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Genetic Programming as a tool for identification of analyte-specificity from complex response patterns using a non-specific whole-cell biosensor

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ABSTRACT

Whole-cell biosensors are mostly non-specific with respect to their detection capabilities for toxicants, and therefore offering an interesting perspective in environmental monitoring. However, to fully employ this feature, a robust classification method needs to be implemented into these sensor systems to allow further identification of detected substances. Substance-specific information can be extracted from signals derived from biosensors harbouring one or multiple biological components. Here, a major task is the identification of substance-specific information among considerable amounts of biosensor data. For this purpose, several approaches make use of statistical methods or machine learning algorithms. Genetic Programming (GP), a heuristic machine learning technique offers several advantages compared to other machine learning approaches and consequently may be a promising tool for biosensor data classification.

In the present study, we have evaluated the use of GP for the classification of herbicides and herbicide classes (chemical classes) by analysis of substance-specific patterns derived from a whole-cell multi-species biosensor. We re-analysed data from a previously described array-based biosensor system employing diverse microalgae (Podola and Melkonian, 2005), aiming on the identification of five individual herbicides as well as two herbicide classes. GP analyses were performed using the commercially available GP software 'Discipulus', resulting in classifiers (computer programs) for the binary classification of each individual herbicide or herbicide class.

GP-generated classifiers both for individual herbicides and herbicide classes were able to perform a statistically significant identification of herbicides or herbicide classes, respectively. The majority of classifiers were able to perform correct classifications (sensitivity) of about 80–95% of test data sets, whereas the false positive rate (specificity) was lower than 20% for most classifiers. Results suggest that a higher number of data sets may lead to a better classification performance.

In the present paper, GP-based classification was combined with a biosensor for the first time. Our results demonstrate GP was able to identify substance-specific information within complex biosensor response patterns and furthermore use this information for successful toxicant classification in unknown samples. This suggests further research to assess perspectives and limitations of this approach in the field of biosensors.

1. Introduction

Whole-cell biosensors are the focus of increasing interest as portable and cost-effective devices for in situ monitoring of environmental pollution (recent reviews: Pasco et al., 2011; Su et al., 2011; Xu and Ying, 2011). A whole-cell biosensor integrates an immobilized microorganism with a physical transducer to generate, after amplification, a signal that is proportional to the concentration of the analyte. Most whole-cell biosensors, for principal reasons, are non-specific and therefore capable of detecting multiple analytes. This property is an advantage for applications dealing with the multitude of mostly unknown substances present in the natural environment (Belkin, 2003). A promising approach to increase the specificity of whole-cell biosensors for in situ environmental monitoring has been the introduction of genetically engineered bacteria, in which the fusion of promoters, responsive to a particular environmental condition, is coupled to reporter genes that mostly generate a fluorescent or electrochemical signal (reviews: Robbens et al., 2010; Ron, 2007; Shin, 2011; van der Meer and Belkin, 2010). However, non-specific whole-cell biosensors mostly do not extend beyond the function of an early-warning system and do not act as an all-in-one monitoring device for detecting and quantifying diverse analytes (e.g. Brayner et al., 2011). To

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overcome this disadvantage, a non-specific biosensor needs to be equipped with a reliable classification method that allows it to function as a full identification system for the detection of individual substances or classes of substances based either on substance-specific signals or on signal patterns arising from the biological component(s) of the biosensor. For this purpose, an essential prerequisite is to detect the complex substance-specific signals or characteristic signal patterns within the large set of data recorded with an online sensor system, a task likely to be unfeasible for a human data analyst.

In data-mining, computer-based machine learning approaches provide effective solutions for such problems (Weiss and Indurkhya, 1998). In brief, supervised machine learning approaches aim to generate programs capable to predict an output from a previously characterized input. Introduced by Koza (1990), Genetic Programming (GP) is a heuristic machine learning approach to develop computer programs based on Darwinian evolutionary principles. A population of randomly generated individual computer programs is modified stepwise by genetic crossover and mutation in a computational "breeding" process (Banzhaf et al., 1997). In between these modification steps during the design process, an evaluation is performed based on how well each program performs the pre-defined task. A selection process then reduces the total set of programmes to the most successful ones as a basis for further 'genetic' modifications. As a supervised machine learning process, GP has a powerful potential for classification (Espejo et al., 2010). Compared to other machine learning-based classification techniques such as artificial neural networks (ANNs), supported vector machines (SVMs) or *k*-nearest-neighbour or compared to statistical classification methods, the accuracy, flexibility and interpretability of GP for classification tasks was shown to be predominantly better or at least as good as that of other techniques (Espejo et al., 2010; Morrison et al., 2010).

The use of GP for practical applications has increased during the recent years. For example, GP is applied for classification tasks in the Life sciences to predict survival rates of patients by evaluation of medical data (Bojarczuk et al., 2004), to classify cancer subtypes based on analysis of molecular data sets (Worzel et al., 2009), in analysis of microarrays (Liu and Xu, 2009) or for the recognition of epileptic patterns in electroencephalographic signals (Lopes, 2007). Furthermore, GP is a tool for predictions in financial applications (Elazouni and Metwally, 2005) or for evaluation of software quality (Afzal and Torkar, 2011).

In the field of biosensors, GP has not been introduced until now. There are several successful approaches in pattern recognition for the classification of substances applying ANNs (e.g. Gholmieh et al., 2003; Talaie and Romagnoli, 1996; Torrecilla et al., 2008) or SVMs (Sadik et al., 2004). Recently, Wedge et al. (2009) constructed an organic field-effect transistor (OFET)-based electronic nose and were able to classify four organic solvents by means of GP analysis of data sets derived from the chemical sensor.

In previous work, we introduced a biosensor employing multiple species of immobilized microalgae on a biochip (Podola and Melkonian, 2005; Podola et al., 2004). Volatile organic compounds as well as herbicides were identified by statistical analysis of toxicant-specific response patterns, arising from individual toxicant-induced reactions of each species on the chip. Although we were able to prove the functionality of this novel approach, it became obvious that a statistical search for substance-specificity by a human data analyst already reached a limit with the simultaneous application of four herbicides (Podola and Melkonian, 2005). Therefore, in the present study, we re-analysed our herbicide data sets by means of GP to evaluate the applicability of this machine learning technique for classification of biosensor data.

2. Materials and methods

2.1. Biosensor system and herbicide exposure experiments

Biosensor data sets for GP analysis were derived from earlier herbicide exposure experiments described previously by Podola and Melkonian (2005) employing a chlorophyll fluorescence-based biosensor system [For detailed information on biosensor system as well as full experimental setup, see Podola and Melkonian (2005)]. The biological component is represented by nine microalgal strains immobilized in parallel in the biosensor array (species: *Chlamydomonas* sp., *Chlorella vulgaris*, *Cryptomonas* sp., *Eudorina elegans*, *Haematococcus pluvialis*, *Pseudokirchneriella subcapitata*, *Scherffelia dubia*, *Staurodesmus convergens*, *Synechocystis* sp., *Tetraselmis cordiformis*). Herbicide exposure experiments were carried out using five herbicides from three chemical classes (triazines: atrazine, simazine; phenylurea derivates: diuron, isoproturon; quaternary ammonium: paraquat). Nine concentrations between 0.05 and 500 $\mu g\,L^{-1}$ for each herbicide and a herbicide-free control were applied in six replicates. Each exposure experiment was carried out for 20 min and fluorescence was recorded every 2 min using a pulse-amplitude-modulated (PAM) imaging chlorophyll fluorometer (IMAGING-PAM, H. Walz GmbH, Effeltrich, Germany). The biosensor signal was calculated based on the herbicide-induced shift of the chlorophyll fluorescence in steady state of photosynthesis (F_t) compared to a toxicant-free control recorded in parallel.

Additionally, fluorescence was recorded following the procedure described above for individual concentrations of atrazine (10 $\mu g\,L^{-1}$), simazine (50 $\mu g\,L^{-1}$), diuron (2 $\mu g\,L^{-1}$) and isoproturon (10 $\mu g\,L^{-1}$), which were used by Podola and Melkonian (2005) as a 'reference data set' to evaluate the performance of herbicide classification.

2.2. Data preparation and Genetic Programming

In the present approach of toxicant identification, a herbicide-specific chlorophyll fluorescence signal is based on specific differences in reactions of nine microalgal strains on toxicant exposure. By our definition, these response patterns have to be (1) specific for one herbicide (or herbicide class), (2) independent of the herbicide concentration applied, and (3) independent of the duration of exposure. One data set used for pattern recognition by GP consists of the biosensor responses of the nine microalgal strains in the biosensor array (recorded in parallel) to one specific exposure condition (herbicide, concentration, exposure time) and is available in six replicates, each.

The biosensor responses from the nine algal strains were passed to the GP software as a feature matrix consisting of nine features (feature = microalgal strain) and can be visualised as a response pattern containing the substance specific information. Considering the experimental setup used, for each herbicide, a maximum of 2760 data sets is available for GP analysis. However, a response pattern can only be postulated if a significant signal from at least one out of the nine biological components (microalgal strains or features) of the biosensor system can be detected. To identify response patterns for further analysis, statistically significant biosensor signals were analysed by comparison against a toxicant free control.

Pattern recognition applying Genetic Programming was performed using 'Discipulus Professional v 4.0' Software (RML Technologies Inc., Littleton, USA). The GP approach introduced here is based on the generation of seven herbicide-specific binary classifiers (computer programmes) by the GP software. Each specific classifier aims on identification of one individual herbicide or one chemical class, respectively. For the GP training of a herbicide-specific or class-specific binary classifier,

Table 1
Parameter settings of Genetic Programming using 'Discipulus v4' GP software.

Parameter	Value	Parameter	Value
Generations without improvement	300	Population size	500
Maximum number of runs	300	Mutation frequency %	95
Block mutation rate %	30	Crossover frequency %	50
Instruction mutation rate %	30	Target subset size %	50
Instruction data mutation rate %	40	Selection by age %	50
Homologous crossover %	95	Selection by difficulty %	50
Initial program size	80	Stochastic selection %	0
Max program size	512	Frequency (in generation equivalents)	1

the GP software is provided with two types of data sets: (1) data sets derived from exposure experiments regarding the specific herbicide or chemical class to be detected and showing a significant biosensor signal (class 1 data) and (2) data sets from all other herbicides displaying a significant biosensor response and/or data sets without a significant biosensor response (class 0 data). According to this definition, in preparation for classifier generation, classes 1 and 0 are assigned to the overall feature matrix containing the entire (2760) data sets. This was done separately in seven specific datasheets for each of the seven classifiers, respectively. The entire matrix was randomly mixed using Microsoft Excel's rand function and subsequently partitioned into three groups. Two groups, each consisting of 40% of the total number of data sets, were used by the GP software as 'training' and 'validation' data during the GP process. 20% of the data sets were used as 'applied' data to finally evaluate the generated classifier with data sets which were not used during the GP process. For each classifier, a five-fold cross validation is performed to minimize the effect of artefacts within the data sets. To evaluate the classifiers, classification results are expressed as 'hit rates' (true positive rate), indicating the number of positive classifications when applying test data sets. Ideally, an optimal classifier should be able to display hit rates of 100% when class 1 data are applied and 0% in case of class 0 data.

GP analyses were performed using the standard settings of the Discipulus software (see Table 1 for details) with the following exceptions: In 'Advanced Options' Stepping was disabled. In 'Single Run Advanced Parameters' the value for 'Weight for Rate of Positive Hits' as well as 'Weight for Rate of Negative Hits' was set to $50/n$, where n is the number of class 1 (for positive hits) or class 0 data (for negative hits) in the training data set, respectively.

2.3. Statistical analyses

The significance of the biosensor response signal was evaluated using student's *t*-test performed by Microsoft Excel 2007. The performance of classifiers in terms of a significant differentiation between class 1 and class 0 data sets was evaluated by the Kruskal–Wallis *H*-test prior to the Mann–Whitney *U*-test using GraphPad Prism v5.02 (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Performance of GP-generated classifiers

The performance of GP-generated herbicide classifiers for five individual herbicide and for two herbicide classes (triazines, phenylurea herbicides) were evaluated using two types of test data sets: (1) The data used as 'GP applied data' during GP training and (2) the 'reference data' used as 'test response pattern RP2' by Podola and Melkonian (2005). Both data sets had not been used for GP training of classifiers before. Table 2 shows the percentages of positive classifications (hit rates) by the classifiers when test data were applied. For both test data sets, classifiers always showed the highest hit rate when data of the trained class 1 herbicide were used. With exception of the DIU and ISO classifier in 'reference data' identification, class 1 identification resulted in positive hits of about 80% or higher, whereas class 0 data were (falsely) positively classified in only 0–40% of the test data. Comparable results were observed for the classifiers for all individual herbicides and for herbicide classes (Table 2).

Statistical analysis revealed significant differences with a $p < 0.05$ between hit rates of class 1 and class 0 data sets of each individual classifier for both of the two types of test data sets ('GP

Table 2
Hit rates (positive classifications) [%] of classifiers for different test data sets and number of class 1 data sets (data sets displaying a significant biosensor response) [*n*] used for training for the individual classifiers, respectively. Hit rates are displayed ± standard deviation ($n = 5$, based on five-fold cross validation). GP applied data: data used as 'Applied data' in Discipulus GP training. Reference data: data used as 'test response pattern RP2' in: Podola and Melkonian (2005). Both datasets had not been used for GP training of classifiers before, i.e. can be regarded as 'unknown samples' for the evaluation of the GP-derived classifiers. Abbreviations: ATR, atrazine; SIM, simazine; DIU, diuron; ISO, isoproturon; PAR, paraquat; ATR/SIM, triazines; DIU/ISO, phenylurea derivates; upper-case letters indicate classifiers, lower-case letters label test data sets.

Test data		CLASSIFIER obtained by GP training for						
		ATR	SIM	DIU	ISO	PAR	Triazines ATR/SIM	Phenylurea herbicides DIU/ISO
n of class 1/class 0 datasets for GP analyses		*330/2430*	*354/2406*	*402/2358*	*462/2298*	*174/2586*	*684/2076*	*864/1896*
GP applied data	atr	**84.3 ± 5.6**	22.2 ± 15.6	5.2 ± 2.3	24.7 ± 13.6	1.1 ± 1.9	**89.8 ± 2.1**	23.0 ± 12.7
	sim	27.5 ± 18.4	**88.0 ± 4.9**	3.1 ± 2.4	6.0 ± 5.1	4.7 ± 5.1	**84.5 ± 5.5**	4.9 ± 3.1
	diu	8.7 ± 3.7	4.8 ± 4.8	**94.6 ± 1.3**	7.8 ± 6.9	0.5 ± 1.1	5.3 ± 3.7	**93.8 ± 4.3**
	iso	20.9 ± 5.2	7.9 ± 6.0	3.2 ± 2.0	**79.7 ± 5.5**	0.7 ± 1.5	17.7 ± 9.9	**82.5 ± 10.1**
	par	3.5 ± 4.1	3.4 ± 3.6	0.4 ± 1.0	0.0 ± 0.0	**88.4 ± 7.8**	1.6 ± 2.3	0.0 ± 0.0
Referemce data	atr	**81.3 ± 4.3**	21.0 ± 22.4	3.2 ± 1.9	9.0 ± 4.3	2.0 ± 4.5	**80.7 ± 5.1**	13.0 ± 3.8
	sim	40.3 ± 33.8	**80.7 ± 18.8**	3.2 ± 4.6	4.3 ± 1.9	0.0 ± 0.0	**96.3 ± 4.0**	5.3 ± 4.6
	diu	1.3 ± 0.7	9.3 ± 20.0	**52.7 ± 30.2**	27.7 ± 33.6	0.0 ± 0.0	0.0 ± 0.0	**95.0 ± 3.5**
	iso	22.0 ± 3.0	27.3 ± 21.6	4.0 ± 3.2	**71.7 ± 32.0**	0.0 ± 0.0	14.7 ± 8.1	**81.3 ± 7.8**

Values in bold and italic indicate significance.

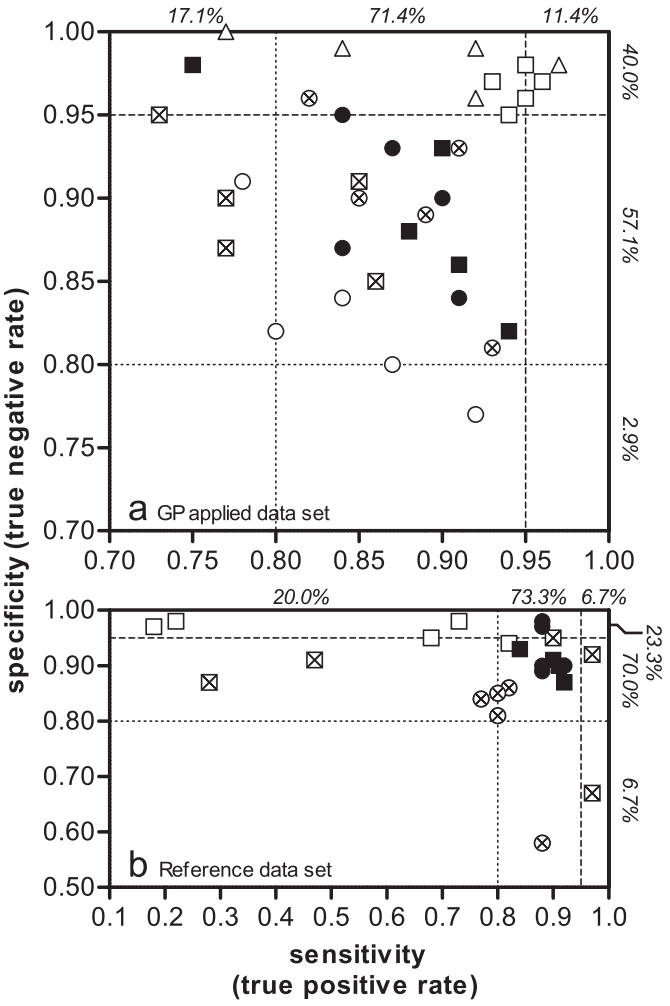

Fig. 1. Performance of herbicide classifiers in terms of sensitivity (true positive rate) and specificity (true negative rate): The figure shows sensitivity and specificity for each of the 35 classifiers generated (7 herbicides or herbicide classes in five replicates, each). For each classifier, sensitivity and specificity were calculated based on the 'GP applied' data set (a) and 'Reference' data set (b). Classifiers are labeled as follows: ○, atrazine; ⊗, simazine; □, diuron; ⊠, isoproturon; △, paraquat; ●, triazines: atrazine, simazine; ■, phenylurea derivates: diuron, isoproturon. The average numbers *n* of data sets used for the evaluation of the seven types of classifiers for the 'GP applied' data set (a) were (*n* used for sensitivity/*n* used for specificity): atrazine (66/278), simazine (71/274), diuron (81/264), isoproturon (92/252), paraquat (35/310), triazines: atrazine, simazine (137/208), phenylurea derivates: diuron, isoproturon (173/193). For the 'Reference' data set (b), *n* was (60/180) for individual herbicides and (120/120) for herbicide classes. Average *n* was calculated from five individual replicates used for five-fold cross validation. For sensitivity and specificity, percentile values given in the figure indicate the proportion of classifiers (from total *n* of classifiers) in categories 1.00–0.95, <0.95–0.80 and <0.80.

applied data' and 'reference data'), respectively. This demonstrates that classifiers were able to significantly differentiate between class 1 and class 0 data.

Fig. 1 shows the classification performance in terms of sensitivity (true positive rate) and specificity (true negative rate) for all classifiers generated (7 classifier types for herbicides or herbicide classes in five replicates, each). For each classifier, sensitivity and specificity were calculated based on the 'GP applied' data set used during GP training. With respect to specificity, more than 90% of classifiers evaluated showed comparable results in correct negative classifications at a level of 80% or higher for both types of test data. However, classification of the 'GP applied' data set led to better results in the category of a classification success of 95% or higher: 40% of classifiers were able to provide this accuracy, compared to 23.3% of classifiers when the reference data set was applied. Generally, sensitivity is displaying a slightly lower accuracy of correct classification compared to the specificity. For both data sets, about 80% of classifiers were able to provide correct positive classifications at the 80%-level, whereas only a few classifiers performed a correct positive classification of 95% or higher.

Even though this study has a general character and results cannot be transferred on a specific application at the present state of development, it is obvious that some application might suffer from a false positive/negative rate of about 20% or from low sample numbers as may occur during routine application of the sensor system. However, our results demonstrate successfully that a high rate of >95% of correct for positive as well as for negative classification can be achieved by the present GP approach. The number of data sets used for classifier generation might hold potential for the improvement of correct classification rates: For evaluation of classifiers in terms of sensitivity and selectivity, the number of data sets tested (i.e. *n* of measured biosensor samples) ranged from 31 up to a few hundred, depending on data class and herbicide (Fig. 1, see figure legend). From the present data, no relationship between low sample numbers and the accuracy of classification in terms of sensitivity and selectivity during the evaluation procedure can be observed. However, a relationship between classification performance and data sets available for GP training purposes can be postulated: Defined by the experimental setup dealing with an overall data set of 2760 biosensor samples for GP analyses, for classifiers for individual herbicides the number of class 0 data available for GP training is in average about 8 times higher than class 1 data. It can be assumed that the higher amount of class 0 data could explain the higher performance in specificity compared to sensitivity, since a significantly higher *n* of negative (class 0) data sets potentially improves the classifiers ability in correct classification of negatives (true negative rate) during GP training. In contrast, for the training of classifiers for herbicide groups, the *n* of class 1 and class 0 data is more balanced at a factor of 2.5. This leads to a more balanced relationship between sensitivity and specificity with values between 80 and 95% for most of the classifiers. Therefore, it can be concluded that the increase of data sets for training purposes may clearly improve the accuracy and reliability of the classifiers. Unpublished data confirm this assumption.

Furthermore, increasing the diversity of biological components may lead to a higher sensitivity of a classification method. Chaplen et al. (2007) were able to increase sensitivity of a statistical classification approach up to 95% by combining a microalgal and fish chromatophore sensor for classification, whereas both individual sensors which showed a true positive rate of about 70%, each. With respect to our array-based algal biosensor system, this aspect could be implemented during strain selection by a higher weighting of biological diversity of the very diverse groups of microalgae compared to the present sensitivity-based selection strategy (sensitivity here: sensitivity against toxicants). Also the use of selectively resistant mutants in the biosensor array as described by Altamirano et al. (2004) may be an interesting option in this context.

3.2. Potential of Genetic Programming in a whole-cell multisensor approach

The advantages offered by whole-cell biosensors are apparent (Eltzov and Marks, 2011; Pancrazio et al., 1999): Cells can provide the higher stability of a self-maintaining system compared to some isolated biological components (e.g. enzymes, DNA or antibodies). A higher cost-efficiency can be obtained since downstream processes such as isolation/production of the biological components are not required. Moreover, when exposed to harmful environmental conditions, the reactions observed can be considered as

Table 3
Whole-cell biosensors for the detection and classification of multiple analytes.

Biological component(s)	Signal	Toxicant-specific pattern	Classification method	Reference
Rat hippocampal neurons	Neuronal action potential	Shape of neuronal action potential	Nearest neighbour algorithm	Prasad et al. (2006)
Hybrid cell line Mouse neuroblastoma × rat glioma)			Unconstrained nonlinear optimization	Akanda et al. (2009)
Human alveolar epithelial cells	Raman scattering	Raman spectrum	Principal component analysis and linear discriminant analysis	Notingher et al. (2004)
Microalgal cells and fish chromatophores	Fluorescence and optical density	Combined biosensor signals	Linear discriminant analysis	Chaplen et al. (2007)
E. coli carrying *lux*-genes fused to different stress-responsive promoters	Luminescence	Luminescence pattern arising from different promoter-activated *lux*-genes	Discriminant analysis	Ben-Israel et al. (1998)
			Bayesian decision theory and nearest neighbour algorithm	Elad et al. (2008)
Rat hippocampal tissue	Quantification of short-term plasticity	Laguerre coefficient and first order kernel (Volterra modeling)	Artificial neural networks (ANNs)	Gholmieh et al. (2003)

overall effects of the perturbations on a living. Although whole-cell biosensors are mostly non-specific and therefore most promising for multi-analyte detection, many proposed whole-cell systems lack this property (Eltzov and Marks, 2011). Furthermore, multi-analyte detection only develops its full potential in a combination with a powerful classification method allowing the identification of the detected substances. During the last decade a few methods were successfully introduced to address this problem (Table 3). Here, several cell types and detection techniques were applied and classification relied on mathematical/statistical methods as well as on machine learning approaches. In addition to the biosensor used in our study, only two systems (the *lux*-gene sensor and the combined microalgal/fish chromatophore sensor) make use of toxicant-specific reaction patterns arising from a multisensor for classification (Table 3).

In comparison to our previously used statistical approach (Podola and Melkonian, 2005), the present investigations indicate GP can be effectively employed to identify herbicides with higher significance and furthermore classify herbicide classes using the same biosensor setup. With the present data sets, training (or generation) of classifiers requires a few minutes to a few hours each, depending on the complexity of the substance-specific patterns. However, the classification of unknown samples (here: test data) by the computer programmes (classifiers) that emerged from the training process can be performed in real-time and potentially can be technically integrated in the biosensor system. Our approach employed GP for data analysis in combination with multi-analyte whole-cell biosensors. Whereas a multisensoric (or array-based) approach is required to provide a substance-specific pattern for classification, the method may not restricted to whole-cell biosensors only and could be applied to other more specific multisensoric biosensors.

One problem of particular interest in environmental monitoring is the detection of substances in mixtures (Silins and Hogberg, 2011). Using ANNs for classification of substances in mixtures, studies by Bachmann et al. (2000) and Baronas et al. (2004) demonstrated that these tasks can be potentially solved by a machine learning method. Establishing a substance-specific classifier library for the identification of important toxic substances (e.g. Priority substances under the EU Water Framework Directive; see http://ec.europa.eu/environment/water/water-dangersub/pri_substances.htm; European Union 2001) could be envisaged in the medium to long-term.

3.3. Conclusions

In the present work, we successfully demonstrated the application of Genetic Programming for pattern recognition and significant analyte classification in complex biosensor signals for the first time. With respect to the advantages of this machine learning technique in combination with non-specific biosensors, further investigations appear to be promising to evaluate the potentials and limitations of GP in this field. To meet the requirements of several specific practical applications, improving sensitivity and specificity of this new approach may be necessary. Here, we suggest focusing further efforts on the number of training data sets required for an optimal GP training as well as on the increase of biological diversity within the multisensor array.

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