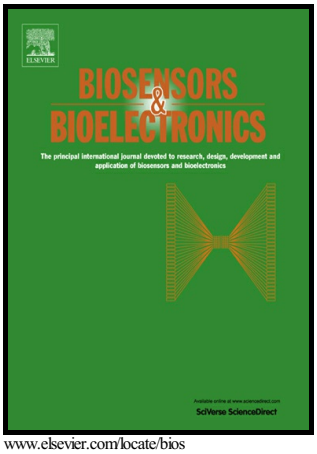

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Fluorescence-based biosensor for monitoring of environmental pollutants: From concept to field application

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Abstract

An advanced optical biosensor was developed based on the enzymatic reaction with halogenated aliphatic hydrocarbons that is accompanied by the fluorescence change of pH indicator. The device is applicable for the detection of halogenated contaminants in water samples with pH ranging from 4 to 10 and temperature ranging from 5 to ~~50~~ 60 °C. Main advantages of the developed biosensor are small size (60×30×190 mm) and portability, which together with short measurement time of 1 min belong to crucial attributes of analytical technique useful for routine environmental monitoring. The biosensor was successfully applied for the detection of several important halogenated pollutants under laboratory conditions, e.g., 1,2-dichloroethane, 1,2,3-trichloropropane and γ-hexachlorocyclohexane, with the limits of detection of 2.7, 1.4 and 12.1 $mg \cdot l^{-1}$, respectively. The continuous monitoring was demonstrated by repetitive injection of halogenated compound into measurement solution. Consequently, field trials under environmental settings were performed. The presence of 1,2-dichloroethane (10 $mg \cdot l^{-1}$) was proved unambiguously on one of three potentially contaminated sites in Czech Republic, and the same contaminant was monitored on contaminated locality in Serbia. Equipped by Global Positioning System, the biosensor was used for creation of a precise map of contamination. Concentrations determined by biosensor and by gas chromatograph coupled with mass spectrometer exhibited the correlation coefficient of 0.92, providing a good confidence for the routine use of the biosensor system in both field screening and monitoring.

Keywords

Dehydrochlorinase, environmental monitoring, field-testing, haloalkane dehalogenase, halogenated pollutant, optical biosensor

1. Introduction

The large-scale manufacture and use of xenobiotic compounds have resulted in worldwide contamination of ~~air, water and terrestrial ecosystems. Halogenated hydrocarbons are one of the largest groups of industrially produced chemicals that are widespread contaminants (Pries et al., 1994)~~ the environment. Many of these compounds are halogenated aliphatic compounds ~~comprised of different haloalkanes, halocycloalkanes, haloalkenes, haloalkynes or haloalcohols~~ that are commonly used as pesticides, solvents, cleaning agents, flame retardants, gasoline additives, degreasers and intermediates for chemical syntheses (Bhatt et al., 2007). ~~Biotic and abiotic processes can also be natural sources of these chemicals (Gribble, 2003). Halogenated aliphatic hydrocarbons~~ These compounds exhibit undesirable negative effects on human health and ecosystem functioning. ~~A wide range of these compounds are currently classified as potentially mutagenic, carcinogenic and genotoxic~~ (Akers et al, 1999; Doherty et al., 1996; Mehendale, 2010) coupled with high. ~~The~~ persistence ~~of halogenated chemicals in the environment together with their~~ and tendency to accumulate in the living organisms ~~may result in a higher incidence of tumors and adverse birth outcomes (Bove et al., 2002)~~. As a consequence, further production and applications of several halogenated aliphatic compounds have been restricted and the problematic chemicals have been replaced by other compounds. However, many of them are prevalent groundwater contaminants and significant components of hazardous wastes and landfill leachates (Vogel et al., 1987). ~~Halogenated aliphatic compounds~~ They can also be found in drinking and surface water, the atmosphere and various trophic food chains (Adriaens et al., 2014).

The widespread distribution of halocarbon pollution ~~with adverse effects and the stricter regulations for the detection of pollutants set by legislative bodies~~ have prompted great interest in fast, accurate and cost-effective technique for pollution control and early warning (Long et al., 2013). ~~Because the majority of halogenated aliphatic hydrocarbons are relatively volatile compounds,~~ Quantitative analyses of environmental samples are generally performed with traditional analytical methods, such as gas chromatography and mass spectrometry. This ~~multi-step~~ labor-intensive procedure involves time-consuming sampling and transport ~~to a specialized laboratory~~ coupled with the storage of the samples and sample pretreatment followed by ~~separation and~~ detection of the target contaminant (Andreescu and Sadik, 2004). ~~The most time-consuming and labour-intensive task is sample preparation (i.e., isolation and preconcentration of the analyte).~~ Although highly accurate and sensitive, conventional methods require sophisticated and expensive equipment~~s~~ and highly trained staff, moreover, it may take a few days to a few weeks to obtain the results of these tests (Eltzov et al., 2011; Wanekaya et al., 2008).

Biosensors appear to be a simple ~~and powerful~~ alternative or a complement to the current analytical techniques for various environmental monitoring applications (Rodriguez-Mozaz et al., 2006a). These devices enable highly sensitive, real-time and high-frequency detection of pollutants without extensive sample preparation and complex equipment (Long et al., 2013). Further advantages provided by biosensors are the possibility of portability, miniaturization and automatization ~~which may be particularly useful for the implementation of biosensors for the screening and monitoring of contaminated sites at competitive costs~~ (Rodriguez-Mozaz et al., 2006b). Although different biosensors have been proposed for the detection of environmental pollutants, most are able to operate only under the controlled laboratory environment; thus, many challenges remain for their use. The main obstacles comprise technical issues concerning the fragility of the immobilized biorecognition element, the

reproducibility and stability of the devices containing a biological component, inadequate electronics and software for data processing and insufficient sensitivity for the field measurements (Orellana et al., 2011; Scognamiglio et al., 2010). Additional challenges are connected with the diversity of compounds and the complexity of matrices in environmental samples, variability in data quality requirements and the broad range of possible environmental monitoring applications (Sadana, 2006). Moreover, biosensors must face the obstacles to commercialization common to all field analytical methods.~~, such as relatively high development costs, a limited market for the analysis of individual compounds or compound classes, the inertia of historical environmental sampling and analysis practices, legislative frameworks.~~ Consequently, only a small proportion of all developed biosensors have reached the market (Bahadir and Sezgintürk, 2015; Orellana et al., 2011). The above-mentioned barriers are all valid for biosensors detecting halogenated aliphatics. Thus, although several biosensors based on different sensing schemes were previously documented for the detection of halogenated aliphatic hydrocarbons (Bidmanova et al., 2014), none were reported for field applications.

The aim of this study is to introduce an advanced optical biosensor that is useful for the screening and monitoring of halogenated contaminants under field conditions. The previously reported sensing concept utilizing the release of protons from enzymatic reaction and the creation of a signal response from the pH optode proportional to the analyte concentration (Bidmanova et al., 2010) provided space for further development and optimization. The sensing properties of the substantially fine-tuned device were characterized under laboratory conditions. Initial trials of on-site detection of halogenated contaminants were documented in detail, and the lessons learned from the biosensor operation in an environmental settings were discussed. As a result of this study, the optimized version of the handheld biosensor was used for rapid on-site quantification of 1,2-dichloroethane contamination in water and the mapping

of contamination distribution in a selected locality using an interlink to the Global Positioning System (GPS). Our study presents a case for transferring a prototype laboratory biosensor to a real world application and its use for on-site monitoring of environmental pollution.

2. Experimental Section

2.1. Chemicals and Materials. The haloalkane dehalogenases LinB from Sphingobium japonicum UT26 (Nagata et al., 1994), DhlA from Xanthobacter autotrophicus GJ10 (Keuning et al., 1985), ~~a~~ DhaA mutant from Rhodococcus rhodochrous NCIMB 13064 (DhaA31 with five amino acid substitutions: I135F, C176Y, V245F, L246I and Y273F, Pavlova et al., 2009) and γ-hexachlorocyclohexane dehydrochlorinase LinA from Sphingobium japonicum UT26 ~~(kind gift of Prof. Lal from the University of Delhi, India)~~ were heterologously expressed in Escherichia coli BL21 and purified using metal affinity chromatography (procedure described in Supplementary Information). Conjugates of 5(6)-carboxyfluorescein and 5(6)-carboxynaphthofluorescein with bovine serine albumin (CF-BSA and CNF-BSA, respectively) were prepared as previously described (Bidmanova et al., 2012). Ampicillin sodium salt and isopropyl-β-D-thiogalactopyranoside were obtained from Duchefa (the Netherlands). 2-(N-morpholino)ethanesulfonic acid was purchased from Carl Roth (Germany). Nitric acid was purchased from Lach-Ner (Czech Republic). Ethanol, methanol, acetone and toluene were purchased from Chromservis (Czech Republic). All other chemicals were purchased from Sigma-Aldrich (USA). All reagents were of analytical grade and used without purification. Solutions were prepared with deionized water with a resistivity of 18.2 MΩ·cm using a Millipore Milli-Q water purification system (Millipore Inc., USA).

2.2. Instrumentation. The bionsensor consisted of two attached fluorometers (Photon Systems Instruments, Czech Republic) with a solid-state LED containing a 590 nm band limiting filter as a light source and PIN photodiode with a 670 nm band limiting filter as a detector. Glass

sticks (Photon Systems Instruments, Czech Republic) 80 mm in length and 8 mm in diameter were used as transducers. Glass discs (GLASSBEL, Czech Republic) with a diameter of 8 mm and height of 1 mm were mounted in front of the glass sticks. The output signal was sent to a computer and recorded using the FluorPen 1.0 software (Photon Systems Instruments, Czech Republic).

2.3. Preparation of biosensor tips. The lyophilized enzymes LinB, DhlA, DhaA31 and LinA (2 mg) and lyophilized conjugate CNF-BSA (4 mg) were dissolved in 25 % (v/v) glycerol (32 µl). This mixture (5 µl) was applied to the glass discs by pipetting into a thin layer. The discs were exposed to 70 % (v/v) glutaraldehyde vapors for 30 min at 23 °C. Reference discs were prepared in the same manner using bovine serum albumin (BSA, 2 mg) instead of the lyophilized enzyme. Prepared biosensor tips were stored in 50 mM phosphate buffer (pH 7.5) at 4 °C overnight or in lyophilized form followed by hydration using 50 mM phosphate buffer (pH 7.5) prior to use.

2.4. Biosensor measurements. Laboratory experiments were performed in a stirred glass vial at 23 °C. The biosensor tip was immersed into 5 ml of 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES, pH 9.0). After recording the steady signal, the biosensor was immersed into 1 mM HEPES buffer (pH 9.0) spiked with the analyte. The signal was recorded for 1 min. The biosensor tip was washed with deionized water prior to further use. A slope from the linear signal response to the analyte was calculated, and the biosensor response was evaluated as the difference between the reference and the signal. The concentrations of analytes used were determined by gas chromatography as described in detail in the Supplementary Information. The calibration data were fitted using Origin 6.1 (OriginLab, USA). The detection limit (a signal three times the standard deviation of the noise related to the intercept of the linear function) was determined by linearizing the plot using a log-logit function (Mikkelsen and Cortón, 2004).

Biosensor on-site analyses began by immersion of the biosensor into 50 mM phosphate buffer (pH 9.0) before each measurement. Then, the biosensor tip was immersed into the water body or a beaker with a water sample (in case the access to water was complicated). The biosensor signal was recorded for 1 min, and the response was calculated as a slope from the linear signal. New biosensor tips were used for each measurement.

Water samples were collected from all sites into dark gas-tight glass bottles for laboratory-based verification of the presence of halogenated hydrocarbons. Extraction of the samples was performed using hexane with an extraction ratio of 9:1 (water sample:hexane). A gas chromatograph–mass spectrometer GC7890A-MS5975C (Agilent Technologies, USA) with a FFAP 30 m×0.25 mm×0.25 µm column (Phenomenex, USA) was used. The temperature program began with an isothermal period at 40 °C for 1 min, followed by a temperature increase to 220 °C at a rate of 20 °C per min; this temperature was held for 20 min.

The physico-chemical parameters of water, such as temperature, pH and dissolved oxygen, were determined on site (sampling sites in the Czech Republic) using the portable multi-parameter instrument Multi 350i (WTW, Germany). Further physico-chemical parameters were determined in the laboratory. The total hardness of the water was determined by the RATAJ Hardin test of GH according to the enclosed manual (Rataj, Czech Republic). The buffering capacity of the samples was calculated as a slope from the dependence of pH changes on the addition of 8.8, 17.6 and 35.1 µmol hydrochloric acid into 5 ml of the water sample.

2.5. Statistics. All of the experiments were performed minimally in triplicate. The means and standard errors of the data were calculated. Analysis of variance (one-way ANOVA) and a correlation analysis were performed for data obtained from the repeatability and field-testing experiments, respectively, using the Analysis ToolPak in Microsoft Excel 2010 (Microsoft,

USA) and Statistica version 12 (StatSoft, USA). A p value < 0.05 indicated statistical significance.

3. Results and Discussion

3.1. Design, preparation and optimization of the biosensor. The advanced optical biosensor is composed of test and reference channel, glass sticks used as optical transducers, a biosensor and a reference tip. The biosensor tip consists of a disposable glass disc with an immobilized biorecognition element, i.e., enzyme releasing protons as products of enzymatic reaction with the target analyte. The reference tip is occupied by BSA instead of the enzyme. Both tips contain fluorescent pH indicator co-immobilized in one layer with the enzyme or BSA. After excitation of fluorescent pH indicator by light source from fluorometer, the fluorescent light is produced in dependence on the concentration of protons. The light is directed into the detector located in the fluorometer where it is transformed into an electrical signal.

A key step in the development of the sensing system is the selection of materials including the biological recognition element and pH indicator, because they govern the selectivity and sensitivity. Haloalkane dehalogenases (EC 3.8.1.5) and γ-hexachlorocyclohexane dehydrochlorinases (EC 4.5.1.B1) belong to enzyme families that have been intensively studied for their catalytic activity towards significant halogenated pollutants which is accompanied by a release of protons. Among them, the haloalkane dehalogenase LinB from Sphingobium japonicum UT26 (Nagata et al., 1994) exhibits one of the highest conversion rates. This enzyme has been biochemically characterized and its production is an established process. Therefore, it was selected for the preparation of the biosensor and its optimization. The enzyme was immobilized on a ~~disposable~~ glass disc to ensure close contact of the enzyme with ~~the glass stick used as~~ an optical transducer. Various immobilization methods were previously tested for the immobilization of LinB (Bidmanova et al., 2013), and cross-linking

was determined to be the most successful immobilization technique for this enzyme. Here, we used the cross-linking reagents glutaraldehyde, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and dextran polyaldehyde (detailed information on the immobilization is provided in the Supplementary Information). Cross-linking of the enzyme by glutaraldehyde and EDC based on the utilization of surface primary amines and carboxyls, respectively, led to similar activity retention (approximately 40 %; Fig. S1). The formation of water-insoluble particles such as cross-linked enzyme aggregates (CLEAs) resulted in a substantial decrease in activity followed by a further activity drop after entrapment into polyvinyl alcohol. Because immobilization via cross-linking with glutaraldehyde was simple and cheap and resulted in adequate enzyme activity, this procedure was further tested for immobilization of the pH indicator.

The pH indicator used in the first prototype of the biosensor was 5(6)-carboxyfluorescein conjugated with BSA, which could be easily co-immobilized with the enzyme on the optical transducer (Bidmanova et al., 2010). Although this procedure ensured the tight contact of both recognition elements, a 2-min delay in the biosensor response was observed in the presence of the analyte due to insufficient pH sensitivity of the indicator ~~(Fig. 1)~~. A new pH indicator was selected, which would promptly reflect the change in the local pH accompanying the biosensor response. The indicator 5(6)-carboxynaphthofluorescein fulfilled the selection criteria concerning pH sensitivity, excitation and emission wavelengths and quantum yield. Because it possessed the same functional groups as the previously used 5(6)-carboxyfluorescein, the immobilization could be performed using the same method (Bidmanova et al., 2012). Conjugates of CNF-BSA cross-linked onto the glass discs using glutaraldehyde exhibited sensitivity in the pH range from 7.0 to 10.0 (calculated pKa of 8.56, Fig. 1), and thus provided a perfect match with the common pH profile maximum of the haloalkane dehalogenases (pH 8.0-10.0 determined for 13 enzymes, Koudelakova et al., 2012)

and the prevalent pH of environmental samples (pH 6.5-8.5 for surface waters and pH 6.7-8.7 for groundwater determined from 32 sampling sites at 7 different localities; Bidmanova and Kotlanova, unpublished results).

Fig. 1 here

The thorough optimization of the instrumentation also significantly affected the final performance of the novel biosensor. The first prototype (Bidmanova et al., 2010) was a rather large instrument that was dependent on incessant electric power. Additionally, the application of the biosensor in field-testing was compromised by a low signal to noise ratio and low resistance of the device to interference from the power supply. Therefore, a new optoelectronic hardware unit utilizing a LED as a light source and a photodiode as a signal detector was constructed. The scale-down of the newly built instrument to a size of 60×30×190 mm and a weight of 300 g was directed towards a field application requiring easy manipulation, high robustness, compactness and portability. The device was equipped with an electronic control board for data read-out, processing and storage and two buttons and a display to set-up the programmable parameters. Measured data were transferred to a PC immediately via a USB connection. Moreover, a battery option for the device based on sequential storage of the data in the internal memory was successfully tested. The developed software provided visualization of the data in a graph and a data transfer routine into Microsoft Excel. GPS positioning was enabled by the connection of the device with a GPS module. This advanced version of the biosensor offered increased sensitivity and a shorter measurement time at significantly reduced costs compared to the original prototype.

To monitor the technical improvements of the biosensor, the signal stability of the fine-tuned device was determined by measuring the biosensor response for 60 min (Fig. S2). Both the

biosensor and reference discs exhibited a stable signal, with oscillations lower than 1 %. To further improve the performance of the biosensor by strengthening the signal, the effect of the light intensity of measuring light on the biosensor response towards the model compound 1,2-dibromoethane was tested (Fig. S3). Low measuring light intensity provided the lowest response from the tested variants, because the biosensor response increased significantly with increasing of the measuring light intensity. The highest response was observed for the maximum achievable measuring light intensity. Because the maximum light intensity allowed by instrumental setting was reached, the hardware modification needed to be performed. The current going through the LED was doubled, resulting in a significant increase of both light and signal intensity. On other hand, the calculated biosensor response was similar to the response of biosensor without improvement of LED current (data not shown).

A very important feature of a biosensor system intended for environmental monitoring is the measurement time, which should be as low as possible to enable fast screening of different sampling sites or to detect any changes in the analyte concentration in time. The measurement time of the biosensor is dependent upon the frequency of data collection and the evaluation method in addition to other factors. While the first prototype and subsequently updated biosensor collected data at intervals of every 5 and 30 s, respectively, the latest device recorded data every 1 s. This innovation enabled us to modify the data evaluation procedure from a steady-state to a kinetic method (i.e., from the determination of the difference between the initial and stable end-point signal to the determination of the slope from the biosensor response). The measurement time was reduced from the 30 min required by the prototype to 1 min for the recent device, which ranked the biosensor among one of the fastest systems for the detection of halogenated aliphatic hydrocarbons compared to previously published devices (Bidmanova et al., 2010; Campbell et al., 2006; Peter et al., 1996; Peter et al., 1997; Prathap et al., 2012; Reardon et al., 2009).

3.2. Characterization of the biosensor. Biosensor performance is strongly affected by the environmental pH and temperature. The effect of both factors was studied with the biosensor utilizing the LinB enzyme as the biorecognition element (Fig. 2). More than 60 % of the biosensor response was retained in the pH range from 4.0 to 10.0, with a maximum at pH 8.0. The response of the biosensor dropped rapidly below pH 4.0, which is in strong contrast to the soluble enzyme exhibiting only 40 % of its activity at pH 7. Additionally, the pH optimum was slightly shifted compared to the pH of 8.8 determined as the optimum for the soluble enzyme (Koudelakova et al., 2012). The temperature profile of the biosensor was in agreement with the finding of a greater than 60 % response at temperatures ranging from 15 to 50 °C. The temperature of 5 °C represents the lowest temperature applicable ~~was limiting~~ for the biosensor measurements due to the broad scatter in the response, but still approximately 20 % of the response compared to the optimum was observed. This portion of activity was observed for the soluble enzyme at 20 °C. In contrast, the maximum biosensor response was observed at 40 °C, which was in good agreement with the temperature profile of the soluble dehalogenase enzyme (Koudelakova et al., 2012). The shift in the pH optimum and broadening of the pH and temperature range for the enzyme after immobilization were previously described in the literature (Chen et al., 2000). This phenomenon might be attributed to the stabilization of the enzyme molecules via immobilization, the diffusion limitations of the immobilized layer and the preincubation of the biosensor in alkaline buffer at 22 °C using the advantage of a short-time measurement.

Fig. 2 here

3.2.1. Reproducibility and repeatability. The critical parameters of the biosensor that influence its applicability are reproducibility, repeatability and the possibility of continuous use.

Biosensor tips prepared from three different batches of enzyme and conjugates of pH indicator were tested to determine the reproducibility of the biosensor preparation. Furthermore, the same tips were used in ten consecutive measurements with the model compound 1,2-dibromoethane (Fig. S4). Differences in the response of the biosensor prepared from the individual tips were not significant for a significance level of α=0.05, which indicated a good reproducibility of the biosensor preparation. The repetitive use of tips led to moderate oscillations in the biosensor response, with a relative standard deviation of 7.3 %. The applicability of the biosensor for continuous monitoring was tested by studying the biosensor response to repeated injections of the model compound (Fig. S5). A steep decrease in the response was observed immediately after exposure of the biosensor to the measurement solution containing the analyte. Subsequent regeneration in HEPES buffer for 10 min returned the biosensor response to 85 % of the initial value, whereas, 95 % of the recovered signal was observed after 20 min of regeneration.

3.2.2. Quantification of analytes. Finally, the biosensor response to several significant environmental contaminants was tested (Fig. 3). The biosensor discs based on the wild-type LinB haloalkane dehalogenase enzyme were used for the detection of the toxic halogenated hydrocarbons 1,2-dibromoethane and 3-chloro-2-(chloromethyl)-1-propene. The probable human carcinogen 1,2-dichloroethane was detected using the biosensor based on the wild-type DhlA enzyme. The exposure of the biosensor to increasing concentrations of these analytes led to the release of more protons from the enzymatic reaction, thereby resulting in a higher biosensor response. A plateau was reached at a saturating concentration of the analytes. The calibration curve followed the Michaelis-Menten profile, indicating an enzyme-catalyzed process with a correlation coefficient of 0.99 for all three compounds. The limits of detection were 2.4, 1.4 and 4.6 $mg \cdot l^{-1}$ for 1,2-dibromoethane, 3-chloro-2-(chloromethyl)-1-propene and 1,2-dichloroethane, respectively. However, the biosensor based on the haloalkane

dehalogenase LinB is not able to distinguish 1,2-dibromoethane and 3-chloro-2-(chloromethyl)-1-propene in a real sample as it was demonstrated with the first biosensor prototype (Bidmanova et al., 2010). The selectivity of the biosensor is dependent on the substrate specificity of selected biorecognition element and the device allows quantitative detection of compounds within the class of substrates. Specific detection of individual compounds would be possible by utilizing engineered enzyme variants with narrow substrate specificity or by simultaneous employment of more enzyme variants differing in their substrate specificity. Effect of substrate specificity on the biosensor response was also demonstrated in following measurement with the biosensor discs based on the wild-type LinB enzyme. They were used for the detection of the highly hazardous compound 1,2,3-trichloropropane in the preliminary experiments. However, the biosensor was insensitive to this analyte due to the low catalytic performance of the enzyme (Hrdlickova et al., unpublished results). A comparison with published data revealed that the natural enzyme providing sufficient catalytic activity with 1,2,3-trichloropropane was missing. Thus, protein engineering (rational design in combination with saturation mutagenesis) was applied to obtain a variant of the haloalkane dehalogenase DhaA from Rhodoccoccus rhodochrous NCIMB 13064 that displayed a 26-fold higher catalytic efficiency for 1,2,3-trichloropropane than the wild-type enzyme (Pavlova et al., 2009). Subsequent utilization of DhaA31 in the biosensor provided a calibration curve determined in the range from 0 to 40 $mg \cdot l^{-1}$ of 1,2,3-trichloropropane. The calibration followed the same profile as the case mentioned above for the analytes, with a limit of detection of 1.4 $mg \cdot l^{-1}$ and correlation coefficient of 0.99. Another halogenated analyte detected by the biosensor was γ-hexachlorocyclohexane, which belongs to the persistent organic pollutants banned under the Stockholm Convention (Website of the Stockholm Convention, retrieved 2015). This chemical is hydrolytically dechlorinated by the haloalkane dehalogenase LinB after the initial dehydrochlorination catalyzed by the γ-

hexachlorocyclohexane dehydrochlorinase LinA (Lal et al., 2010). Therefore, two variants of the biosensor discs were tested: with a single enzyme (LinA) and a mixture of LinA and LinB in a 1:1 ratio. The response of the biosensor based on the LinA enzyme alone was faster and more reproducible compared to the second variant. The calibration of the LinA biosensor was determined in the concentration range of 0 to 100 $mg \cdot l^{-1}$, with a limit of detection of 12.1 $mg \cdot l^{-1}$ and correlation coefficient of 0.96.

Fig. 3 here

This study demonstrated the successful improvement of the detection limit of the biosensor. While the first prototype exhibited a detection limit of 25 $mg \cdot l^{-1}$ for 1,2-dibromoethane (Bidmanova et al., 2010), systematic research and substantial development resulted in the more recent device utilizing a different indicator dye and fine-tuned optoelectronics with a limit of detection lower by one order of magnitude (reaching 2.4 $mg \cdot l^{-1}$). The currently determined values were within the range from 4 $ng \cdot l^{-1}$ to 25 $mg \cdot l^{-1}$ of previously reported enzymatic biosensors detecting halogenated aliphatic hydrocarbons (Bidmanova et al., 2010; Campbell et al., 2006; Peter et al., 1996; Peter et al., 1997; Prathap et al., 2012; Reardon et al., 2009). These concentrations are well-suited for the measurement of environmental pollutants at contaminated localities (e.g., superfund sites typically require monitoring in the gram or milligram range) However, because agricultural run-off monitoring or the measurement of drinking water may require measurements in the microgram range or lower, different limits of detection are required (Scognamiglio et al., 2010). These low values were reported for a few biosensors detecting halogenated aliphatics (Peter et al., 1996; Prathap et al., 2012; Reardon et al., 2009); however, these devices were developed and optimized for the detection of one specific analyte. Herein, the biosensor was used to monitor several hazardous halogenated

contaminants due to the proper selection of specific haloalkane dehalogenases according to their substrate specificity. Employment of a genetically modified dehalogenase provided a biosensor specific for an analyte that could not be detected using the conventional device based on the wild-type enzyme. Moreover, the proposed biosensor concept met the requirement for versatility in terms of the applied interchangeable biorecognition elements, which enabled broadening of the spectrum of detected analytes. This desired feature was demonstrated by the successful monitoring of γ-hexachlorocyclohexane based on the replacement of haloalkane dehalogenase with γ-hexachlorocyclohexane dehydrochlorinase without further optimization.

Recovery experiments with different concentrations of 1,2-dichloroethane spiked in HEPES buffer were performed to evaluate the analytical reliability and application potential of the developed biosensor (Fig. S6). The device exhibited the recovery between 78.5 % and 160.9 %, which indicated applicability of the biosensor for detection of halogenated contaminants during environmental monitoring. Here, biosensor can be used as a cheap alternative to conventional methods during prescreening of potentially contaminated sites. Subsequently, accurate concentrations of halogenated pollutants at selected spots need to be evaluated using laboratory-based traditional methods such as gas chromatography.

3.3. Field-testing in the Czech Republic. After successful laboratory measurements, the biosensor was ready for pilot testing under field conditions. Several potentially contaminated localities in the Czech Republic were considered; however, chromatographic measurements confirmed contamination by halogenated aliphatic hydrocarbons at only one of them. This locality was in the vicinity of a manufacturing plant for ethylene-based polymers with previously reported leakage of 1,2-dichloroethane, chloroethylene, trichloroethylene and trichloromethane (Website of Ministry of Environment of Czech Republic, retrieved 2015). The biosensor was tested at three different sites at this location. Site 1 was a gutter with

presumable contamination by halogenated aliphatic hydrocarbons due to the efflux of ground water from the industrial area. Sites 2 and 3 were located at the bank of the river upstream and downstream of the plant, respectively. The flow rate of approximately 100 $m^3 \cdot s^{-1}$ in this part of the river reduced the probability of detecting the contaminants due to substantial dilution. First, properties of the water samples (i.e., pH and temperature) were characterized immediately using a portable multi-parameter instrument (Tab. S1). Water from sites 2 and 3 exhibited an alkaline pH and approximate temperature of 20 °C, which provided optimal conditions for field-testing of the biosensor. Water from site 1 exhibited a near-neutral pH and lower temperature compared to sites 2 and 3. These characteristics could diminish the biosensor response to the contaminants. However, more than 50 % of the biosensor response was observed under these conditions when measuring the pH and temperature profiles.

The determination of halogenated chemicals by the biosensor based on the haloalkane dehalogenases LinB and DhlA was performed on-site. The biosensor employing LinB did not exhibit positive response at any of the sampled sites. In contrast, a strong positive response was observed for the biosensor employing DhlA at sampled site 1 (Fig. 4). This referred to the presence of a halogenated aliphatic chemical (most likely 1,2-dichloroethane) because DhlA exhibited enzymatic activity with this compound while LinB did not (substrate specificities of haloalkane dehalogenases described by Koudelakova et al., 2012).

Verification of the biosensor response was performed by gas chromatography coupled with mass spectrometry. Laboratory analysis of the collected water samples confirmed the positive response obtained from the biosensor measurements at sampled site 1. The chemical 1,2-dichloroethane was identified as the most abundant pollutant present in collected water (Fig. 4). Trace concentrations of other contaminants (e.g., 1,1,2-trichloroethane, 1,1,2,2-tetrachloroethane, 3-chloro-2-chloromethyl-1-propene, 2,2-dichloroethanol, and 2,2,2-trichloroethanol) were revealed in the sample. However, their concentrations of

approximately 0.1 $mg \cdot l^{-1}$ were negligible in comparison to the 10 $mg \cdot l^{-1}$ concentration of 1,2-dichloroethane. Furthermore, concentrations of this chemical were insignificant at sampled sites 2 and 3, where they were below the detection limit of the biosensor and the highest concentration allowable for drinking water (Website of United States Environmental Protection Agency, retrieved 2015).

Fig. 4 here

This pilot study demonstrated that the biosensor was able to unambiguously detect halogenated aliphatic chemicals in the water matrix. Moreover, the selection of enzymatic recognition elements enabled the identification of the contaminant based on a comparison of substrate specificities. The aim of the following test was to verify whether the biosensor was applicable for the mapping of contaminants. The study was performed in the gutter with a previously confirmed contamination by 1,2-dichloroethane (sampled site 1 in Fig. 4). The biosensor response was measured at six different sites situated downstream (Fig. 5) (e.g., site 1 was the source, an additional inflow was located between sites 4 and 5, and sites 5 and 6 were stagnant water due to the partition of the stream). The location of the sites affected physical and chemical variables of the water, such as water speed, temperature, pH (Tab. S2), turbidity and dissolved oxygen. Although a rough correlation between the biosensor and gas chromatographic-mass spectrometric results was observed, the accuracy of the biosensor measurements was significantly impaired by the above-mentioned factors. Further discrepancies could also be attributed to the unequal initial sensitivity of the different biosensor discs. Therefore, calibration of newly introduced discs based on a record of the biosensor response in the calibration solution with a defined pH difference was included into the measurement procedure and used for correction of the measured data.

Fig. 5 here

3.4. Field-testing in Serbia. Finally, the biosensor was tested at a location in Serbia. Sixteen sampled sites (Fig. 6) were located near a large industrial complex with reported leakages of 1,2-dichloroethane into the soil and wastewater (Martinovic-Vitanovic and Kalafatic, 2009). Fourteen measurements were performed with samples collected from monitoring wells reaching a depth of 5 m (sites 1-7 and 10-16) and two with samples of surface water (sites 8 and 9). The application of the biosensor was compromised by the extremely low temperature (10 °C in the freshly collected water that dropped to 0 °C during measurement). Thus, both biosensor and chromatographic measurements were bound to laboratory conditions. The integration of calibration into the measurement procedure enabled the quantification of the concentration of the halogenated contaminant 1,2-dichloroethane. The correlation coefficient for the concentrations determined by the biosensor and conventional method was 0.92. This significant correlation indicated that the biosensor developed in the study could be used to determine halogenated aliphatic hydrocarbons quantitatively at environmental sites. For these applications, we are working on the development of a methodology for the fast adjustment of the initial pH and temperature of the samples due to the previously observed effects on the biosensor response.

Fig. 6 here

To the best of our knowledge, the testing of a biosensor at the real contaminated localities described in this study is the first application of a biodevice for the detection of halogenated aliphatic hydrocarbons under field conditions. In previous reports, biosensors have been used

for the detection of halogenated aliphatic hydrocarbons in buffer solutions (Bidmanova et al., 2010; Campbell et al., 2006; Peter et al., 1996; Peter et al., 1997; Prathap et al., 2012; Reardon et al., 2009) or alternatively in spiked water samples (Anirudhan and Alexander, 2015). The demonstration of the proper performance of the biosensor under field conditions with subsequent validation through the comparison of results with those obtained by the traditional method is usually the last necessary step in the development of a new device (Rodriguez-Mozaz et al., 2006a). In view of this, the developed biodevice succeeded and exhibited a high potential to become an indispensable tool for high-throughput or continual analyses, including the spatial and temporal characterization of halogenated contaminants. Specific examples of particularly suited applications might include the identification of hot spots at the contaminated site or concentration changes of a contaminant on a short timescale, on-site monitoring of a process control stream to determine the efficiency of hazardous waste site remediation, monitoring agricultural run-off during a peak application period or monitoring wells to determine whether concentrations of target analytes during a remediation procedure or after site closure are in compliance.

4. Conclusion

The biosensor described in this article allowed the fast and simple detection of halogenated aliphatic hydrocarbons, which are common contaminants in the environment. The activities of the haloalkane dehalogenase and γ-hexachlorocyclohexane dehydrochlorinase were well-retained after immobilization. The pH indicator immobilized on a glass disc represents a viable transducer for the detection of local changes in pH values that occur as a result of enzymatic reactions. The biosensor enables the rapid detection of important environmental pollutants 1,2-dibromoethane, 1,2,3-trichloropropane, 1,2-dichloroethane, 3-chloro-2-(chloromethyl)-1-propene and γ-hexachlorocyclohexane, with detection limits of 2.4, 1.4, 2.7,

1.4 and 12.1 $mg \cdot l^{-1}$, respectively. The device was tested for the detection of 1,2-dichloroethane at two contaminated locations in the Czech Republic and Serbia, respectively. A map of contamination was created using on-site biosensor measurements with GPS localization. Conceivably, the method may be extended to continuous monitoring of the target compounds and unknown, structurally related chemicals, thereby providing an early warning system of water pollution and reducing the ecological and human health risks.

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Figures

Fig. 1. Fluorescence response of conjugates of 5(6)-carboxyfluorescein (CF-BSA) and 5(6)-carboxynaphthofluorescein (CNF-BSA) with bovine serine albumin to pH changes. Conjugates were immobilized onto the glass discs using glutaraldehyde. Measurements were performed in 50 mM phosphate buffer at 23 °C. The standard errors were calculated from three independent measurements.

Fig. 2. Effect of pH and temperature on the biosensor response to 1,2-dibromoethane. The concentration of 1,2-dibromoethane was 64 $mg \cdot l^{-1}$. Measurements were performed in 1 mM HEPES buffer at 22 °C. The standard errors were calculated from three independent measurements.

Fig. 3. Calibration plots of the biosensor for selected halogenated hydrocarbons. The measurements were carried out in 1 mM HEPES buffer (pH 9.0) at 22 °C with the limits of detection (LOD). Concentrations of analytes were determined by gas chromatography. The standard errors were calculated from three to four independent measurements.

Fig. 4. Field-testing of the biosensor for monitoring 1,2-dichloroethane contamination in the Czech Republic. The table shows the comparison of the 1,2-dichloroethane concentration in water determined by gas chromatography-mass spectrometry vs. the biosensor response. The map illustrates the sampled sites with numbering consistent with the table.

Fig. 5. Mapping contamination using on-site biosensor measurements in the Czech Republic. The table shows the comparison of 1,2-dichloroethane concentrations in the water determined

by gas chromatography-mass spectrometry vs. the biosensor response. The map illustrates the sampled sites with numbering consistent with the table.

Fig. 6. Comparison of 1,2-dichloroethane concentrations in environmental water samples from Serbia measured with the biosensor vs. gas chromatography (GC). The map illustrates the sampled sites with numbering consistent with the table.

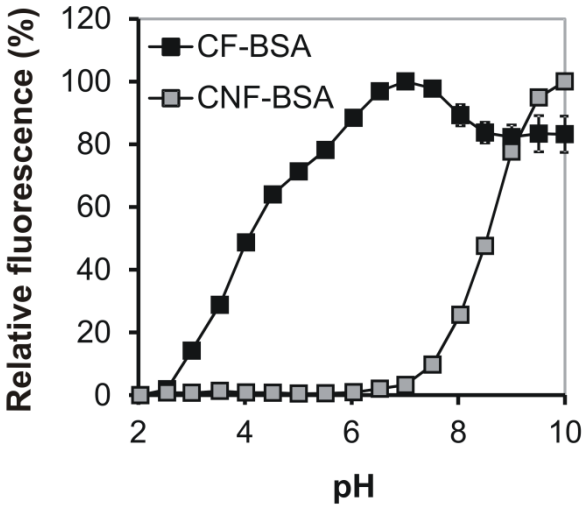

Fig. 1.

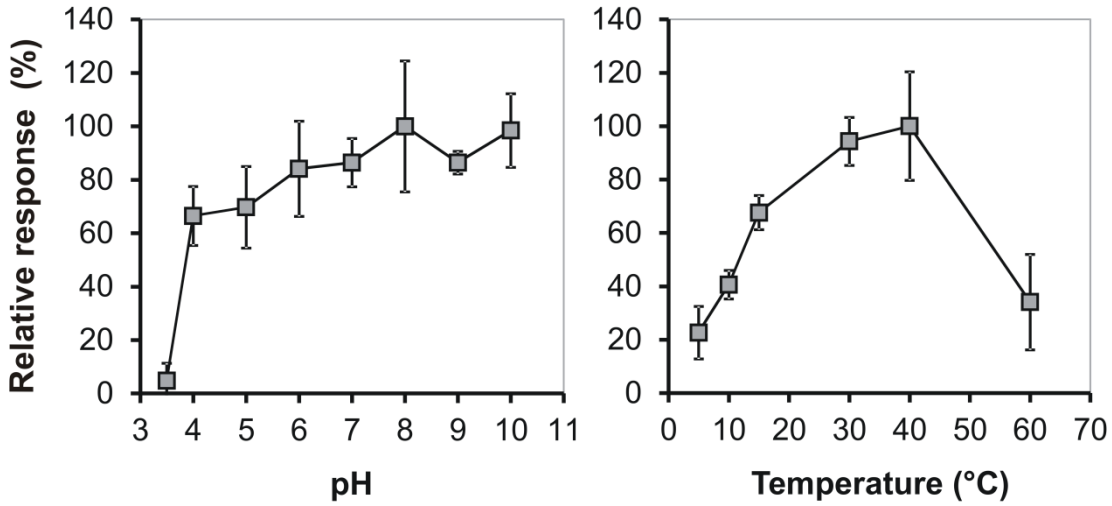

Fig. 2.

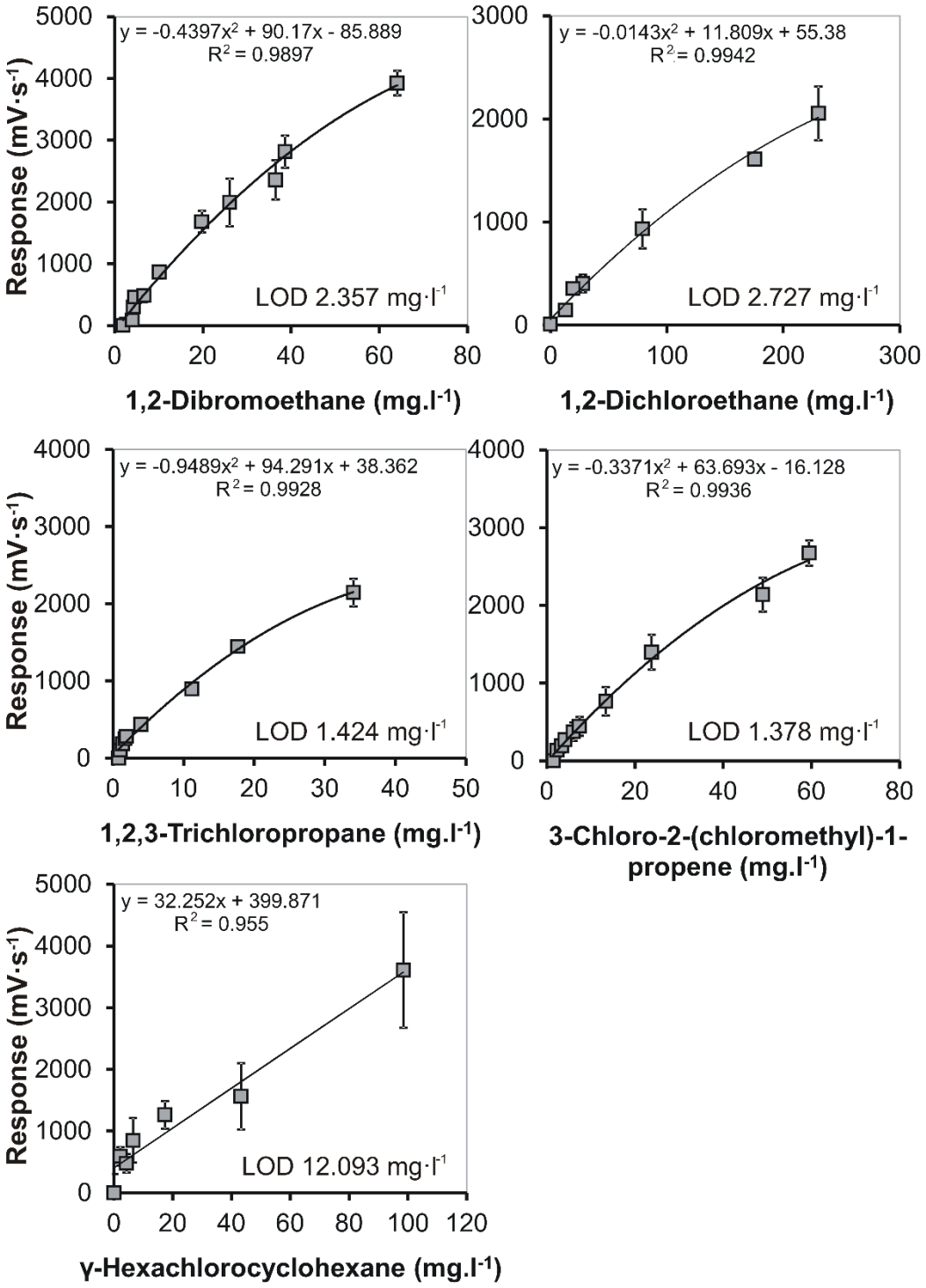

Fig. 3.

Sampled site	Concentration GC ($mg \cdot l^{-1}$)	Response sensor ($mV \cdot s^{-1}$)
1	9.62	2222.45
2	0.82×10^{-3}	111.18
3	0.76×10^{-3}	0

Fig. 4.

Sampled site	Concentration GC ($mg \cdot l^{-1}$)	Response sensor ($mV \cdot s^{-1}$)
1	1.68	2003.8
2	1.60	956.3
3	1.46	670.2
4	1.13	529.9
5	0.96	752.4
6	1.08	901.6

Fig. 5.

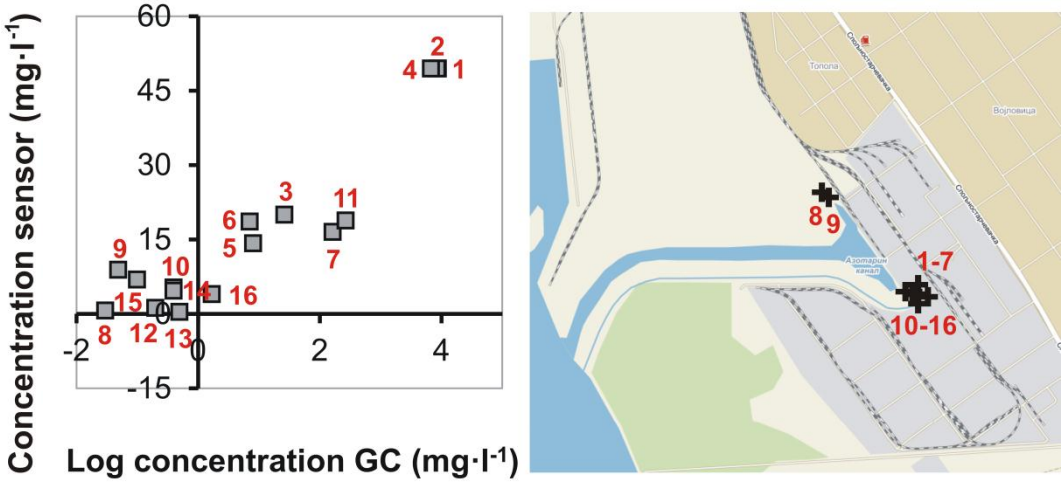

Fig. 6.

Highlights

- Biosensor using enzymatic reaction of dehalogenase and dehydrochlorinase developed
- Detection of halogenated pollutants under laboratory conditions demonstrated.
- Field trials under environmental conditions successfully performed.