Toxicity assessment of chlorinated secondary effluents by the *Vibrio fischeri* bioluminescence assay

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Abstract

The objective of this manuscript was to assess the toxicity formation on biological treated wastewater from a municipal wastewater treatment plant, after a chlorination-dechlorination disinfection process. Water was monitored by means of physicochemical and ecotoxicological parameters, with the use of *Vibrio fischeri* bioassay. Since a dechlorination process removed residual chlorine, the positive correlation between toxicity values and chlorine concentrations suggested the formation of toxic disinfection by-products (DBPs). Statistically significant differences could be observed for toxicity values according to total carbon, total inorganic carbon, total nitrogen, chlorine, and pH. However, total organic carbon, chemical oxygen demand, electrical conductivity and turbidity had no statistical significance on toxicity formation. Three second-order polynomial equations were fitted between with total carbon and total nitrogen concentrations as independent variables, and EC50 as a dependent one.

Keywords: bioluminescence, disinfected wastewater, reclaimed water, chlorination.

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1 Introduction

Due to the shortage of water resources, great attention is being paid to the reclamation and reuse of wastewater from municipalities, mainly for irrigation [1]. For this and other purposes, disinfection treatment is considered to be the most efficient mechanism for the inactivation or destruction of pathogenic organisms in secondary effluents, preventing the spread of waterborne diseases to downstream users and to the environment [2]. Although chlorination has been the traditional and most common wastewater disinfection system used all over the world [3], it soon became clear that the reaction of chlorine with organic and inorganic substances in the water formed some toxic disinfection by-products (DBPs) [4]. The monitoring of water quality by means of physicochemical parameters is a limited procedure, because all these values may indicate the nature of the pollutants but do not yield any information about the biological effects. In this sense, an ecotoxicological screening can be used as a global evaluation of effects that cannot be done by specific chemical analysis [5], a fact included in the new European regulatory framework [6]. The aim of this paper was to assess the ecotoxicity formation after a chlorination-dechlorination disinfection process of biological treated wastewater from a municipal wastewater treatment plant (WWTP), where both domestic and industrial wastewater were being discharged. Treated effluent was monitored by means of physicochemical and ecotoxicological parameters, with the use of the biological response of the bioluminiscent marine bacterium *Vibrio fischeri*. From this evaluation and analysis, quadratic polynomial models were chosen for toxicity prediction.

2 Materials and methods

2.1 Sample collection and chemical treatments

The study was carried out with 30 secondary effluent samples collected at the WWTP of Cartagena, a Mediterranean city situated in the Southeast of Spain. These samples were collected after the biological treatment and clarification but prior to disinfection. The wastewater samples were immediately delivered to the laboratory and filtered using a 1.2 μm glass fibre filter (Millipore, Bedford, MA, USA) to eliminate suspended solids. Chlorine disinfection was carried out with 100 mL of filtered water at four different concentrations of available chlorine, i.e. 5, 10, 25, and 50 mg/L, from a sodium hypochlorite solution 5% w/v (Panreac, Barcelona Spain). The experiments were performed into sealed glass bottles incubated in an isotherm dark chamber (20ºC) during 30 minutes with slow agitation (20 rpm). The dechlorination experiments were performed with sodium sulfite 0.5% w/v (Panreac, Barcelona, Spain) prepared with ultra-pure water from an Elix 3 Milli-Q system (Millipore, Bedford, MA, USA). Based on the residual chlorine concentration of each experiment, a one-to-one ratio of Na_2SO_3 was used. All chemicals were analytical grade.

2.2 Chemical analyses

Total organic (TOC) and inorganic carbon (TIC) and total nitrogen (TN) were analysed in a TOC analyzer (Shimadzu TOC-V CSH). Ammonia nitrogen (NH4-N) was measured by liquid chromatography (Metrohm 861). Electrical conductivity (EC) was determined with a Crison GLP 32 conductimeter (Barcelona, Spain) and turbidity (NTU) with the Hach 2100N laboratory turbidimeter (Hach Company, Loveland, USA). Measures of pH were conducted in a Crison GLP 22 pH meter (Barcelona, Spain). Chemical oxygen demand (COD) was determined using the Spectroquant NOVA 30 from Merck (Darmstadt, Germany).

2.3 Toxicity test with luminescent bacteria *Vibrio fischeri*

Acute toxicity was assessed by determining the luminescence inhibition of the marine Gram-negative bacterium *Vibrio fischeri* (strain NRRL B-11177), which is purchased in freeze-dried form from Strategic Diagnostics Inc. (SDI, Newark, DE, USA) and reconstitutes by rehydration. Reconstituted bacteria were exposed to chlorinated-dechlorinated wastewater samples for 5, 10, and 15 min. The light emission was measured using the Microtox® 500 analyzer. Light production was directly proportional to the metabolic activity of the bacterial population, and basic test protocol was followed for the toxicity determination, using the Microtox Omni Software. The concentration of the sample (mg/L) which produces a 50% decrease in light after exposure for *"T"* minutes is designated as the Effective Concentration (EC50). Results are presented in EC50-5min, EC50-10min, and EC50-15min, respectively.

2.4 Statistical analyses

Statistical analyses were carried out using the software Statistical Package for Social Sciences (SPSS 15.0). The Kolmogorov-Smirnov test was used for testing the goodness-of-fit to the normal distribution of studied variables. Pearson's correlation coefficient (r) and analysis of variance (ANOVA) were computed between toxicity and physicochemical variables. The toxicity formation as a function of different parameters was analysed by fitting data to different models, and the fitting performance of each model was measured by means of F-values, correlation coefficient (R), multiple determination coefficient (R^2) and adjusted R^2 ($adj\text{-}R^2$). Residual values were evaluated for independence by means of the Durbin-Watson test, and predicted *vs*. measured values allowed us to test the accuracy of each model.

3 Results and discussion

3.1 General considerations

Table 1 depicts the main statistic parameters for secondary effluents before the chlorination-dechlorination treatments. Because all the variables approached to

the normal distribution, as indicated by the Kolmogorov-Smirnov tests (p values > 0.05), they were analysed with their raw forms. The same fact was found for all parameters after the treatments. For that reason, the distribution and further ANOVA analyses were carried out with the mean value, the number about which the sum of the squares of the residual deviations is a minimum.

Table 1: Statistic description of quality physicochemical parameters of secondary effluent before the chlorination-dechlorination treatment.

Parameters	Minimum	Maximum	Mean	S.D.
TC (mg/L)	72.74	103.20	87.38	10.94
TOC (mg/L)	8.61	11.89	9.74	1.23
TIC (mg/L)	60.85	92.20	77.64	11.18
TN (mg/L)	36.99	51.56	42.72	4.72
NH4-N (mg/L)	5.62	41.24	15.96	13.15
EC (μS/cm)	2,480.00	3,280.00	2,838.57	327.89
pH	7.57	7.86	7.71	0.09
NTU	1.92	3.92	2.83	0.72
COD (mgO_2/L)	23.00	84.00	48.17	22.83

Most part of carbon was in the inorganic form (TIC), experimenting an important and statistically significant decrease after the chlorination-dechlorination processes (F=8.01, p<0.001). The average value for raw samples was 77.64 mg/L, meanwhile for chlorination treatments with 5, 10, 25, and 50 ppm of chlorine was 39.02, 42.46, 42.85, and 45.81 mg/L, respectively, all of them below the minimum value of not-processed wastewater samples: 60.85 mg/L.

This fact can be explained by a mineralization, i.e., an important degradation of the carbon compounds to form water and carbon dioxide that will be emitted to the atmosphere, as previously reported both for water ozonation [7] and in chlorinated swimming pool water [8].

The highest TN value was displayed by a raw secondary effluent sample (51.56 mg/L) meanwhile the lowest one was for a 50 mg/L chlorinated sample (28.59 mg/L). The analysis of Pearson's correlation coefficient for this chemical parameter according to chlorine levels was -0.581 (p<0.01), indicating a possible reaction between hypochlorous acid originated during aqueous chlorination and ammonia, to finally generate gaseous nitrogen [9]. The generation of this hypochlorous acid would also be an alternative explanation of the relative but significant decrease in pH values when samples were chlorinated (F=10.70, p<0.001). However, no significant changes in pH were observed in our previous studies when disinfection was carried out with ultraviolet light [2].

ANOVA analysis showed a significant increase of turbidity values when comparing raw water with chlorinated samples (F=7.92, p<0.01). These results are in agreement with other studies that have found a higher and significant turbidity value after the chlorination process [2, 10]. The conversion of

particulate carbon forms into dissolved organic carbon could be an explanation for this fact [11] that could only be avoided by a combined chlorination-filtration treatment [12].

3.2 Effect of different parameters on toxicity formation

There appears to be an important effect on toxicity values that can be related to chlorine concentrations in the wastewater, since a statistically significant positive correlation could be observed for this parameter. It is clear that the toxicity of the wastewater increased with the addition of chlorine disinfectant. Since the dechlorination process removed residual chlorine from disinfected wastewater prior to toxicity analyses, the positive correlation suggested the formation of DBPs. This fact has been pointed by several authors, and it is a common concern in the legislation of industrialized countries, especially for drinking water [13]. In order to observe the toxic impact of each chlorine dosage in an easier way, the toxicity impact indices (TII) were calculated as TII_{50}=100/EC50 for each incubation time. These indices are illustrated in Fig. 1 and, as reported by Farré *et al.* [14], they are related to an amount of a mixture of unknown composition. It is clear that a higher $TIII_{50}$ is achieved for a more toxic sample.

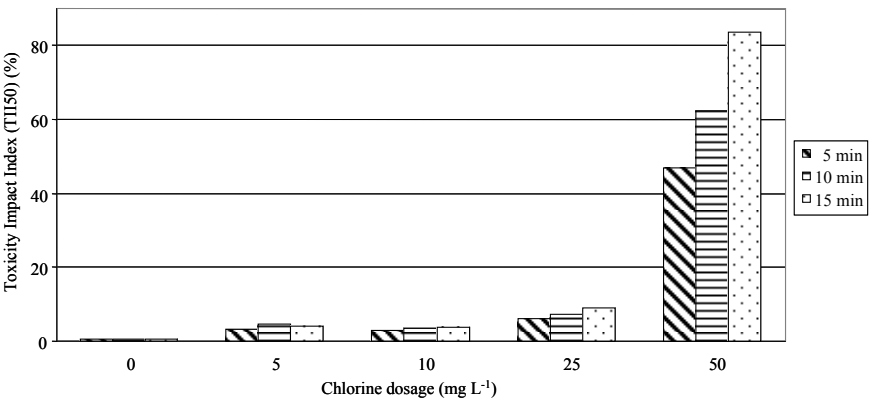

Figure 1: TII_{50} assigned to different chlorine dosages and exposure times.

Analysis of variance for TC according to chlorine dosage displayed statistically significant differences, as presented in Figure 2, corresponding the maximum value to a raw sample (103.20 mg/L) and the minimum one to a wastewater sample chlorinated with 25 ppm (21.06 mg/L) n ($F=8.12$, $p<0.001$). The differences of the decreasing trend of TC for the first chlorine dosage (5 mg/L) and the increasing tendency for all other dosages may lie in the complex reactions taken into account. Initially, chlorine would act as an oxidant, transforming organic and inorganic carbon into carbon dioxide. A further increase of chlorine levels in treated wastewater would promote halogenation reactions of intermediate products and the formation of DBPs, leading to an increase in TC values.

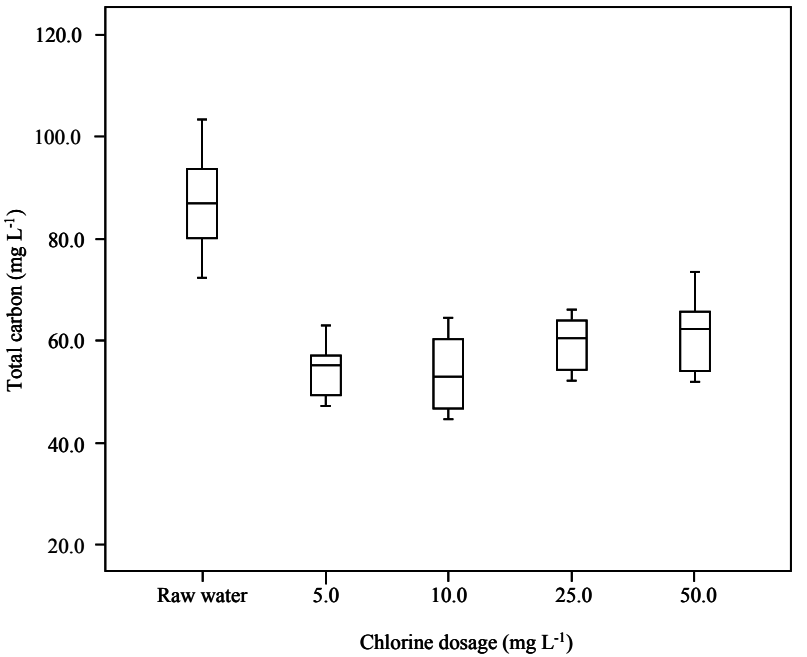

Figure 2: Box-plot of TC *versus* chlorine dosage (F=8.12, p<0.001).

On the other hand, a strong and positive correlation was obtained for TC and TIC for 5, 10, and 15 min EC50, as presented in Table 2. However, a weak and negative correlation was obtained for TOC values. In agreement with other reports, organic carbon can be a broad indicator of the amount of organic matter in the wastewater, but it does not contain specific information about the potential of this organic matter to serve as DBP precursor [15–17].

As seen in Figure 3, TN mean concentrations in wastewater samples were directly affected by chlorine dosage, with the highest average value for raw wastewater samples (42.72 ± 4.72 mg/L) and the lowest one for 50 ppm chlorinated samples (31.96 ± 3.14 mg/L).

Together with this fact, Table 2 shows a statistically significant decrease on toxicity levels with the increase in TN values. The consumption of chlorine by ammonia nitrogen forms deals to the formation of chloramines, which produce less DBPs, because the lower reactivity of combined chlorine than free chlorine. Sorlini and Collivignarelli [18] reported similar results in artificial lakes and rivers. Wang *et al.* [19] have also reported a decrease in the toxicity of disinfected wastewater when ammonia nitrogen increased from 2 to 30 mg/L.

The effect of chlorine to ammonia ratios *versus* toxicity formation was also studied. The one-way ANOVA test displayed a decreasing tendency for EC50 at any considered experiment time with the increase of Cl_2/NH_4-N ratios. This fact would suggest a higher DBPs formation, as previously reported by Zhang *et al* [20].

With the increasing of Cl_2/NH_4-N ratios, chlorine or chloramines previously formed would hydrolyze to form hypochlorous acid (HOCl), which would clearly increase DBP formation. Inversely, Diehl *et al.* [21] reported a decrease in DBP formation during a chloramination experiment when the chlorine-to-ammonia nitrogen ratio increased.

Table 2: Pearson's correlation coefficient of chemical and toxicity parameters in secondary effluent wastewater samples (*p<0.01).

Parameters	EC50-5 min	EC50-10 min	EC50-15 min
TIC	0.771*	0.750*	0.735*
TC	0.763*	0.743*	0.727*
TN	0.629*	0.679*	0.670*
Chlorine	-0.546*	-0.486*	-0.514*
pH	0.725*	0.713*	0.694*
TOC	-0.273	-0.107	-0.163
COD	0.151	0.248	0.084
EC	-0.118	-0.093	-0.121
NTU	-0.253	-0.274	-0.311

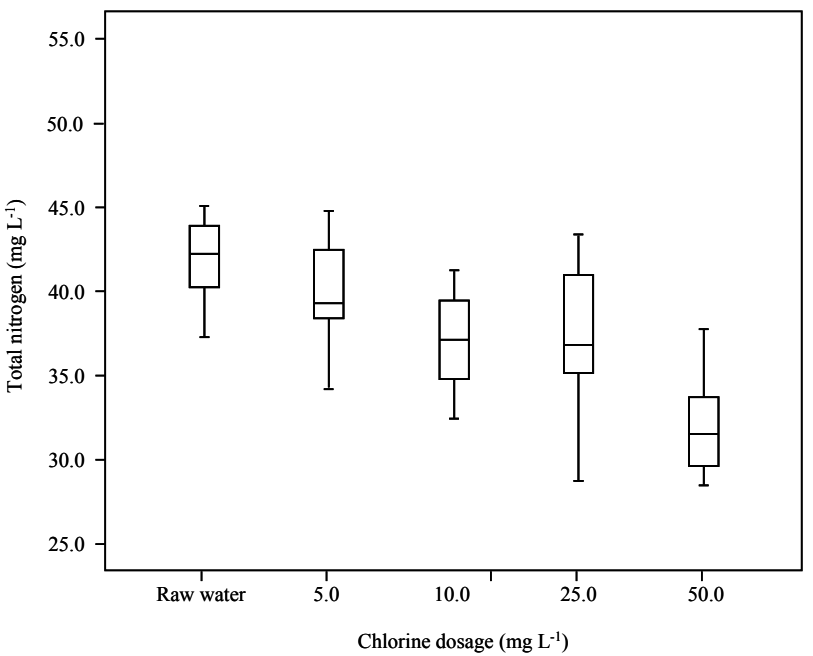

Figure 3: Box-plot of TN *versus* chlorine dosage (F=6.15, p<0.01).

Also the toxicity formation in chlorinated-dechlorinated wastewater samples was affected by pH, indicating an increase in toxic results with the decreasing tendency of pH (Table 2). This physicochemical parameter affects the distribution of hypochlorous acid (HOCl) and hypochlorite (OCl^-) species. At low pH values, HOCl is the predominant specie, which is responsible for the formation of DBPs because a higher oxidative capacity than OCl^- anion. On the other hand, an increase in pH also enhances the degradation of these DBPs [20, 22]. Diehl *et al.* [21] reported similar results, showing a decrease on different DBP formation with an increase in the pH from 6 to 10, and Yang *et al.* [22] found a marked difference in DBP formation in Suwannee river water, as judged by the range in pH from 4 to 9; the maximum yields of DBPs occurred at pH 5-6.

For all these reasons, a pH control would contribute to a significant reduction of DBPs formation [23].

3.3 Multiple regression models

The selected variables in our study for the regression analysis were those presenting statistically significant differences in the one-way ANOVA test according to toxicity formation, i.e., TIC, TC, TN, chlorine, and pH (Table 2). The toxicity formation as a function of these parameters was studied by fitting data to different models, and the fitting performance of each model was measured by means of residual values and multiple determination coefficient (R^2). Besides, the adjusted R^2 ($adj\text{-}R^2$) was a useful index for comparing the explanatory power of models with different number of predictors [24].

By applying multiple regression analysis on the experimental data, three second-order polynomial equations were fitted between EC50-5min, EC50-10min, and EC50-15min, as dependent variables, and TC and TN values as independent variables, according to the following equation:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{12}X_1X_2$$

The coefficients of the regression model were calculated by the least-square method and are shown in Table 3. The equation should contain a constant term (β_0), two linear coefficients (β_1 and β_2), two quadratic terms (β_{11} and β_{22}), and one interaction (β_{12}). However, the interaction effect of TC and TN (β_{12}), as well as the block term (β_0), were found to be not-significant, because a level of significance over 0.05 of their *t*-values. For that reason, these terms were excluded from three equations. On the contrary, terms for TC, TN, TC*TC, and TN*TN proved to be significant because a significance of *t*-values below 0.05. Hence, the fitted equations for each effective concentration were formulated as follows:

$$EC50\text{-}5min = -18.601*TC+30.415*TN+0.150*TC^2-0.381*TN^2 \quad (1)$$

$$EC50\text{-}10min = -25.000*TC+40.285*TN+0.204*TC^2-0.512*TN^2 \quad (2)$$

$$EC50\text{-}15min = -26.504*TC+43.442*TN+0.214*TC^2-0.558*TN^2 \quad (3)$$

All the equations displayed an important and negative influence of TC values, and an important and positive influence of TN values. The opposite significant trend was found for the quadratic effects of both parameters, i.e. TC^2 and TN^2,

Table 3: Second-order polynomial equations and statistic evaluation of EC50-5min, EC50-10min, and EC50-15 min models.

EC50-5 min: [$F = 47.292 > F_{0.05\ (4,26)} = 2.743$; R = 0.963; $R^2 = 0.927$; $R^2_{adj} = 0.907$; Durbin-Watson statistic = 1.901]					
Variable	Coefficient	Coefficient value	S.E.	*t*-value	*p*-value
Constant	β_0	4.823	-	1.429	0.636
TC	β_1	-18.601	4.123	-4.512	0.000
TN	β_2	30.415	7.783	3.908	0.001
TC*TC	β_{11}	0.150	0.029	5.147	0.000
TN*TN	β_{22}	-0.381	0.108	-3.536	0.003
TC*TN	β_{12}	0.184	-	1.231	0.178
EC50-10 min: [$F = 50.328 > F_{0.05\ (4,26)} = 2.743$; R = 0.967; $R^2 = 0.935$; $R^2_{adj} = 0.916$; Durbin-Watson statistic = 1.887]					
Variable	Coefficient	Coefficient value	S.E.	*t*-value	*p*-value
Constant	β_0	9.219	-	2.458	0.651
TC	β_1	-25.000	4.680	-5.342	0.000
TN	β_2	40.285	8.877	4.538	0.000
TC*TC	β_{11}	0.204	0.034	5.957	0.000
TN*TN	β_{22}	-0.512	0.126	-4.059	0.001
TC*TN	β_{12}	0.182	-	1.493	0.428
EC50-15 min: [$F = 49.222 > F_{0.05\ (4,26)} = 2.743$; R = 0.964; $R^2 = 0.929$; $R^2_{adj} = 0.910$; Durbin-Watson statistic = 2.239]					
Variable	Coefficient	Coefficient value	S.E.	*t*-value	*p*-value
Constant	β_0	9.399	-	2.336	0.675
TC	β_1	-26.504	4.643	-5.709	0.000
TN	β_2	43.442	8.812	4.930	0.000
TC*TC	β_{11}	0.214	0.034	6.301	0.000
TN*TN	β_{22}	-0.558	0.125	-4.459	0.000
TC*TN	β_{12}	0.164	-	1.256	0.471

S.E.: *Standard Error*

although with a weak relationship. However, the inclusion of these quadratic terms in the equations was necessary, as the models without them resulted in a lower adj-R^2, i.e. 0.579, 0.561, and 0.534, for EC50-5min, EC50-10min, and EC50-15min, respectively.

The multiple determination coefficient (R^2) for each model allowed us to measure the proportion of the variance of the response variable, i.e. EC50-5min, EC50-10min, and EC50-15min, explained by the independent variables. The closer R^2 approaches 1.0, the better the model fits the data. The value of this coefficient for equations (1), (2), and (3) was 0.927, 0.935, and 0.929, respectively, which means that only 7.3, 6.5, and 7.1% of the variance could not be explained by each equation. These multiple determination coefficients were higher than those reported in previous studies [25]. Golfinopoulos and

Arhonditsis [26] have also reported the use of second-order equations, including first and second-degree terms in the independient variables and without a constant term, for the prediction of THM concentrations in dinking water.

The Durbin-Watson statistic test was used to detect the absence of autocorrelation in the residuals from the regression models. When Durbin-Watson value converges to zero, there is a strong correlation between the regression residuals. On the contrary, a Durbin-Watson value converging to 2.0 indicates a weak correlation or random distribution between successive points [27]. As shown in Table 3, Durbin-Watson statistic for these three models were found to be 1.901, 1.887, and 2.239, respectively, demonstrating that the residuals were independent. Uyak *et al*. [28] have reported similar results in the development of multiple regression models for predicting the concentrations of different DBPs in drinking water.

Finally, the comparison of measured and predicted ecotoxicity values reported very high Pearson's correlation coefficients, i.e. 0.938, 0.956, and 0.961 for EC50-5min, EC50-10min, and EC50-15min, respectively, always with a significance level of 99.9% (p-value<0.001).

4 Conclusions

The Microtox® test has been used for the assessment of acute toxicity of treated wastewater samples after a chlorination-dechlorination process. Toxicity values were demonstrated to be strongly associated with chlorine levels, and a significant but negative relationship was established with TIC, TC, TN, and pH. However, there was a weak association between TOC, COD, EC, and NTU with toxicity of the disinfected wastewater samples.

When significant factors were included in a regression analysis, only TC and TN remained in two-order polynomial equations for toxicity prediction (EC50), after an exposure of 5, 10, and 15 minutes. Models proved to explain a high variance proportion of the response variable, because multiple determination coefficients of 0.927, 0.935, and 0.929, respectively. Further surveys could allow us to develop fuzzy rule-base models, in order to improve the predictive capacity.

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