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Immunoassay for rapid on-site detection of glyphosate herbicide

E. Viirlaid · M. Ilisson · S. Kopanchuk · U. Mäeorg · A. Rinken · T. Rinken

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Abstract Glyphosate is the most widespread herbicide and its global use is steadily increasing. Although glyphosate is considered to have low toxicity, its wide application has raised concerns about its effects on human health. The extensive use of glyphosate has risen a need of its continuous monitoring in drinking and surface waters to assure in accordance with the set standards. Within the present study, we have developed a novel assay for the on-site detection of glyphosate by combining flow-through technology with the high specificity of immunorecognition. The proposed biosensing system was based on the detection of fluorescence signal generated by the quantitative replacement of glyphosate in antigen-antibody complex with IgY-type anti-glyphosate antibodies on microbeads by synthetic 5-carboxytetramethylrhodamine (5-TAMRA) conjugated glyphosate. The working range of this assay was in low millimolar range and the time required for glyphosate detection around 0.5 h. The applicability of the immunoassay for glyphosate detection in surface water was tested and the biosensor results were validated with high-performance liquid chromatography.

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E. Viirlaid (✉) · M. Ilisson · S. Kopanchuk · U. Mäeorg · A. Rinken · T. Rinken
Institute of Chemistry, University of Tartu, Ravila 14A, 50411 Tartu, Estonia
e-mail: edith.viirlaid@ut.ee

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Introduction

Glyphosate (*N*-(phosphonomethyl)glycine) (Fig. 1) is the most common herbicide throughout the world (Duke and Powles 2008) and its formulations are extensively used in agriculture, forestry, and gardening, and also on *railway* tracks and aquatic systems (Torstensson et al. 2005; Van Bruggen et al. 2018). The widespread trade names of glyphosate include Roundup®, Glypro®, Rodeo®, Aquamaster®, etc. (Tu et al. 2001).

The global volume of glyphosate use is increasing steadily (Van Bruggen et al. 2018). For example, the amount of glyphosate used by farmers in the USA has increased over 10 times in the last two decades: from less than 10 million kg in 1992 to more than 136 million kg in 2015 (USGS 2015). Such intensive use of glyphosate leads to the increase of its residuals in the environment (Van Bruggen et al. 2018) and in several countries, glyphosate has been detected in surface and groundwater levels up to 430 μg L^{-1} (Coupe et al. 2011; Mörtl et al. 2013; Van Bruggen et al. 2018).

The average half-life of glyphosate in water varies from a few to 91 days (Henderson et al. 2015). It absorbs strongly to soil particles due to its high adsorption capacity; the half-life of glyphosate in different soils can be up to 197 days (Giesy et al. 2000). The main degradation product of glyphosate is aminomethylphosphonic acid (AMPA), which toxicity

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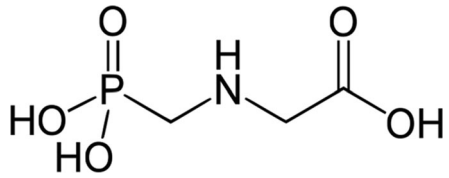

Fig. 1 Structural formula of glyphosate

is comparable to that of glyphosate and which is of similar ecological concern as glyphosate itself (FAO/WHO Meeting 1997; Van Bruggen et al. 2018). AMPA has even a greater environmental persistence than glyphosate as the half-life for AMPA in the soil is estimated to be 76–240 days (Giesy et al. 2000). Rain and erosion transport soil particles with adsorbed glyphosate and AMPA into surface water (Todorovic et al. 2014; Van Bruggen et al. 2018), where they can undergo desorption and be found far away from the original site of application.

Although glyphosate is considered to have low toxicity, its wide use and accumulation potential have raised concerns about its effects on human health and there are numerous reports of its carcinogenicity (EFSA 2015; FAO/WHO Meeting 2016; Guyton et al. 2015). International Agency of Research on Cancer (IARC) has classified glyphosate as "probably carcinogenic to humans" (Guyton et al. 2015). The current acceptable daily intake level in the USA (referred to as chronic reference dose cRfD) is 1.75 mg glyphosate per kilogram of body weight per day (mg kg^{-1} day^{-1}); the corresponding level in the EU is more than 5-fold lower at 0.3 mg kg^{-1} day^{-1}, indicating the diverging opinions of authorities (EFSA 2015; FAO/WHO Meeting 2016; Myers et al. 2018).

To eliminate any potential risk to human health, maximum residue limits (MRL) have been set for glyphosate in drinking water and food. In drinking water, the most stringent level has been set in the EU (0.1 μg L^{-1}) (EC 1998), while in Canada, the MRL value is almost 3000 times higher (280 μg L^{-1}) (Government of Canada 2017) and even 7000 times higher (700 μg L^{-1}) in the USA (Hamilton et al. 2003). In food, the allowed MRL values depend on product, ranging from 0.05 mg kg^{-1} for milk, eggs, and honey up to 30 mg kg^{-1} for grain (EC 2018; USDA 2018). For fodder, the MRL value is 500 mg kg^{-1} (FAO/WHO 2019). Some countries have also set allowed glyphosate residue levels for the protection of water biota. In the Netherlands, the maximum admissible risk level of glyphosate in surface waters is 77 μg L^{-1} (Luijendijk et al. 2003); in Canada, the maximum long- and short-term exposure levels in freshwater are at 800 μg L^{-1} and 27,000 μg L^{-1} respectively (Government of Canada 2017).

The extensive and unregulated use of glyphosate has risen the need for its regular and operative monitoring in drinking and surface waters to assure the accordance with the set standards. The most frequently employed method for the detection of glyphosate residues in water and aqueous fluids is liquid chromatography coupled with UV-Vis, fluorescence, and/or mass spectrometric detection (Stalikas and Konidari 2001). Chromatography methods are sensitive, allowing to achieve a limit of detection (LOD) 0.025 μg L^{-1} (Royer et al. 2000), but sophisticated equipment and unavoidable sample pretreatment do not enable their on-site and real-time application. Besides chromatography, methods with lower sensitivity like spectrophotometrical (LOD is 9.9–1000 μg L^{-1}) (Glass 1981; Bhaskara and Nagaraja 2006; Zheng et al. 2013), capillary electrophoresis (LOD is 100–7600 μg L^{-1}) (Rojano-Delgado et al. 2010; Silva et al. 2013), and electrochemical sensors (LOD is 8450 μg L^{-1}) (Martínez Gil et al. 2013) have been applied.

As an alternative, several immunoassays in which IgY- or IgG-type anti-glyphosate antibodies are used for selective glyphosate detection have been proposed. Clegg et al. (1999) pioneered the first studies on indirect competitive enzyme-linked immunosorbent assay (ELISA) for the detection of glyphosate, obtaining LOD value of 76 μg L^{-1} (Clegg et al. 1999). A few years later, direct competitive ELISA with added analyte derivatization step with succinic anhydride resulted in LOD as low as 0.1 μg L^{-1} (Lee et al. 2002). A competitive ELISA system using analyte derivatization step with acetic anhydride and magnetic particles with bioselective anti-glyphosate antibodies with LOD of 0.6 μg L^{-1} was proposed by Rubio et al. (2003). Using a fully automated setup with online immunocomplex capture of glyphosate onto a protein A/G-specific immunoglobulin-binding support preceding the glyphosate derivatization step with acetic anhydride, González-Martínez et al. (2005) achieved even lower LOD value of 0.021 μg L^{-1} for ELISA. All previously described ELISA methods have shown high specificity towards glyphosate and the cross-reactivity is less than 0.1% (Clegg et al. 1999; Rubio et al. 2003; González-Martínez et al. 2005). The main drawback of ELISA is the comparably long time required for analyses (1–4 h) (Clegg et al. 1999; Lee et al. 2002; Rubio et al. 2003;

González-Martínez et al. 2005), which prevents the utilization of ELISA for on-site applications.

The principle of immunorecognition is also used in glyphosate biosensors. Two different sandwich-type immunosensors coupled either with gold or Co-B/SiO_2/dye nanoparticles have been proposed by Lee et al. (2010, 2013). These systems are based on the detection of fluorescence with LOD values of 10 μg L^{-1} and 0.046 μg L^{-1} respectively. Glyphosate detection in competitive mode with carbon dot labeled antibody attached to magnetic beads had LOD 8 μg L^{-1} (Wang et al. 2016). For the biorecognition of glyphosate in surface plasmon resonance (SPR) assay, Ding and Yang (2013) replaced anti-glyphosate antibodies with oligopeptides, which are more stable and resistant to environmental condition. This SPR biosensor showed high glyphosate specificity, but the LOD value was relatively high (98 μg L^{-1}) (Ding and Yang 2013). The analysis time with the above-described biosensors is 1–2 h (Lee et al. 2010, 2013). In addition, amperometric biosensors have been proposed for the detection of glyphosate. The main problem of these sensors is their low selectivity towards glyphosate as they follow the inhibition of peroxidase by glyphosate. The amperometric biosensors exhibit LOD values from 0.16 to 30 μg L^{-1} (Songa et al. 2009a, b; Oliveira et al. 2012).

The aim of the present study was to develop an assay for the detection of glyphosate combining the high specificity of immunorecognition and the flow-through technology to allow on-site glyphosate detection within less than 1 h. The proposed biosensing system was based on the detection of fluorescence signal generated by the quantitative replacement of glyphosate in antigen-antibody complex with IgY-type anti-glyphosate antibodies on microbeads by synthetic 5-carboxytetramethylrhodamine (5-TAMRA) conjugated glyphosate. The measurements were carried out on a flow injection analysis setup with a single-use bioactivated beads microcolumn for the attachment of glyphosate from the sample.

Materials and methods

Reagents and equipment

Polyclonal anti-glyphosate IgY-type antibodies were purchased from Agrisera AB (product AS132739, 5 mg mL^{-1}). Glyphosate (95%) originated from Shandong Binnong Technology Co., Ltd. Epichlorohydrin (99%) was from Acros Organics and ethanolamine (99%) was from AppliChem, both were used for the immobilization of antibodies on Sephadex gel (G50 Medium, GE Healthcare). All solvents (purity at least 99.8%), used for the synthesis of 5-TAMRA-glyphosate, were purchased from Lach-Ner and used without further purification, except dichloromethane (DCM) and dimethylformamide (DMF), which were dried on 0.3 nm molecular sieves prior to use. 5-TAMRA-N_3 (98%) originated from Baseclick GmbH and *N*,*N*-diisopropylethylamine (DIPEA) from Fluka. All other reagents used for the synthesis of 5-TAMRA-glyphosate were obtained from Sigma-Aldrich and were at least 97% purity (except for 6-heptynoic acid, which had 90% purity). Dulbecco's phosphate-buffered saline (D8537, without Mg^{2+} and Ca^{2+}, pH 7.4) and Pluronic F-127, used in fluorescence anisotropy (FA) and biosensor measurements, were obtained from Sigma-Aldrich. For HPLC analyses, glyphosate (99.7%) and isotope-labeled (IL) glyphosate (13C 99%, 15 N 98%) were purchased from Sigma-Aldrich. 9-Fluorenylmethyl-chloro-formate (Fmoc-Cl) (98%) was purchased from Acros Organics; MeCN with the purity of pesticide residue analysis (99.99%), diethyl ether (99.8%), acetic acid (≥ 99.8%), formic acid (98%), boric acid (≥ 99.8%), and sodium hydroxide (≥ 98%) from Honeywell. MeOH with the purity of residue analysis (> 99.8%) was purchased from Acros Organics. Isopropanol (≥ 99.8%), ammonium acetate (≥ 99.99%), and ammonium hydroxide solution (28.0–30.0% NH_3) were purchased from Sigma-Aldrich. Milli-Q ultrapure water (18.2 MΩ/cm) was used in all of the experiments.

Flash chromatography was performed on Isolera One (Biotage, Sweden) with normal-phase silica gel or reverse-phase C18-silica gel columns as specified. NMR spectra were recorded with Bruker Avance III HD spectrometer. ^{1}H and ^{13}C spectra were measured at 700 and 176 MHz correspondingly. Chemical shifts are presented in parts per million, decoupling constants in hertz. Structural assignments were performed by measuring ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC spectra. HRMS spectra were measured on Thermo Electron LTQ Orbitrap spectrometer with the ESI method. HPLC-MS was performed with Shimadzu LC Solution (Prominence) system connected to LC-MS-2020 ESI-MS. The analysis was performed with RP-HPLC equipped with a Phenomenex Gemini C18 analytical column (5 μm, 250 × 4.6 mm) maintained at 40

°C, and a photodiode array detector SPD-M20A. Acetonitrile-Milli-Q water gradient with 0.1% TFA additive at a flow rate of 1 mL min^{-1} was used.

Protocol for glyphosate detection

The measurements were carried out with FIAlab 3500B system (FIAlab Instruments). The protocol of measurement was adapted from Peedel and Rinken (2014) and modified according to the specific characteristics of glyphosate measurements. The optimal protocol was the following: first, 20 μL suspension of bioactivated beads (produced as described afterwards) was injected from a constantly shaken microcentrifuge tubes into phosphate-buffered saline (PBS; 0.1 M, 0.15 M NaCl; pH 7.2) stream at flow rate 2 μL s^{-1} into a partially closed flow channel to form a single-use microcolumn; the column was packed with 30 μL PBS at rate 2 μL s^{-1} (Fig. 2a). Next, the glyphosate-containing sample was added at flow rate 1 μL s^{-1} (Fig. 2b) and incubated for 30 min to assure the attachment of glyphosate onto beads (Fig. 2c). For the displacement of attached glyphosate, 30 μL of 0.5 μM 5-TAMRA-glyphosate was added at a flow rate of 1 μL s^{-1} and incubated for 10 s (Fig. 2d); after that, the unbound 5-TAMRA-glyphosate was removed with 40 μL PBS at flow rate 5 μL s^{-1}. The bound 5-TAMRA-glyphosate was detected by measuring the fluorescence intensity at λ_{em} = 640 nm perpendicular to the excitation (λ_{ex} = 546 nm). The assay response was calculated as the difference of the fluorescence intensity in the presence and absence of 5-TAMRA-glyphosate (Fig. 2e). For each sample, the average signal was calculated from 2–4 identical measurements. After each measurement, the flow channel was opened, beads were discarded, and the system was ready for the next analysis after washing with PBS (3 mL) at a flow rate 100 μL s^{-1} (Fig. 2f).

Preparation of bioactivated beads for the attachment of glyphosate

For the attachment and preconcentration of glyphosate from the sample, we used anti-glyphosate antibodies immobilized onto Sephadex G50 medium beads. The immobilization of anti-glyphosate antibodies onto gel was carried out with the help of epichlorohydrin in 0.5 M Na_2CO_3 buffer (pH 9.50) according to the scheme given in Fig. 3, as described by Juronen et al. (2018), with slight modifications.

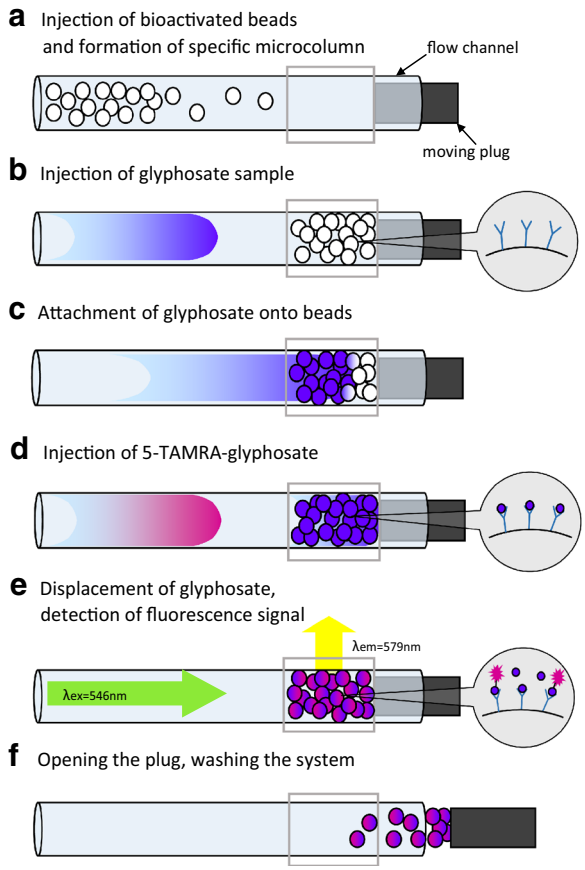

Fig. 2 The principle of glyphosate detection with BIA-based biosensing system

First, to swell the dry beads, 0.5 mL water was added to 25-mg beads and incubated at room temperature for 24 h. To attach the anti-glyphosate antibodies (0.3 mL with the concentration of 2.5 mg mL^{-1}), the swollen beads were activated with 50 μL of 5% epichlorohydrin, which via nucleophilic attack forms a covalent bond with the amine nitrogen of the antibody (Ehlers et al. 2007). The presence of the attached protein on the gel was examined by staining it with Coomassie blue. The bioactivated with anti-glyphosate antibodies gel was stored at 4 °C for up to 30 days with no detectable loss of activity of the attached antibody.

Synthesis of 5-TAMRA-glyphosate

The glyphosate detection was carried out as competitive immunoanalysis with a 5-TAMRA-glyphosate conjugate, which was synthesized according to the scheme given in Fig. 4.

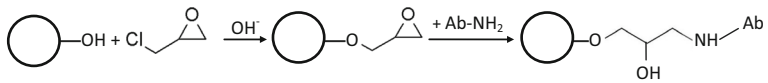

Fig. 3 Production of bioactivated beads

First, *N*-(hept-6-ynoyl)-*N*-(phosphonomethyl)glycine was synthesized. *N*,*N*-Diisopropylethylamine (DIPEA) (0.5 mmol, 87 μL, 2 eq) and 6-heptynoyl chloride (prepared as described previously; Hensle et al. 2014) (0.5 mmol, 72 mg, 2 eq) were added with a microsyringe to tetrabutylammonium salt of glyphosate (0.5 mL of 0.5 M, 1 eq) (prepared as described previously; Romieu et al. 2013) in *N*-methyl-2-pyrrolidone (NMP) and the mixture was stirred at room temperature under argon atmosphere for 24 h. The excess acyl chloride was quenched with water (100 μL). The mixture was diluted with ethyl acetate (2 mL) and purified directly with flash chromatography (ethyl acetate-methanol 0–100% gradient). *N*-(hept-6-ynoyl)-*N*-(phosphonomethyl)glycine (37 mg, 0.132 mmol, 53%) identity was analyzed and confirmed with nuclear magnetic resonance spectroscopy (NMR) and high-resolution mass spectrometry (HRMS). NMR spectra are available in the Supplementary Information (Figures 1S–5S).

^{1}H NMR (D_2O, 700 MHz): δ = 4.19/4.08 (s, 2H, PCH_2), 3.54–3.58 (m, 2H, NCH_2COOH), 2.48 (t, *J* = 7.5 Hz, 1.3H, $NCOCH_2$), 2.25–2.28 (m, 1.6H, $NCOCH_2$, and CCH), 2.11–2.17 (m, 2H, CH_2CCH), 1.55–1.64 (m, 2H, $NCOCH_2CH_2$), 1.41–1.50 (m, 2H, $NCOCH_2CH_2CH_2$)

^{13}C NMR (D_2O, 176 MHz): δ = 176.64/176.08 (COOH), 174.60/173.88 ($NCOCH_2$), 85.94/85.87 (CCH), 69.44/69.42 (CCH), 51.38/49.85 (NCH_2COOH), 47.71/46.88/44.79/43.96 (PCH_2), 32.05/31.89 ($NCOCH_2$), 27.23/27.19 ($NCOCH_2CH_2CH_2$), 23.77/23.65 ($NCOCH_2CH_2$), 17.37/17.29 (CH_2CCH)

HRMS (ESI): [M+H$^+$]: calculated 278.0788 Da, found 278.0785 Da; [M+DIPEA+H$^+$]: calculated 407.2266, found 407.2299; [M+2*DIPEA+H$^+$]: calculated 536.3784, found 536.3822

For the synthesis of 5-TAMRA-glyphosate, a magnetically stirred 1-mL round bottom flask was charged with 0.41 mg (1.48 μmol, 1 eq) of *N*-(hept-6-ynoyl)-*N*-(phosphonomethyl)glycine. It was dissolved in 0.1 mL of *t*-BuOH:water 2:1 mixture. 2.4 μL (24 μg, 0.15 μmol, 0.1 eq) of $CuSO_4$ solution (10 mg mL^{-1}) and 5.9 μL (59 μg, 0.30 μmol, 0.2 eq) of sodium ascorbate solution (10 mg mL^{-1}) were then added with a microsyringe. Finally, 0.84 mg of 5-TAMRA-azide (1.63 μmol, 1.1 eq) was added to the flask and then, it was flushed with argon, closed with a septum, and covered with aluminum foil. The mixture was stirred at room temperature for 3 h. Afterwards, the mixture was purified directly with reverse-phase flash chromatography (water acetonitrile 5–100% gradient + 0.1% TFA). Fractions were analyzed with HPLC-MS; the fraction-containing 5-TAMRA-glyphosate was identified (m/z = 788 and 396, see HPLC-MS chromatograph in the Supplementary data Figure 6S) and the solvents were evaporated in vacuo. Then, the product was dried further overnight under high vacuum. The yield of 5-TAMRA-glyphosate was 0.95 mg (1.20 μmol, 81%).

The synthesized 5-TAMRA-glyphosate was stored at − 18 °C and dissolved in assay buffer on the day of the experiment.

Characterization of anti-glyphosate antibody affinity towards 5-TAMRA-glyphosate with fluorescence anisotropy

The FA measurements were carried out at 25 °C in the kinetic mode with PHERAstar (BMG Labtech, Germany) microplate reader, using an optical module with filters of 540 nm (slit 20 nm) and 590 nm (slit 20 nm) for excitation and emission, respectively. The dual emission detection mode allowed simultaneous recording of emission intensities parallelly ($I\parallel$) and perpendicularly ($I\perp$) to the plane of excitation light. Sensitivities of the detection channels (G factor) were adjusted with a gain correction of the photomultiplier tubes (PMTs) using erythrosine B as a standard (Veiksina et al. 2014). Black 96-well flat bottom polystyrene nonbinding (NBS) surface microplates (Corning, Product No.3993) were used in all experiments.

The specific binding of 5-TAMRA-glyphosate to antibodies was measured in the presence (nonspecific binding) or absence (total binding) of an excess (2.5 mM) of non-labeled glyphosate, and the difference between these values was defined as specific binding. The reaction was started by addition of antibodies to the probe and reaction was monitored for 100 min. For

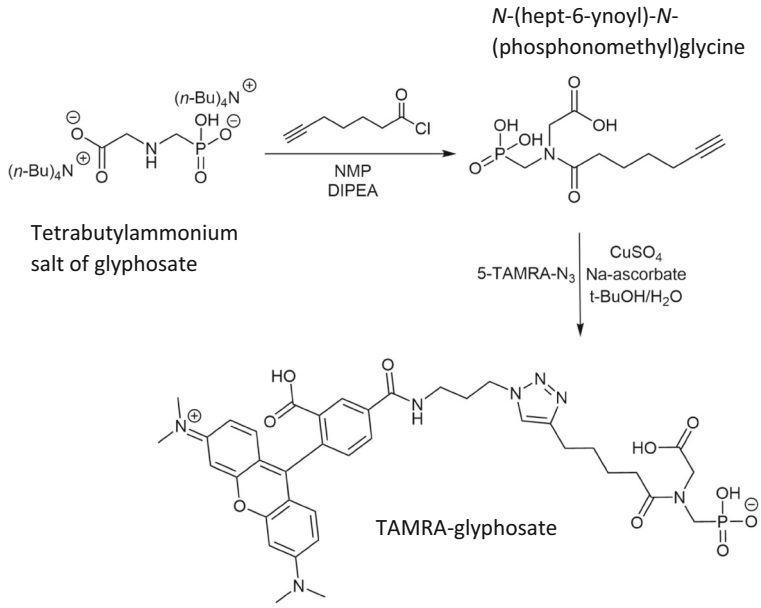

Fig. 4 Synthesis of 5-TAMRA-glyphosate

measurement of dissociation, the excess of non-labeled glyphosate (final concentration 2.5 mM) was added to the reaction medium after the equilibrium was reached, and the reaction was monitored in the kinetic mode as described above. The binding affinity of 5-TAMRA-glyphosate to antibody was calculated from binding experiments, where the probe concentration was kept fixed at 10 nM and the antibody concentration was varied from 0 to 1.5 μM.

The FA signal at time moment t was calculated from the following equation:

$$\mathrm{FA}(t) = \frac{I(t)_{II} - I(t)_{\perp}}{I(t)_{II} + 2 \bullet I(t)_{\perp}} \quad (1)$$

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

The K_D value, characterizing the affinity of 5-TAMRA-glyphosate binding, was calculated with GraphPad Prism™ 5.04 (GraphPad Software, Inc., San Diego, CA, USA) using equations proposed earlier and taking into account ligand depletion (Veiksina et al. 2010). The data is presented as the mean ± the standard deviation of at least two independent measurements.

HPLC analysis

Stock solutions of glyphosate 1 mg mL^{-1} were prepared in an amber glass vial and stored at 4 °C up to 12 months. A stock mixture solution of glyphosate was prepared in water at 10 μg mL^{-1} in an amber glass vial and stored at 4 °C up to 3 months. Calibration solutions with concentrations 0.05 to 1 μg L^{-1} were prepared daily. Stock solution of IL glyphosate was prepared at 1 mg mL^{-1} in an amber glass vial and stored at 4 °C. A working solution was prepared by diluting the stock solution to 10 μg mL^{-1}.

For derivatization, 50 μL of IL glyphosate (20 μg L^{-1}) and 500 μL of boric acid (0.45 M, pH 9 adjusted with 10 M NaOH) were added to 3 mL of sample or calibration solution. After 15 min, 3 mL of diethyl ether was added and the mixture was strongly shaken. After 15 min, the aqueous phase was removed to a new tube and 250 μL of MeCN and 250 μL of Fmoc-Cl 0.1 mg mL^{-1} in MeCN solution were added and shaken. After 1 h, 40 μL of formic acid was added along with 2 mL of diethyl ether. After 1 h, the aqueous phase can be moved into a chromatographic vial for analysis.

Chromatographic analyses were performed using tandem mass spectrometer with Agilent Jet stream electrospray ionization source (Agilent 6490) coupled to a liquid chromatograph (Infinity 1290, Agilent Technology). MassHunter Workstation Software B.08.00 by Agilent Technologies was used for data collection and

evaluation. The chromatographic column used was Zorbax Eclipse Plus C18 (RRHT) 2.1 × 50 mm, 1.8-μm packing with a corresponding 2.1 mm × 5 mm precolumn (Agilent Technology).

The mobile phases used were as follows: (A) 0.1 mM ammonium acetate and 0.01% formic acid in the water, titrated to pH = 5 with ammonium hydroxide solution and (B) 0.005% formic acid in a mixture of MeCN and water (95:5 v/v). The gradient program during direct analysis was as follows: 0–3.25 min, 5–40% B; 3.25–5 min, 40–75% B; 5–6 min, 75–100% B; 6–8 min, 100% B. The outlet of the analytical column was directed to waste by column switching valve for the first 2 min of the run. The eluent flow rate was 0.4 mL min^{-1} and the column was maintained at 40 °C; the injection volume was 50 μL. The following MS parameters were used: drying gas temperature 200 °C, drying gas flow 15 L min^{-1}, nebulizer gas 30 psi, sheath gas temperature 300 °C, and sheath gas flow 12 L min^{-1}. The cone voltage for every compound was 5 V and the collision energy was optimized for every compound separately by changing the values manually.

The method is validated and accredited in the Estonian Environmental Research Center. The limit of detection is 0.056 μg L^{-1} and the limit of quantitation is 0.093 μg L^{-1}. Recovery is corrected with internal standards. Repeatability of the method is 16%, reproducibility is 32%, and measurement uncertainty is 50%.

Water samples

Glyphosate surface water blank samples were collected according to standard ISO 5667-6 into a 500-mL plastic bottle from the river Valgejõgi in Estonia (April 2019 with air temperature 7 °C at depth 0.3 m, middle of the river) and spiked for testing purposes. The glyphosate concentrations used for the correlation study were 5, 13, 18.3, and 23 mM.

Results and discussion

Synthesis of glyphosate conjugates

The proposed immunoassay for glyphosate detection follows the change of fluorescence intensity of the replacement of glyphosate in complex with antibody by its synthetic-labeled conjugate. This conjugate should mimic the glyphosate immunogenic complexes used for the production of antibodies to be able to interact with the anti-glyphosate antibody since small size molecules like glyphosate (M = 169 g mol^{-1}) are conjugated with a carrier protein to generate sufficient immune response in animals (Clegg et al. 1999b). For immunization, the glyphosate molecule is commonly conjugated with bovine serum albumin (BSA) or thyroglobulin via the carboxylic or secondary amino groups of the glyphosate molecule (Clegg et al. 1999; Bhullar 1998; González-Martínez et al. 2005). The latter has also been used for the derivatization of glyphosate with succinic anhydride (Lee et al. 2002) or acetic anhydride (Rubio et al. 2003) to increase the affinity of the glyphosate molecule towards anti-glyphosate antibody and the sensitivity of glyphosate immunosensors. So, for the synthesis of glyphosate conjugates with a fluorescent marker, we used two different strategies: attaching marker via the carboxylic group or via the secondary amino group to imitate the immunogenic conjugates used for the production of anti-glyphosate antibodies.

Two different fluorophores, 5-carboxytetramethylrhodamine (5-TAMRA) and cyanine 3 modification B (Cy3B), both showing outstanding performance in fluorescence-based assays due to good photostability, high quantum yield, and low nonspecific binding were selected to carry out the labeling. In addition, the emission wavelengths of 5-TAMRA (573 nm) and Cy3B (572 nm) lie towards the red end of the visible spectra allowing to minimize the experimental noise, which is typically higher at lower wavelengths (Cooper et al. 2004; Aneja et al. 2008).

For the production of 5-TAMRA-glyphosate, we used 6-heptynoyl chloride as a linker, which was attached to the glyphosate by amidation of glyphosate secondary amino group. The yield of 5-TAMRA-glyphosate was relatively high (81%) and the purity of the conjugate was at least 95%, measured by HPLC (Fig. S1). In addition, we also tried to conjugate glyphosate with Cy3B (*N*-hydroxysuccinimidyl ester of Cy3B) using hexamethylenediamine as a linker via carboxylic group, but due to the poor solubility of glyphosate in organic solvents, this synthesis route failed. So, in glyphosate immunoassay, we used 5-TAMRA-glyphosate as a detectable competitive antigen. The conjugation of a fluorophore can also change its emission maximum (Aneja et al. 2008). We found that the emission maximum for 5-TAMRA-glyphosate was shifted to 640 nm, which is somewhat higher than the emission wavelength of 5-TAMRA-Azide (λ_{em} =

579 nm in DMF, DMSO). Therefore, in our further measurements, we detected the fluorescence of the bound 5-TAMRA-glyphosate at 640 nm to obtain the maximum output of the biosensor.

The affinity of 5-TAMRA-glyphosate towards anti-glyphosate antibody

The conjugation of the glyphosate molecule affects also its binding properties to the anti-glyphosate antibody. The binding properties of 5-TAMRA-glyphosate to the anti-glyphosate antibody were studied using the fluorescence anisotropy (FA) method where the light which is emitted by a fluorophore has dissimilar intensities along different polarization axes (Rinken et al. 2018). FA was a suitable tool for this kind of affinity studies, as the binding of 5-TAMRA-glyphosate to the antibody, which molecular weight is ~ 250 times bigger than that of the fluorophore molecule, significantly slows down the self-rotational frequency of the unbound probe (accordingly, fluorescence anisotropy increases).

The time courses of the increase of FA signal caused by the binding of 5-TAMRA-glyphosate to anti-glyphosate antibody at some selected antibody concentrations are shown in Fig. 5. In all antibody concentrations studied, the FA signal reached its steady-state level within 100 min. After this time, the reaction was reversed by the addition of excess non-labeled glyphosate (final concentration 2.5 mM), which caused a fast decrease of anisotropy (Fig. 5).

The FA data was used for the determination of steady-state values of total and nonspecific binding affinities of 5-TAMRA-glyphosate at different antibody concentrations and building of binding curves, as described earlier (Veiksina et al. 2010). These binding curves of the total and nonspecific binding (Fig. 6) allowed to estimate the K_D value for 5-TAMRA-glyphosate 2.9 ± 0.2 μM. Comparing the obtained K_D value with common nanomolar affinities of antigen-antibody interaction, the affinity of 5-TAMRA-glyphosate is quite moderate, probably due to the relatively small size of the antigen.

The direct determination of the affinity of the glyphosate itself was complicated, as it was not possible to follow its interaction with the antibody. In our preliminary FA experiments (data not shown), we found that the presence of 0.5 mM glyphosate was not enough to produce any observable decrease in the total binding of 10 nM 5-TAMRA-glyphosate within 100-min incubation, in contrast to 2.5 mM glyphosate, generating the dissociation of 5-TAMRA-glyphosate. This indicates that the value of K_D for glyphosate is between those limiting concentrations. The observed difference in the affinities of labeled and non-labeled glyphosate to the anti-glyphosate antibody is probably caused by the antibody generating process, as antibodies are produced against an antigen-carrier protein conjugate.

This relatively low affinity allows sensing of glyphosate in millimolar concentration range.

Optimization of the glyphosate immunoassay

First, we modified the existing assay protocol used for pathogen immunoassays in a similar BIA-based biosensor setup (Peedel and Rinken 2014; Juronen et al. 2018). The four-crucial features for the proposed competitive immunoassay were the following: (a) incubation time for the attachment of glyphosate to anti-glyphosate antibodies immobilized on microbeads; (b) amount of 5-TAMRA-glyphosate for the replacement of bound glyphosate; (c) incubation time of 5-TAMRA-glyphosate to generate a detectable glyphosate-dependent signal; (d) amount of PBS and its flow rate for removing the unbound 5-TAMRA-glyphosate.

Due to the relatively low affinity of glyphosate towards anti-glyphosate antibody, we injected glyphosate-containing sample to the system first and incubated it for 30 min to achieve quantitative concentration-dependent attachment of glyphosate to bioactivated beads and produce distinguishable signals. In comparison with a similar BIA-based biosensor setup, where the affinity of the attachable compound is in the nanomolar range, the optimal incubation time of glyphosate was 10 times longer (Juronen et al. 2018), slowing down the analysis.

The optimal concentration of 5-TAMRA-glyphosate to obtain a detectable change in the biosensor signal was found by varying the concentration of 5-TAMRA-glyphosate from 0.1 to 1.9 μM. Within this concentration range, the signal rose linearly along with the concentration of 5-TAMRA-glyphosate with the slope 154.5 ± 5.8 μV μM^{-1} and the background signal, measured in the absence of 5-TAMRA-glyphosate, was 134.3 ± 1.7 μV. Based on the obtained results, it was found that the signal of 5-TAMRA-glyphosate was reasonably quantifiable at a 5-TAMRA-glyphosate concentration of 0.5 μM and this concentration was used in the following measurements.

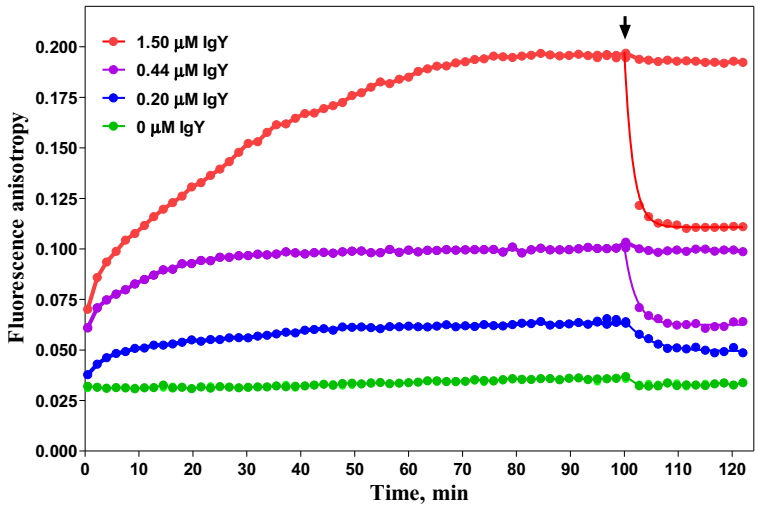

Fig. 5 Time course of anisotropy change caused by the binding of 5-TAMRA-glyphosate (10 nM) to anti-glyphosate antibody. After 100-min incubation, the dissociation of the formed antigen-antibody complex was initiated by the addition of glyphosate (final c = 2.5 mM, lines correspond to the one-phase exponential decay fits). FA (t) values were calculated according to Eq. (1)

Resulting from the different affinities of glyphosate and 5-TAMRA-glyphosate, the determination of the optimal incubation time of 5-TAMRA-glyphosate for the replacement of glyphosate was of major importance. So, the incubation time was varied from 10 to 600 s. In our setup, the shortest incubation time of 10 s was found to be optimal, leading to the quantitative partial displacement of glyphosate and setting the biosensor detection limit at 2.4 mM.

Last, but not least, we optimized the process of removing the unbound 5-TAMRA-glyphosate from the system. It was found that washing with 40 µL buffer (2.5 column volumes) was sufficient to remove all unbound 5-TAMRA-glyphosate. We used flow rate 5 µL s^{-1} as optimal, as at lower flow rates, the efficiency of washing did not improve and at higher flow rates (up to 150 µL s^{-1}), the microcolumn became unstable.

The total time for glyphosate analysis, using the modified optimized protocol, was 37 min.

Detection of glyphosate

The signal of the immunoassay (fluorescence intensity at 640 nm) decreased linearly along with the increase of glyphosate concentration (Fig. 7).

The calibration plot was linear in the whole studied glyphosate concentration range up to 30 mM, with the slope − 1.604 ± 0.070 µV mM^{-1}. The LOD value for glyphosate was 2.4 mM (406 mg L^{-1}), 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^{-1}). The obtained

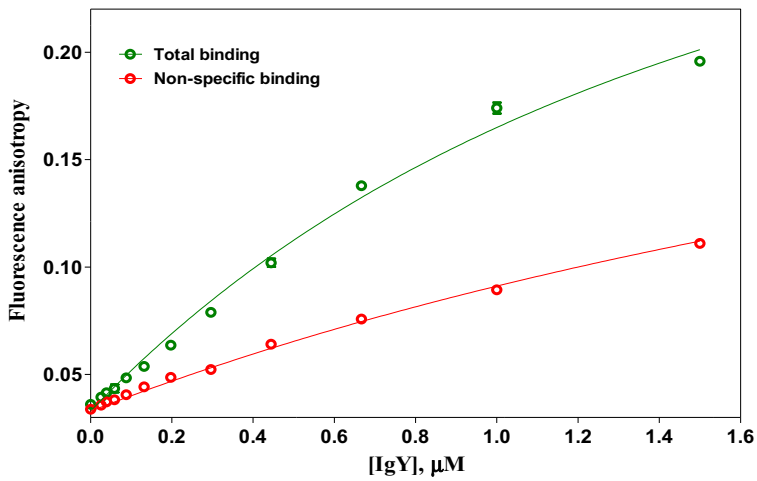

Fig. 6 Saturation curve for the binding of 5-TAMRA-glyphosate to anti-glyphosate antibody. 5-TAMRA-glyphosate (10 nM) was incubated with different concentrations of anti-glyphosate antibody in the absence (total binding) and presence (nonspecific binding) of 2.5 mM glyphosate. After 100-min incubation period at 25 °C, FA (t) values were calculated according to Eq. (1). The data is shown as the mean ± the standard deviation of two independent experiments

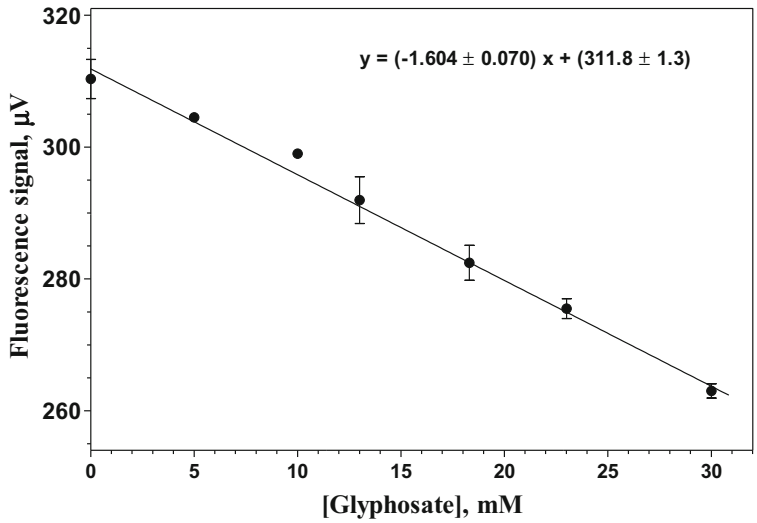

Fig. 7 Dependence of the biosensor signal on glyphosate concentration in PBS (0.01 M, pH 7.2, 0.15 M NaCl); [5-TAMRA-glyphosate] = 0.5 μM

LOD and LOQ values for direct glyphosate detection are considerably higher than corresponding values gained with standard methods (LOD and LOQ values with HPLC are 0.025 and 0.05 μg L^{-1} respectively (Royer et al. 2000)). 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 in the course of its application.

The applicability of the immunoassay for the assessment of glyphosate in surface water

In order to evaluate the performance of the glyphosate immunoassay and validate the obtained results with a standard method (HPLC), the same surface water samples, spiked with different concentrations of glyphosate, were analyzed with both methods. The results of these analyses are shown in Table 1.

Table 1 Analyses of glyphosate in surface water with immunoassay and HPLC

Actual concentration of glyphosate (mM)	LC-MS results (mM)	HPLC bias (%)	Immunoassay results (mM)	Immunoassay bias (%)
0	< LOD		< LOD	
5	4.67	− 7%	6.73	35%
13	11.2	− 14%	11.1	− 14%
18.3	12.7	− 31%	16.7	− 9%
23	14.4	− 37%	21.7	− 6%

Most of the obtained results were smaller than the actual glyphosate concentration in the spiked samples. This can be due to the fast degradation of glyphosate, the absorbance of glyphosate onto the vials, or simply due to the uncertainty of dilution procedures and spiking. However, all biases with both methods were below 50% which is an acceptable limit for such a difficult analyte as glyphosate and indicate the applicability of the glyphosate immunoassay for the detection of glyphosate in surface water.

Conclusions

An immunoassay for glyphosate detection, based on the displacement of glyphosate in its complex with anti-glyphosate antibody by 5-TAMRA-glyphosate, has been proposed for on-site analyses. The main advantage of the method is that no pre-treatment of samples is required and the time required for analyses is approximately half an hour. The limit of detection of this biosensing setup, determined by the relatively low affinity of glyphosate towards anti-glyphosate antibody, is 2.4 mM, which 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 results of glyphosate detection in surface

water simultaneously with the immunoassay and the HPLC method were in good correlation.

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