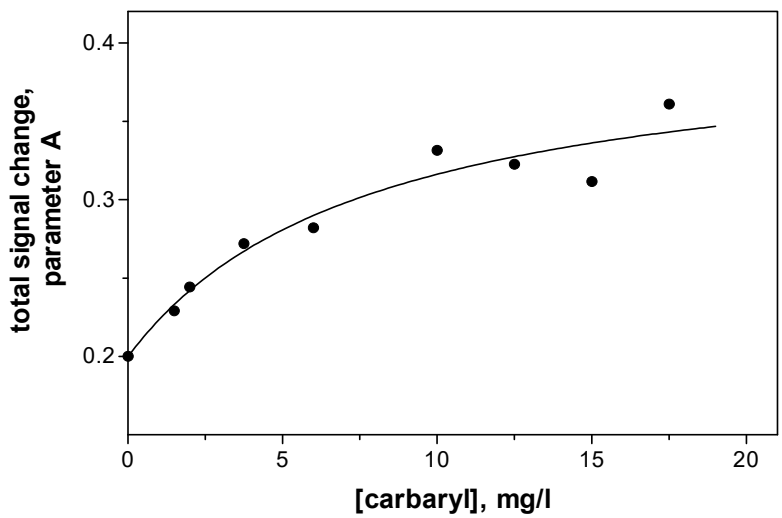


Figure 6. Calculated total signal change at different carbaryl concentrations. Experiments were carried out under constant stirring at 25 °C at tyrosinase concentratsion of 7 μg/ml and tyrosine concentration of 0.15 mM in PBS (0.1 M, pH 6.5).

For the determination of carbaryl concentrations it is reasonable to use these two calculated independent parameters simultaneously. From Figure 5 it can be seen that for concentrations of carbaryl below 1 mg/l and above 15 mg/l, the

calculated kinetic parameter B value is less than 6×10^{-4} sec^{-1} and higher at carbaryl concentrations between 1 and 14 mg/l. So, if $B<6\times10^{-4}$ sec^{-1}, then it is necessary to analyse parameter A (Fig. 6). If the concentration of carbaryl in the solution is about 1 mg/l based on both calculated parameters, the concentration of carbaryl is determined from the initial slope of the curve of kinetic parameter as a function of carbaryl concentration (Fig. 5) [I]. The scheme shown in Figure 7 illustrates data analysis and determination of carbaryl concentration.

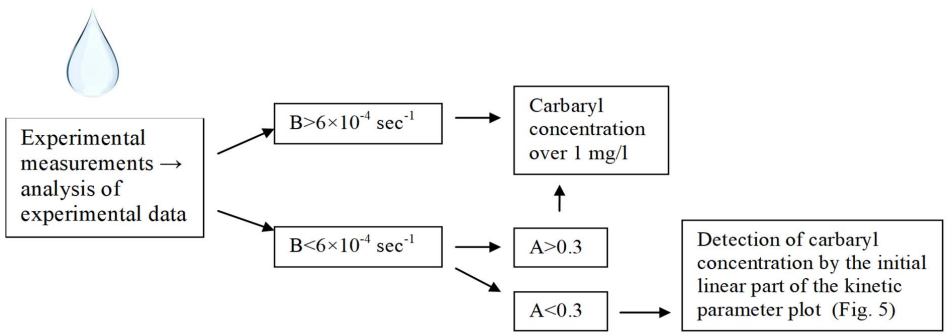


Figure 7. The scheme for the determination of carbaryl.

The limit of detection for carbaryl was 0.2 mg/l calculated as the carbaryl concentration corresponding to the signal, exceeding the background signal by 3 standard deviation values. The biosensor allowed to measure carbaryl residues in drinking water on level, suggested by The US Health Advisory Program (0.4 mg/l) and in livestock water on level, which is recommended in Canada (1.1 mg/l) [41,43]. The process of analysis is quite quick (< 1 h) and simple, and can be automated.

4.2. Glyphosate biosensor

The glyphosate biosensor was based on a totally different detection principle – competitive immunoanalysis, which unlike inhihibition-based biosensors allows very selective detection of the selected analyte.

For the attachment and preconcentration of glyphosate from samples we used anti-glyphosate antibodies immobilized onto microbeads. Glyphosate in its complex with anti-glyphosate antibody was replaced by synthetic labeled conjugate. For this purpose 5-TAMRA-glyphosate was synthesized [II]. The fluorescence marker was conjugated to the secondary amino group of glyphosate to mimic the immunogenic glyphosate conjugates used for the production of anti-glyphosate antibodies.

As conjugation affects also antigen's binding properties to antibody, the binding properties of 5-T-g to the anti-glyphosate antibody were studied using the FA method (Fig. 8) [II].

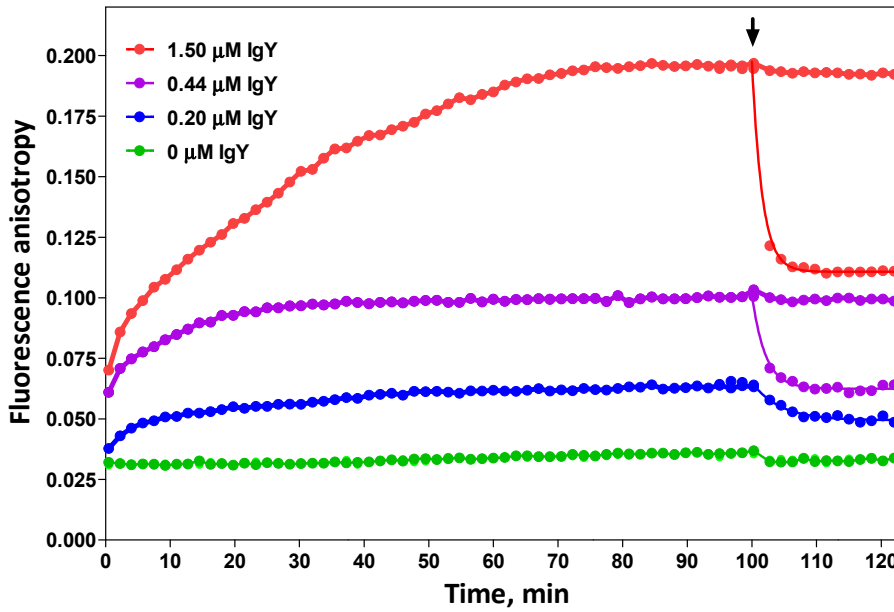


Figure 8. Time course of anisotropy change caused by the binding of 5-T-g (10 nM) to anti-glyphosate antibody. After 100 min incubation, the dissociation of the formed antigen-antibody complex was initiated by the addition of glyphosate (final c=2.5 mM, lines correspond to the one-phase exponential decay fits). FA(t) values were calculated according to Eq. 2.

The FA data was used for the determination of steady state values of total and nonspecific binding affinities of 5-T-g at different antibody concentrations and building of binding curves, as described earlier [123]. These binding curves (Fig. 9) allowed to estimate the K_D value 2.9±0.2 µM for 5-T-g which is quite moderate compared to common nanomolar affinities of antigen-antibody interaction, probably due to the relatively small size of the antigen [II].

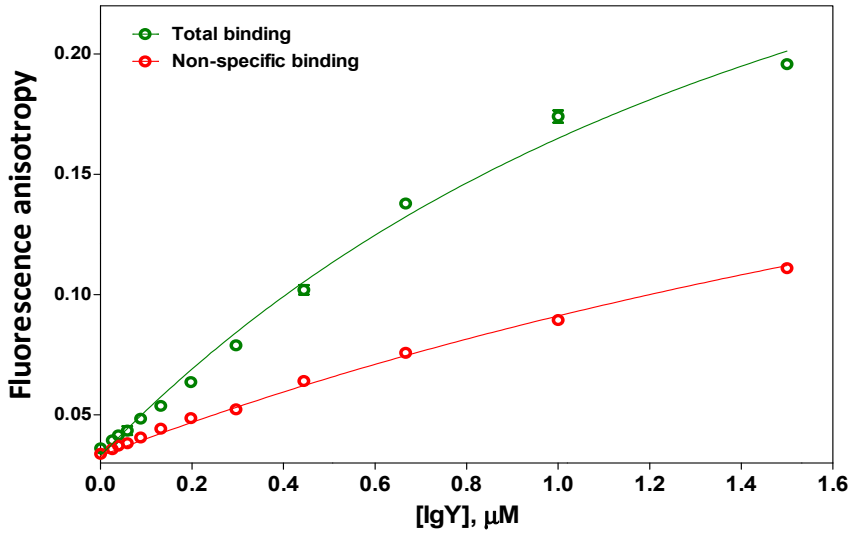


Figure 9. Binding curves of 5-T-g to anti-glyphosate antibody. 5-T-g (10 nM) was incubated with different concentrations of anti-glyphosate antibody in the absence (total binding) and presence (non-specific binding) of 2.5 mM glyphosate. FA (t) values were calculated according to Eq. 2 from data collected after 100 min incubation at 25 ºC. The data is shown as the mean ± the standard deviation of two independent experiments.

Lower affinity of glyphosate towards anti-glyphosate antibody compared to 5-T-g was considered for the optimization of the glyphosate immunoassay. So, 30 min incubation time was used for the attachment of glyphosate to the bio-activated beads to produce measureable signals. This was 10 times longer compared to a similar BIA-based biosensor based on nanomolar range affinities [126].

The optimal concentration of 5-T-g was found by varying the concentration of 5-T-g from 0.1 to 1.9 µM. Based on the obtained results, it was found that the signal of 5-T-g was sufficiently quantifiable at 0.5 µM and this concentration was used in the following measurements [II]. Resulting from the different affinities of glyphosate and 5-T-g, the determination of the optimal incubation time of 5-T-g for the replacement of glyphosate was of major importance. The incubation time was varied from 10 to 600 sec. In our set-up, the optimal incubation time of 5-T-g for the replacement of glyphosate was 10 sec, leading to quantitative partial displacement of glyphosate [II].

The amount of PBS and its flow rate for removing the unbound 5-T-g was also optimized. It was found that washing with 40 µl buffer with flow rate 5 µL s^{-1} was sufficient to remove all unbound 5-T-g [II]. At lower flow rates the efficiency of washing did not improve and at higher flow rates the microcolumn became unstable. The total time for glyphosate analysis using the modified protocol, was 37 min.

The dependence of the biosensor output, which was calculated as a difference of average signal of intensity before adding 5-T-g and after removing unbound 5-T-g, on glyphosate concentration is shown in Fig. 10 [II].

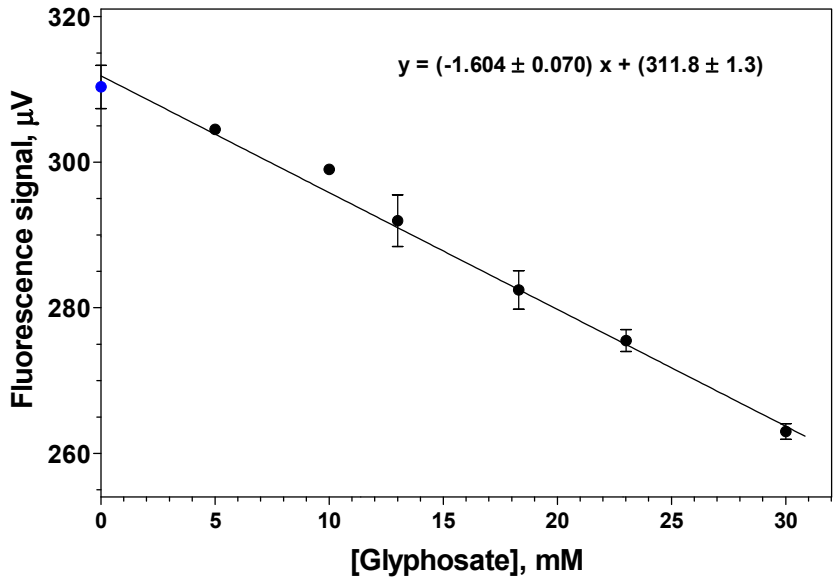


Figure 10. Dependence of the biosensor signal on glyphosate concentration in PBS (0.01 M, pH 7.2, 0.15 M NaCl); [5-T-g]=0.5 µM. • – background signal ([5-T-g]=0.5 µM) with no glyphosate present.

The calibration plot was linear in the whole studied glyphosate concentration range up to 30 mM. Relatively low affinity which was evaluated by the FA method allows sensing of glyphosate in millimolar concentration range. The LOD value for glyphosate was 2.4 mM (406 mg/l), calculated as the glyphosate concentration corresponding to the signal, exceeding the background signal by 3 standard deviation values. The limit of quantification, which signal exceeds the background by 10 standard deviation values, was 8.1 mM (1369 mg/l). The obtained LOD (406 mg/l) and LOQ (1369 mg/l) values for direct glyphosate detection are considerably high, however the proposed immunoassay allows to measure glyphosate residues in water on levels, which are relevant to assure safety on the site of glyphosate application [II].

4.3. Applicability of pesticide biosensors

For the detection of carbaryl with biosensor, preliminary studies were carried out in potatoes, as the major route of carbaryl intake for the general population is food [130]. Potatoes contain tyrosine and also tyrosinase naturally, so the effect of potato test samples on the reaction parameters was measured [127, 131]. First we studied the dependence of the total signal change *A* on the concentration of tyrosine added to the potato, and obtained a linear dependence (Fig. 11 A) [127]. While no potato was added and measurements were made at a specific tyrosinase concentration, the total change in the signal was hyperbolically dependent on the tyrosine concentration (Fig. 11 B) [131].

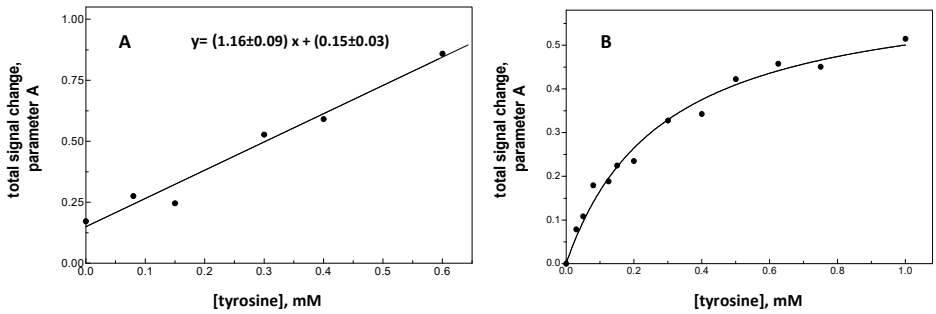


Figure 11. Dependence of total signal change parameter *A* on tyrosine concentration. Experiments were carried out under constant stirring at 25 °C in PBS (0.1 M, pH 6.5). **A**: with grated potato peel (17.3 mg/ml), [tyrosinase]=0 μg/ml, [carbaryl]=0 mg/l; **B**: without grated potato peel, [tyrosinase]=7 μg/ml, [carbaryl]=0 mg/l.

The difference is due to the fact that the potato contains both tyrosine and tyrosinase and the reaction is triggered by the addition of the potato peel to the reaction cell. Based on the change of parameter *A*, it was calculated that the activity of tyrosine and tyrosinase in 1 g grated potato crust corresponded to 0.09 mM tyrosine in the presence of 7 μg/l tyrosinase [127]. The dependence of the calculated total signal change (parameter *A*) on the concentration of potato in the samples was a linear function with a slope $(1.46\pm0.04)\times10^{-2}$ l/g (R=0.95) (Fig. 12) [131].

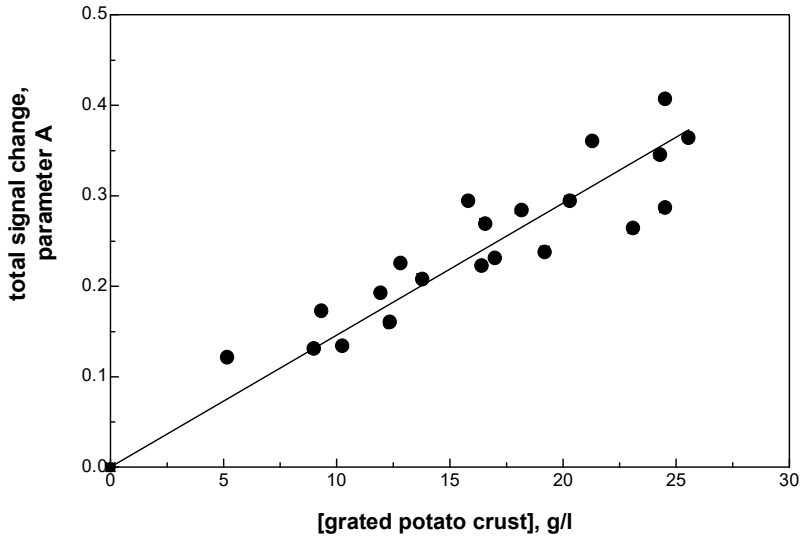


Figure 12. Dependence of total signal change parameter *A* on grated potato mass. Experiments were carried out under constant stirring at 25 °C in PBS (0.1 M, pH 6.5).

The applicability of the glyphosate biosensor was tested in the surface water samples and validated with a standard HPLC method [II]. Both methods resulted in 6–37% less glyphosate in the spiked with glyphosate surface water samples than the actual concentration. This can be explained with the fast degradation of glyphosate and the absorbance of glyphosate onto the vials. Though, all biases with both methods were below 50% which is an acceptable limit for such a problematic analyte as glyphosate. The results of glyphosate detection in surface water with the immunobiosensor and HPLC method were in good correlation and indicate the applicability of the glyphosate immunoassay for the detection of glyphosate in surface water [II].

4.4. Stabilization and concentration of samples

To improve the sensitivity of glyphosate biosensor and enhance the reliability of glyphosate quantitative analyses, we studied options for the stabilization and concentration of glyphosate samples.

This method was based on the attachment of glyphosate onto microparticles. Two different types of aminoactivated solid beads – silica microparticles and magnetic iron oxide nanoparticles were used. Glyphosate immobilization on the aminoactivated beads was carried out using EDC/NHS chemistry. The optimization of the immobilization process was focused on the production of reproducible amounts of bound glyphosate within minimal time. Two operational parameters were optimized: to minimize the incubation time for the attachment

of glyphosate, and achieve the most effective procedure for removing of all unbound glyphosate from the mixture [III].

For the separation of the unsoluble particles with attached glyphosate, two different techniques were used. Magnetic extraction was used for AFeNP-s and vacuum filtering for ASBs. The magnetic separation was very effective and fast, vacuum filtering for ASBs had to be repeated for 10 times [III]. Next we optimized time, required for glyphosate immobilization onto particles. Glyphosate recovery after 1–120 minutes immobilization showed that the maximum immobilization yield was achieved already within 1 minute incubation with ASB. For AFeNPs, the shortest time for detectable quantitative attachment of glyphosate was 10 minutes [III].

Glyphosate binding graphs were prepared for both ASB and AFeNP (Fig. 13).

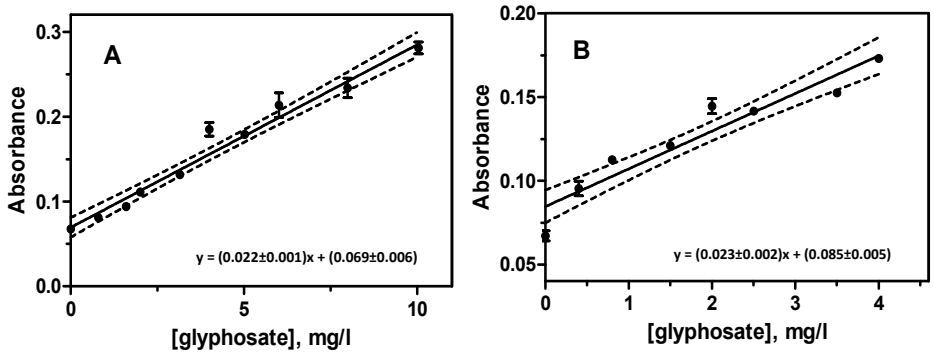


Figure 13. Glyphosate binding onto **A**: aminoactivated silica beads; [ASB]=200 mg/l; **B**: AFeNP; [AFeNP]=20 mg/l. The immobilization of glyphosate was carried out at room temperature in 10 mM HEPES buffer at pH 7.0.

The concentration of ASBs (200 mg/l) used for immobilization was taken as proposed in the literature [111]. The dependence of glyphosate recovery on the concentration of AFeNPs was studied in the AFeNPs concentration range 20–80 mg/l. We found that this function was linear and AFeNPs concentration 20 mg/l was the minimal concentration to get reliable results for glyphosate immobilization studies [III].

Calibration plots for ASB and AFeNP were linear over the range of glyphosate concentrations studied (Fig. 13). The detection limits were quite high, resulting from the detection limit of phosphorus by the applied ascorbic acid method (0.1–1 mg/l) [128]. The slope of calibration curve defines the sensitivity of detection, and it was similar for ASB and AFeNP, although the concentration of aminoactivated silica beads was 10 times higher than that of AFeNPs. In order to increase the sensitivity of the system, the amount of nanoparticles can be increased [III].

To characterize the binding capacity of ASB and AFeNP particles, we assessed the number of amino groups per particle available for glyphosate immobilization. Calculations based on measured maximal recovery of glyphosate revealed that both ASB and AFeNP particles had approximately 1000 amino groups per particle [III].

The study indicated that both types of aminoactivated solid beads can applied for the stabilization and concentration of glyphosate-containing probes. Comparing the total time required for immobilization and washing procedures, this is considerably lower for AFeNPs due to the very effective separation of unbound glyphosate. Accordingly, the application of AFeNPs has a high potential to be integrated with on-line and *in situ* analyses for monitoring of glyphosate [III].

We also studied the stability of glyphosate-AFeNP complexes. These complexes were stable for at least one week at room temperature and pH 7.0, which is sufficient for the transport or suspended analyses of samples [132].

CONCLUSIONS

Two basically different biosensing systems for the detection of 2 problematic pesticides – carbaryl and glyphosate have been developed. In addition, a method for pre-concentration and stabilization of glyphosate from samples analyses onto microbeads has been proposed.

The biosensing system for carbaryl was based on inhibition of tyrosinase by carbaryl. The biosensor output signal was analyzed with the biosensor dynamic model, which allowed rapid calculation of kinetic and steady state parameters from pre-equilibrium data. Study on the alteration of tyrosinase activity by carbaryl indicated that carbaryl acted as an inhibitory substrate of tyrosinase and at low concentrations accelerated tyrosinase-catalyzed oxidation of tyrosine by dissolved oxygen. For the determination of carbaryl, it was necessary to use two independent reaction parameters combined. This combined use of two parameters allowed to use the biosensor for carbaryl detection within concentration range 0.1–20 mg/l. The limit of detection for carbaryl was found to be 0.2 mg/l.

An immunoassay for glyphosate detection, based on the displacement of glyphosate in its complex with anti-glyphosate antibody by 5-TAMRA-glyphosate was proposed. The antigen/antibody complexes were formed on microbeads, with the covalently bound anti-glyphosate antibodies. The beads formed single-use renewable micro-columns for the attachment of glyphosate from flows. The measurements were carried out on flow injection analysis system and the fluorescence intensity of bound 5-TAMRA-glyphosate was detected. Optimal experimental conditions were found by varying the concentration of 5-TAMRA-glyphosate, incubation time, etc. Using the optimized protocol, the dependence of the biosensor signal on glyphosate concentration was studied both in buffer and surface water samples. The limit of detection of this biosensor, determined by the relatively low affinity of glyphosate towards anti-glyphosate antibody, is in millimolar concentration range. This is sufficient to apply the proposed immunoassay for *in situ* glyphosate analysis for timely detection of glyphosate pollution in water reservoirs before glyphosate dilution and degradation and assure relevant safety levels. The main advantage of the method is the fact that the sample pre-treatment step is not required and the results are obtained in about half an hour.

The biosensor applicability was tested in spiked samples and compared with HPLC standard method. Both methods resulted in less glyphosate in the spiked with glyphosate surface water samples than the actual concentration The results of glyphosate detection in surface water obtained with the immunobiosensor and HPLC method were in good correlation and indicated the applicability of the glyphosate immunoassay for the detection of glyphosate in surface water.

The carbaryl biosensor was tested in grated potato mass, which naturally contains both tyrosine and tyrosinase. Activities of these compound should be taken into account when inhibition based biosensor are used for carbaryl determination in potato mass.

In order to increase sensitivity of glyphosate assessment, a preconcentration step is often integrated. Binding of glyphosate to a solid support not only helps to concentrate the samples, but also improves the stability of glyphosate, since glyphosate may degrade during sampling and analysis. Two different types of aminoactivated solid beads – silica microparticles and magnetic iron oxide nanoparticles were used for the attachment of glyphosate. Binding of glyphosate to aminoactivated beads was carried out via carboxylic group of glyphosate using EDC/NHS chemistry. The study indicated the high potential of AFeNPs for the application of stabilization and concentration of glyphosate-containing probes for in situ analysis and continuous monitoring of glyphosate, as all necessary procedures could be carried out with high efficiency within 20 minutes. In addition, the obtained results indicated that the AFeNP/glyphosate complexes were stable for at least one week at room temperature.

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SUMMARY IN ESTONIAN

Biosensorsüsteemid pestitsiidide määramiseks veeproovides

Pestitsiidid on ained, mida kasutatakse peamiselt põllumajanduses, aga ka muudes sektorites kahjurite (putukad, taimehaigused, umbrohud jne) tõrjeks [1,2]. Ülemaailmne pestitsiidide kasutamine kasvab pidevalt: 2017. aastal müüdi kogu maailmas kokku umbes 3.6 miljonit tonni pestitsiide [2,3]. Enamik kasutatud pestitsiide võivad reostada vett ja pinnast ning avaldada kahjulikku mõju elusorganismidele [4]. Võimalike ohtude kõrvaldamiseks elusorganismidele on enamiku pestitsiidide jaoks kehtestatud joogi- ja pinnavees lubatud sisaldused. Euroopa Liidus on näiteks iga üksiku pestitsiidi lubatud piirsisaldus joogivees 0.1 μg/l [5]. Pestitsiidide määramiseks veeproovidest kasutatakse käesoleval ajal peamiselt kromatograafilisi meetoditeid, rakendades UV-Vis, fluorestsents- ja/või massispektromeetrilisi detektoreid. Nende meetoditega saavutatakse küll madalad määramispiirid, kuid need on keerukad ja aeganõudvad ega sobi *in situ* ja on-line monitooringuks [6]. Kromatograafiliste meetodite kõrval on üheks alternatiiviks biosensorite kasutamine. Biosensorid võimaldavad kiiremaid, odavamaid ning reaalajas kohapeal läbiviidavaid analüüse.

Käesoleva doktoritöö eesmärgiks oligi välja töötada biosensorsüsteemid kahe erineva probleemse pestitsiidi – karbarüüli ja glüfosaadi määramiseks veeproovidest, mis võimaldaks nende ühendite kiiret kohapealset määramist. Lisaks uuriti võimalusi glüfosaadi eelkontsentreerimiseks ja stabiliseerimiseks veeproovides, mida on võimalik kasutada koos biosensoritel põhineva glüfosaadi selektiivse detekteerimisega. Läbiviidud uuringute käigus töötati välja biosensorsüsteemid karbarüüli ja glüfosaadi määramiseks. Biosensorisüsteemide konstrueerimisel uuriti nende tundlikkust, tööpiirkonda ja sobivust rakendamiseks looduslikus vees. Lisaks uuriti biosensorsüsteemis toimuvaid reaktsioonimehhanisme.

Karbarüüli biosensorsüsteem koosnes hapnikuandurist ja sellega kontaktis olevast ensüümist – türosinaasist. Karbarüüli määramine põhines türosinaasi inhibeerimisel karbarüüli poolt. Biosensori väljundvoolude analüüsimiseks kasutati biosensorite dünaamilist mudelit, mis võimaldas tasakaalueelse oleku andmetest erinevate reaktsiooni iseloomustavate parameetrite arvutamist. Karbarüüli poolt põhjustatud türosinaasi aktiivsuse muutuse põhjal leiti, et karbarüül käitub türosinaasi suhtes pigem inhibeeriva substraadina ja madalatel kontsentratsioonidel kiirendab türosinaasi poolt katalüüsitavat türosiini oksüdatsiooni lahustunud hapniku toimel. Biosensori dünaamilise mudeli alusel arvutatud kineetilise parameetri *B* sõltuvusel karbarüüli kontsentratsioonist on iseloomulik lame asümmeetriline maksimum ja reaktsioonimehhanism on analoogne mehhanismile, mis tavaliselt iseloomustab mittekonkurentset substraadi inhibitsiooni. Karbarüüli kontsentratsiooni määramiseks ei olnud kineetiline parameeter üksinda piisav ja seetõttu kasutati lisaks teist mudeli alusel arvutatud sõltumatut parameetrit, statsionaarse oleku parameetrit *A*. Kahe parameetri kombineeritud kasutamine võimaldas biosensoriga määrata karbarüüli kontsentratsiooni vahe-

mikus 0.1–20 mg/l. Karbarüüli määramispiiriks saadi 0.2 mg/l [I]. Läbi viidi ka eelkatsed karbarüüli määramise võimaluste uurimiseks riivitud kartulimassis.

Glüfosaadi biosensor põhines konkureerival immunoanalüüsil ja selle määramiseks kasutati antigeen/antikeha bioäratundmissüsteemi kombineerituna graanulsisestusanalüüsiga. Esmalt koguti glüfosaat mikrograanulitele, mille pinnale olid immobiliseeritud glüfosaadi vastased antikehad. Seejärel asendati graanulile seondunud glüfosaat fluorestsentsmärgisega glüfosaadiga, ning mõõdeti selle fluorestsentsintensiivsust. Glüfosaadi määramispiiriks saadi 406±17 mg/l, mis on piisav glüfosaadireostuse *in situ* tuvastamiseks enne glüfosaadi lahjenemist ja lagunemist [II]. Kõrge määramispiiri määras glüfosaadi suhteliselt madal afiinsus glüfosaadi vastaste antikehade suhtes, mida hinnati fluorestsents anisotroopia meetodiga. Väljatöötatud biosensori eelis on see, et analüüsi saab läbi viia ilma proovi eeltöötlemisetapita ja analüüsitulemused saadakse umbes poole tunniga. Meetodi valideerimine näitas, et glüfosaadi määramistulemused pinnaveeproovidest on korrelatsioonis HPLC meetodiga saadud tulemustega [II].

Glüfosaadi biosensori tundlikkuse parandamiseks ja analüüside usaldusväärsuse suurendamiseks uuriti glüfosaadi proovide stabiliseerimise ja kontsentreerimise võimalusi, mida oleks võimalik integreerida glüfosaadi biosensoriga reaalajas ja kohapeal läbiviidavaks seireks. Glüfosaadi sidumiseks kasutati kahte erinevat tüüpi aminoaktiveeritud mikrograanuleid – ränidioksiidi mikroosakesi (ASB) ja magnetilisi raudoksiidi nanoosakesi (AFeNP). Glüfosaadi immobiliseerimine aminoaktiveeritud osakestele viidi läbi kasutades EDC/NHS keemiat. Kogu immobiliseerimisetapp võttis aega ligikaudu 20 minutit [III]. Võrreldes ASB ja AFeNP osakeste kasutamist võib väita, et kokkuvõttes on raudoksiidi nanoosakeste kasutamine tõhusam tänu eelkõige kiirele ja lihtsale osakeste eraldamisele magneti abil. Uuriti ka glüfosaadi-AFeNP komplekside stabiilsust ja leiti, et need kompleksid on toatemperatuuril ja pH 7.0 juures stabiilsed vähemalt ühe nädala, mis võimaldab vajadusel ka proovide transporti ja hilisemat analüüsi [132].

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