Water pollution monitoring by an artificial sensory system performing in terms of *Vibrio fischeri* bacteria

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Abstract

This report describes the application of potentiometric multisensor system for estimation of water samples toxicity in terms of Microtox® analyzer – a wide spread instrument for toxicity evaluation. The working principle of Microtox® analyzer is based on a registration of luminescence from *Vibrio fischeri* bacteria which depends on metabolism conditions and toxicity of the environment; this is associated with certain limitations. Unlike this bioassay procedure the employment of multisensor system does not require the use of living organisms and can provide for faster toxicity evaluation. 54 real and imitated polluted water samples, for which the toxicity was established by bioassay, were studied. The response of multisensor array processed with machine learning techniques allows for prediction of *EC50* (toxicity index) with relative errors of 20-25%. Taking into account the complexity of the task (simulation of complex biological reactions with inanimate instrument) this can be considered as a good promise for further research in this direction in order to develop instrumental alternative for toxicity assessment.

Keywords: Electronic tongue, *Vibrio fischeri*, Water pollution

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1. Introduction

Water pollution, which is really a global problem now, is caused by a constant increase of the number of industries and plants, an accelerated rate of the development of the agriculture and a constant growth of the amount of vehicles. Most of the sources of aquatic pollution are well-known. Around 50% of the total pollution of surface water is accounted for agriculture sector [1]. In this case the major pollutants are ammonium (NH_4^+) [2] and nitrate (NO_3^-) [3] ions, while phosphorous [4] is widely appearing from pesticide input. Domestic and municipal wastes [5, 6] also cause a great damage to ecosystems. Such wastes may contain a wide range of the pollutants, like pathogens [7], organic substances [8, 9], heavy metals [10, 11] and more and more pharmaceuticals [12]. Due to the population growth the amount of wastes produced by people is increasing significantly. About 3 billion people in the world lack access to clean water, according to the World Health Organization. It is supposed, that water pollution will increase at least twice over the next 20 years [13].

One of the most important integral characteristics of the water quality is toxicity. Toxicity characterizes direct biological hazard of a water sample for a living organism. The toxicity is a convenient integral estimate as opposed to, e.g. MAC (maximum allowable concentration) widely applied for water analysis. MAC represents concentrations of chemical elements and their compounds in the environment that would not cause pathological changes or diseases in human body over long-term exposure. It means that there is a limit of the harmful substance content below which it is safe for humans to interact with this compound. However, the amount of the pollutants increases every day, over seventy thousands of contaminants being currently totaled [13, 14]. Therefore the determination of the maximum allowable concentration for each of these pollutants is getting much harder if possible at all. Thus, MAC is far from being the optimal criterion of environmental quality evaluation.

Various methods of biotesting have been developed and legislated for water toxicity evaluation. They are mostly based on the study of the reaction of a living test-object when exposed to an aqueous sample. Different aquatic organisms such as fishes, phytoplankton, zooplankton and bacteria are traditionally used as test-objects.

The Microtox Acute Toxicity Test is one of the most widely used biotesting methods. Microtox was introduced by Beckman Instrument Co. [15] and became the first microscale biomonitoring tool in environmental toxicology.

Luminescent marine bacteria *Vibrio fischeri* also known formerly as *Photobacterium phosphoreum* are used as a test-object in such tests, amount of light emitted by these bacteria being an indicator of metabolism. Therefore a water toxicity parameter can be measured as a reduction of light emission caused by the presence of hazardous component(s) in the sample.

Microtox is one of the most widely used methods of biotesting due to the number of advantages. This approach is rather simple, it is based on very few elements, there is no need for preculturing of test biota, because of the fact that the measurement of light emission begins immediately after bacteria are entered into the water. Microtox employs storage of bacteria in lyophilized state which makes it cost-effective because of the elimination of maintenance cost and long term stability of the culture. Besides the Microtox Acute Toxicity Test is an express method in comparison with other bioassays. Microtox tests are typically completed in 15 or 30 minutes while other biotesting methods, such as test with fish or invertebrates, can often take a few days. The short duration of Microtox analysis significantly increases sampling throughput capability of this test.

Microtox is applied as a screening tool for a wide range of ecotoxicological problems. Over 50% of all applications of the Microtox Acute Toxicity Test are related to assessment of industrial-domestic wastes [16, 17], leachate studies, examination of the toxicity of polymer/alum addition to the aeration tank effluent prior using in a slaughterhouse wastewater treatment plant [18], and also for evaluation of risks, in relation with a simulated oil spill [19]. There was an attempt to estimate an effect of river water, sediment and time on toxicity using Microtox [20]. It was found, that the presence of sediment as well as exposure time affected the toxicity of water sample. The extracted fly ash [21], appearing as a result of incineration of wastes, which is one the most popular waste treatment method, has been also investigated.

One of the prevalent applications of Microtox is the evaluation of toxicity of river sediments; such investigations were carried out in Poland [22], France [23], and Portugal [24]. In Poland a study of toxicity of surface waters of several rivers and lakes was also carried out [22]. These tests revealed that the Microtox assay is a suitable test for estimation of the toxicity of bottom sediments, in which contaminants tend to accrue.

Some of the recent works were devoted to the analysis of soils, containing polycyclic aromatic hydrocarbons [25], biochar and pesticides (2,4-D and dicamba) [26], and pharmaceutical wastewaters [27]. Researches from Italy tried to estimate an ecotoxicological effect of suspensions of basaltic rock, ash and cement dusts, which are often detected on building sites near the volcano Etna, on the luminescent of marine bacteria *Vibrio fischeri* [28].

The main disadvantage of Microtox is the complexity of bringing the lyophilized bacteria in working conditions, which makes this platform hardly compatible with an idea of on-line monitoring. Another problem, common to all biotests, is related to the range of dangerous substances and degree of their toxicity that can vary for different biotests and human beings.

A possible "ideal" toxicity assessment instrument should have fast response allowing for of on-line measurements and should possess broad sensitivity spectra towards various toxicants allowing for reliable alarm performance in all cases.

In this paper we attempt to expand the range of aquatic quality indicators for toxicity estimation with a multisensor system, often called electronic tongue. Currently the electronic tongue became a useful tool for food and drink analysis [29] and pharmaceutical analysis [30]. The operation of such systems is based on the application of an array of cross-sensitive chemical sensors for the analysis of liquids and subsequent processing of sensor signals by multivariate data processing techniques. In our previous works we applied this approach and managed to demonstrate that a sensor array can mimic performance of *Daphnia magna* [31, 32], *Chlorella vulgaris*, *Paramecium caudatum* [32]. Therefore we have applied the same approach for *Vibrio fischeri* in the present research.

2. Materials and Methods

2.1. Water samples

Fifty four water samples were provided by the Center for Research and Innovation in Toxicology of the Technical University of Catalonia located in Terrassa (Spain). These were wastewaters collected from different regions of Catalonia ("real" samples) and aqueous solutions of the model toxicants, prepared in the Center for Research and Innovation in Toxicology ("imitation" samples). The details about the solutions of the model toxicants are presented in Table 1. Each sample was prepared in 500 ml plastic bottle with a screw cap. Samples were stored in the refrigerator between measurements.

Table 1. Samples of model toxicants used in the study.

Apart from the 24 samples shown in Table 1 and 26 samples of wastewater, for which the toxicity data were provided by toxicologists, there were four samples, which composition was not disclosed due to our agreement with Center for Research and Innovation in Toxicology. Thus, these samples were actually unknown for the sensor research team.

2.2. Microtox analysis

The reduction of light emission as a measure of water toxicity was determined by SDI Model 500 Analyzer, which integrates a luminometer with an incubator. The incubator was maintained at two different temperatures: all test samples were kept at 15°C and one stock culture cuvette was stored separately at 5°C. The luminometer measures the light emissions from

luminescent bacteria. The *Analyzer* was connected to a personal computer for data collection and processing.

Freeze-dried bacteria with the *Recon,* the *Diluent* and the *Osmotic Adjusting Solution* were obtained from Microbics Corporation (Carlsbad, CA, USA).

The amount of light reduction (Gamma) is calculated as follows:

$$GAMMA(\Gamma) = \frac{\frac{I_t^0}{I_0^0} \cdot I_0^C - I_t^C}{I_t^C} \quad (1),$$

where I_0^0 is a light intensity of solution with the concentration 0 at time 0, I_t^0 is a light intensity of solution with concentration 0 at time *t*, I_0^C is a light intensity of solution with concentration *C* at time 0, I_t^C is a light intensity of solution with concentration *C* at time *t*.

This function allows to calculate the effective concentration *EC50(t)* which is the concentration of sample causing a 50% light reduction at exposure time of *t* minutes. Different chemicals affect marine bacteria at different rates. The decrease of light emission is complete in 5 minutes for some organic substances, while, e.g. for ammonia this time is not enough for ending the changes of light output. In such case a 15 minutes exposure time may be more appropriate. It was decided to measure light emission after 15 minutes when dealing with unknown samples.

Estimation of Gamma function and finally *EC50* were undertaken using a MicrotoxOmni software program. All *EC50* values were expressed as concentration (mg/l) or percentage (%) with 95% confidence intervals; at least three replicate measurements were taken for each sample.

The lower *EC50* value the greater the toxicity of the sample. The acute toxicity was divided by classes: «high toxic» – *EC50* ≤ 1 mg/L, «toxic» – *EC50* ≤ 10 mg/L, «low toxic» – *EC50* ≤ 100 mg/L. The United States Environmental Protection Agency's (EPA) also distinguishes the category «non-toxic» for the samples with *EC50* > 100 mg/L.

The toxicity of wastewater samples was expressed as EC50 in percentage units, which represent the percentage of sample causing a 50% reduction in light emitted. The EC50 of a non-toxic sample is expressed as EC50 > 100%

An automated system for continuous *in situ* aquatic toxicity determination for water streams based on these principles has been developed [33, 34].

2.3. The sensor array and potentiometric measurements

A multisensor system applied in this study was constructed of 23 cross-sensitive potentiometric sensors. Seven of them were poly(vinylchloride) (PVC)-plasticized anion-sensitive sensors based on anion-exchangers of various structure, 7 other were PVC-plasticized cation-sensitive sensitive sensors similar to those reported in [35], another 8 were chalcogenide glass sensors with pronounced sensitivity towards various heavy metals (Ag, Cu, Cd, Fe, Hg, Pb) [36] and one standard pH glass electrode was also included. All membrane active compounds for PVC-plasticized sensors, i.e. ion-exchangers, plasticizers and PVC were Fluka reagents of Selectophore grade from Sigma–Aldrich (Munich, Germany). All components for chalcogenide glasses synthesis were of the highest available purity from Sigma–Aldrich. The measurements were made against standard Ag/AgCl reference electrode. Sensors array was connected to a 32-channel digital high input impedance voltmeter. All obtained data were measured with 0,1 mV precision and recorded to a PC. At least 5 replicated ET (electronic tongue) measurements were carried out for each sample and these results were averaged for further data processing. Water samples were stirred, but not shaken up during the measurements to avoid contamination of the sensors by precipitates present in some of the solutions. Samples were not treated anyhow or diluted before the measurements and used as is. Measurement time in each 50 ml sample was 3 min. The sensor array was washed with three portions of distilled water between measurements, the total time of washing being about seven minutes. The reported procedure was sufficient to provide for ±3 mV reproducibility of the sensors readings in the replicate measurements.

2.4. Data processing

Projection on latent structures method (PLS1), random k-nearest neighbor algorithm (KNN) and random forest (RF) method were used for data processing. Short description of these methods is presented below.

2.4.1. Partial least squares

PLS1 (partial least squares) regression is one of the most widely used methods in chemometrics [37], the PLS regression model is designed from a training set of N observations with X-variables and Y-variables, here the ET data from sensor array are independent variables to predict toxicity values as dependent variables. PLS models were computed with The Unscrambler® 9.7 (CAMO Software AS, Norway). Evaluation of the quality of the PLS model was conducted using two strategies: full cross-validation and random split test set. For full cross-validation N models were generated, where N is the number of samples, using $N - 1$ samples for training and the remaining one for validation so that there were in total N samples for validations but each one for a different model. For random partition 1/3 of samples were randomly extracted

and used as test set and the remaining 2/3 of the samples were used for constructing a calibration model. This procedure was repeated thirty times and each time the standard error of prediction (RMSEP) was calculated:

$$RMSEP = \sqrt{\frac{\sum_i (y_i^{pred} - y_i^{real})^2}{n}} \quad (2),$$

where n is a number of samples in the test set, y_i^{pred} is the value predicted by the model, y_i^{real} is the reference value.

In this paper samples were divided into two groups: the "real" and the "imitation" ones. The dependent variable was prescribed with the maximum value 100 for the samples where *EC50* was more than a hundred. This procedure was done to preserve the model representation, as the number of samples is quite limited and the removal of samples would lead to the loss of this characteristic.

2.4.2. Random forest

Random forest method was proposed in [38] and is applicable for classification and regression tasks. A number of decision trees (Figure 1) are constructed based on the training data assuming that there are *N* training samples and *M* independent variables. The original Breiman's algorithm [38] described shortly below was focused on the classification problem. To construct a single tree, $n \leq N$ training samples were randomly chosen with replacement to form a training set for this tree. To construct a root node of the tree, $m \leq M$ variables were randomly selected and the best split of the data was chosen based on the value of one of the *m* variables. The best split was chosen usually according to the information or Gini gains [39]. The same procedure was recursively applied to two newly created nodes, the training data being split between these nodes. If all the samples to be split belong to a single class then the node becomes a leaf, which predicts this class. To predict the class attribution of a new sample using a single tree, the sample was moved down the tree towards one of its leaves, and the result was the class of the leaf. The whole forest predicts sample's class based on voting: the class predicted by the most trees was chosen. The variability of different trees allows random forest to overcome overfitting.

Figure 1. An example of a decision tree. Each decision node splits the data according to a single variable (*u* or *v* in this case) and each leaf node predicts one of the classes (A or B).

239 2.4.3. Random KNN

240 Random KNN [40] is an extension of the *k*-nearest neighbor algorithm [41]. In KNN the
241 training data itself is used for prediction. Some distance *d* between samples is considered (e.g.
242 Euclidean distance between their variable vectors). For a sample to be classified, *k* nearest
243 (according to *d*) samples ("neighbors") in the training set are found. In case of classification, the
244 most common class of the neighbors is predicted and in case of regression the dependent variable
245 values of the neighbors are averaged.

246 The idea of random KNN is similar to the one used in random forests. A collection of *r*
247 KNN models forms a single neighbor predictor. Each KNN model is obtained using different,
248 randomly chosen variable subsets. As the models are independent, their construction and usage
249 can be performed in parallel.

250 Both random forest and random KNN calculations were carried out using R software,
251 where packages *randomForest* [42] and *rknn* [43] were used.

252

253 3. Results and discussions

254 3.1. PLS-regression

255 The data from the sensor array of all of the samples were employed for producing two
256 different regression models for real and imitated samples. Since the number of the available
257 samples was rather limited we performed two different verification approaches: full cross-
258 validation and random test with 30 splits. The details of these validation procedures are shown in
259 the Table 2.

260

261 Table 2. The parameters of the multisensor system performance in prediction of water toxicity
262 values in terms of Microtox

263

264 The obtained data allow assuming that the sensor system previously calibrated against
265 *Vibrio fischeri* can be used to assess water toxicity, especially when it comes to "real" samples.
266 This is understandable taking into account the sensitivity of the multisensor system to the range
267 of substances toxic for biological organisms such as heavy metals, pesticides and certain other
268 organic compounds often present in polluted waters.

269 PLS1 regression model was used to predict the toxicity indexes of the four totally
270 unknown samples. The results of this prediction are presented in Figure 2. Two of these samples
271 appeared being real wastewaters from Catalonia and the remaining two belong to the group of
272 imitated samples. This was confirmed after the experiment and calculations by the Center for
273 Research and Innovation in Toxicology, Polytechnic University of Catalonia (Terrassa, Spain).

Figure 2. Prediction of toxicity index in terms of Microtox bioassay from the data of the potentiometric system. Two samples are real wastewaters from Catalonia and two are imitated samples

The system is capable of predicting water toxicity of unknown samples with reasonable precision. It is noteworthy, that regression techniques (such as e.g. PLS employed here) are intended for numerical prediction of the target parameter and can handle situations with parameters expressed as inequalities (e.g. EC>100) with certain stipulations.

3.2. Random forests and KNN

The dependent variable was prescribed with the maximum value 100 for all "real" samples with $EC50 > 100\%$, so it was assumed that it is impossible to predict *EC50* values greater than 100%. Hence, we decided to address the problem in two stages: first, the samples were classified between $EC50 \leq 100$ and $EC50 > 100$ and then, if $EC50 \leq 100$, we predicted this value. There was only one censored sample for the imitated dataset so it was just excluded from the study.

Another issue of the dataset was a small number of samples with relatively large *EC50* values (> 50 for "real" and > 100 for "imitation" datasets), which could lead to poor regression performance on the samples with such values. To overcome the difficulty we decided to use log2-transformed *EC50* values for prediction. Thus, from this point on, the regression errors will be reported in log-scale.

– Experimental evaluation: "real" dataset

As was mentioned above, we first classified the water samples into two classes: $EC50 \leq 100$ ("≤ 100" class) and $EC50 > 100$ ("> 100" class). Both RF and random KNN were tried and the evaluation showed the performance of the former method being better. The number of trees in the forest was equal to 100.

We were able to obtain confusion matrices for random forests using full cross-validation. Since the construction of these classifiers is random such matrices are random as well. Hence, 1000 of such matrices were averaged; the obtained results are shown in Table 3. The number of misclassified samples did not exceed 3 in the vast majority of cases - over 99% of matrices.

Table 3. Averaged confusion matrix for "real" dataset

Next, the regression for samples with $EC50 \leq 100$ (there were fifteen samples of such kind) was calculated. This time random KNN outperformed random forests (we used $r = 100$, $k = 1$ and four variables for each KNN model). Apart from RF and RKNN, we tried to make predictions using ordinary linear regression, but it did not perform better than these two methods. Then a distribution of 1000 full cross-validation errors of random KNN was considered. Some statistics of this distribution are shown in Table 4. It can be noticed from the Table 4 that it is possible to predict the dependent variable with relative errors of about $2^{1.5} \approx 2.83$.

Table 4. Regression error distribution statistics.

– Experimental evaluation: "imitation" dataset

After removing a single right-censored sample, 23 samples were left in the "imitation" dataset. There was an attempt to construct regression models for this dataset using random forests, random KNN and linear regression. All methods failed to produce reasonable prediction accuracy in this case, the lowest averaged cross-validation error was observed for RF but still it was 3.1 which is quite large. The performance of a trivial classifier which always predicted the mean of dependent variable values in a training set was additionally evaluated and the error that it produced was 2.8.

The inability to predict *EC50* for the "imitation" dataset can be related to the lack of sensor data or to the small size of the dataset. This dataset is larger than the $EC50 \leq 100$ part of the "real" one. However, it is more difficult for the following reason. There are no covariates highly correlated with the outcome: the maximum absolute value of the Pearson's correlation coefficient was 0.35 (for variable "G9"). In comparison, the same maximum value in the "real" dataset was 0.7 (for variable "C11").

Thus, water toxicity evaluation by a multisensor system in terms of *Vibrio fischeri* marine bacteria is possible with experimental errors about 20-25 %, which is comparable to the cases of the other biological test objects [31, 32].

4. Conclusion.

The assessment of water quality using living test-objects is one of the leading trends of the current environmental control. However on-line monitoring of such kind is not always possible due to the need of the maintaining appropriate habitat conditions for biological creatures. We managed to carry out the application of the sensor system for prediction of the toxicity values of wastewater samples in terms of response of marine bacteria *Vibrio fischeri*. In this case living organisms are used only for calibration of the sensor array. Although the

obtained accuracy in toxicity prediction with multisensor system may seem not very high at the first glance (20-25%), one should take into account unusual task formulation (imitation of complex biological reactions of living organisms with a set of chemical sensors) and possible advantages of multisensor approach such e.g. possibility of performing the toxicity assessment in on-line mode and simplicity of handling. Based on these considerations we believe that suggested approach shows a good promise for further research in this area.

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