

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

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17 Abstract

18 This report describes the application of potentiometric multisensor system for estimation
19 of water samples toxicity in terms of Microtox[®] analyzer – a wide spread instrument for toxicity
20 evaluation. The working principle of Microtox[®] analyzer is based on a registration of
21 luminescence from *Vibrio fischeri* bacteria which depends on metabolism conditions and toxicity
22 of the environment; this is associated with certain limitations. Unlike this bioassay procedure the
23 employment of multisensor system does not require the use of living organisms and can provide
24 for faster toxicity evaluation. 54 real and imitated polluted water samples, for which the toxicity
25 was established by bioassay, were studied. The response of multisensor array processed with
26 machine learning techniques allows for prediction of *EC50* (toxicity index) with relative errors
27 of 20-25%. Taking into account the complexity of the task (simulation of complex biological
28 reactions with inanimate instrument) this can be considered as a good promise for further
29 research in this direction in order to develop instrumental alternative for toxicity assessment.
30

31 Keywords: Electronic tongue, *Vibrio fischeri*, Water pollution
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37 1. Introduction

38 Water pollution, which is really a global problem now, is caused by a constant increase of
39 the number of industries and plants, an accelerated rate of the development of the agriculture and
40 a constant growth of the amount of vehicles. Most of the sources of aquatic pollution are well-
41 known. Around 50% of the total pollution of surface water is accounted for agriculture sector
42 [1]. In this case the major pollutants are ammonium (NH_4^+) [2] and nitrate (NO_3^-) [3] ions, while
43 phosphorous [4] is widely appearing from pesticide input. Domestic and municipal wastes [5, 6]
44 also cause a great damage to ecosystems. Such wastes may contain a wide range of the
45 pollutants, like pathogens [7], organic substances [8, 9], heavy metals [10, 11] and more and
46 more pharmaceuticals [12]. Due to the population growth the amount of wastes produced by
47 people is increasing significantly. About 3 billion people in the world lack access to clean water,
48 according to the World Health Organization. It is supposed, that water pollution will increase at
49 least twice over the next 20 years [13].

50 One of the most important integral characteristics of the water quality is toxicity. Toxicity
51 characterizes direct biological hazard of a water sample for a living organism. The toxicity is a
52 convenient integral estimate as opposed to, e.g. MAC (maximum allowable concentration)
53 widely applied for water analysis. MAC represents concentrations of chemical elements and their
54 compounds in the environment that would not cause pathological changes or diseases in human
55 body over long-term exposure. It means that there is a limit of the harmful substance content
56 below which it is safe for humans to interact with this compound. However, the amount of the
57 pollutants increases every day, over seventy thousands of contaminants being currently totaled
58 [13, 14]. Therefore the determination of the maximum allowable concentration for each of these
59 pollutants is getting much harder if possible at all. Thus, MAC is far from being the optimal
60 criterion of environmental quality evaluation.

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

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

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

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

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

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

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

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

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

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

117 118 2. Materials and Methods

119 2.1. Water samples

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

127
128 Table 1. Samples of model toxicants used in the study.

129
130 Apart from the 24 samples shown in Table 1 and 26 samples of wastewater, for which the
131 toxicity data were provided by toxicologists, there were four samples, which composition was
132 not disclosed due to our agreement with Center for Research and Innovation in Toxicology.
133 Thus, these samples were actually unknown for the sensor research team.

134 135 2.2. Microtox analysis

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

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

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

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

$$145 \quad \text{GAMMA}(\Gamma) = \frac{\frac{I_t^0}{I_0^0} \cdot I_0^C - I_t^C}{I_t^C} \quad (1),$$

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

149 This function allows to calculate the effective concentration $EC50(t)$ which is the
150 concentration of sample causing a 50% light reduction at exposure time of t minutes. Different
151 chemicals affect marine bacteria at different rates. The decrease of light emission is complete in
152 5 minutes for some organic substances, while, e.g. for ammonia this time is not enough for
153 ending the changes of light output. In such case a 15 minutes exposure time may be more
154 appropriate. It was decided to measure light emission after 15 minutes when dealing with
155 unknown samples.

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

159 The lower $EC50$ value the greater the toxicity of the sample. The acute toxicity was
160 divided by classes: «high toxic» – $EC50 \leq 1$ mg/L, «toxic» – $EC50 \leq 10$ mg/L, «low toxic» –
161 $EC50 \leq 100$ mg/L. The United States Environmental Protection Agency's (EPA) also
162 distinguishes the category «non-toxic» for the samples with $EC50 > 100$ mg/L.

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

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

168

169 2.3. The sensor array and potentiometric measurements

170 A multisensor system applied in this study was constructed of 23 cross-sensitive
171 potentiometric sensors. Seven of them were poly(vinylchloride) (PVC)-plasticized anion-
172 sensitive sensors based on anion-exchangers of various structure, 7 other were PVC-plasticized
173 cation-sensitive sensitive sensors similar to those reported in [35], another 8 were chalcogenide
174 glass sensors with pronounced sensitivity towards various heavy metals (Ag, Cu, Cd, Fe, Hg, Pb)
175 [36] and one standard pH glass electrode was also included. All membrane active compounds for
176 PVC-plasticized sensors, i.e. ion-exchangers, plasticizers and PVC were Fluka reagents of
177 Selectophore grade from Sigma–Aldrich (Munich, Germany). All components for chalcogenide
178 glasses synthesis were of the highest available purity from Sigma–Aldrich. The measurements
179 were made against standard Ag/AgCl reference electrode. Sensors array was connected to a 32-
180 channel digital high input impedance voltmeter. All obtained data were measured with 0,1 mV
181 precision and recorded to a PC. At least 5 replicated ET (electronic tongue) measurements were
182 carried out for each sample and these results were averaged for further data processing. Water
183 samples were stirred, but not shaken up during the measurements to avoid contamination of the
184 sensors by precipitates present in some of the solutions. Samples were not treated anyhow or
185 diluted before the measurements and used as is. Measurement time in each 50 ml sample was 3
186 min. The sensor array was washed with three portions of distilled water between measurements,
187 the total time of washing being about seven minutes. The reported procedure was sufficient to
188 provide for ± 3 mV reproducibility of the sensors readings in the replicate measurements.

189

190 2.4. Data processing

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

194

195 2.4.1. Partial least squares

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

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

$$208 \quad 209 \quad 210 \quad 211 \quad RMSEP = \sqrt{\frac{\sum_i (y_i^{pred} - y_i^{real})^2}{n}} \quad (2),$$

212 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
213 the reference value.

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

219

220 2.4.2. Random forest

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

235

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

238

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).

274

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

278

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

283

284 3.2. Random forests and KNN

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

291 Another issue of the dataset was a small number of samples with relatively large $EC50$
292 values (> 50 for “real” and > 100 for “imitation” datasets), which could lead to poor regression
293 performance on the samples with such values. To overcome the difficulty we decided to use
294 \log_2 -transformed $EC50$ values for prediction. Thus, from this point on, the regression errors will
295 be reported in log-scale.

296

297 – Experimental evaluation: “real” dataset

298 As was mentioned above, we first classified the water samples into two classes:
299 $EC50 \leq 100$ (“ ≤ 100 ” class) and $EC50 > 100$ (“ > 100 ” class). Both RF and random KNN were
300 tried and the evaluation showed the performance of the former method being better. The number
301 of trees in the forest was equal to 100.

302 We were able to obtain confusion matrices for random forests using full cross-validation.
303 Since the construction of these classifiers is random such matrices are random as well. Hence,
304 1000 of such matrices were averaged; the obtained results are shown in Table 3. The number of
305 misclassified samples did not exceed 3 in the vast majority of cases - over 99% of matrices.

306

307

Table 3. Averaged confusion matrix for “real” dataset

308

309 Next, the regression for samples with $EC50 \leq 100$ (there were fifteen samples of such
310 kind) was calculated. This time random KNN outperformed random forests (we used $r = 100$,
311 $k = 1$ and four variables for each KNN model). Apart from RF and RKNN, we tried to make
312 predictions using ordinary linear regression, but it did not perform better than these two methods.
313 Then a distribution of 1000 full cross-validation errors of random KNN was considered. Some
314 statistics of this distribution are shown in Table 4. It can be noticed from the Table 4 that it is
315 possible to predict the dependent variable with relative errors of about $2^{1.5} \approx 2.83$.

316

317 Table 4. Regression error distribution statistics.

318

319 – Experimental evaluation: “imitation” dataset

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

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

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

336

337 4. Conclusion.

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

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

350

351 Acknowledgements

352 This work was partially financially supported by Government of Russian Federation, Grant 074-
353 U01and MINECO Spain project CTM2010-18167.

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