

Multiple linear and principal component regressions for modelling ecotoxicity bioassay response

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The ecotoxicological response of the living organisms in an aquatic system depends on the physical, chemical and bacteriological variables, as well as the interactions between them. An important challenge to scientists is to understand the interaction and behaviour of factors involved in a multidimensional process such as the ecotoxicological response. With this aim, multiple linear regression (MLR) and principal component regression were applied to the ecotoxicity bioassay response of *Chlorella vulgaris* and *Vibrio fischeri* in water collected at seven sites of Leça river during five monitoring campaigns (February, May, June, August and September of 2006). The river water characterization included the analysis of 22 physicochemical and 3 microbiological parameters. The model that best fitted the data was MLR, which shows: (i) a negative correlation with dissolved organic carbon, zinc and manganese, and a positive one with turbidity and arsenic, regarding *C. vulgaris* toxic response; (ii) a negative correlation with conductivity and turbidity and a positive one with phosphorus, hardness, iron, mercury, arsenic and faecal coliforms, concerning *V. fischeri* toxic response. This integrated assessment may allow the evaluation of the effect of future pollution abatement measures over the water quality of Leça River.

Keywords: *Chlorella vulgaris*; ecotoxicological assessment; multiple linear regression; principal component regression; surface water quality; *Vibrio fischeri*

1. Introduction

Pollution of surface water with toxic chemicals and excess of nutrients, resulting from storm water runoff, mains leakage leaching, and groundwater discharges, has been an issue of worldwide environmental concern.[1] The water quality assessment must comprise an ecotoxicological characterization, which allows properly evaluating the potential risks of effluent discharges, especially when they are complex.[2] The ecotoxicity evaluation by means of acute bioassays may bring quick and valuable information.[3,4] However, most of the ecotoxicity test methods were established to measure the toxicity of pure single chemicals and not to be applied to unknown environmental water samples with complex components. Since chemicals are present in environmental water as a complex mixture, their potential ecotoxicological effects are much complicated due to their interactions.[5–9] In addition, even if the toxicity of an environmental sample is tested, there is no guidance on how to evaluate the water quality in terms of protection of aquatic living organisms.[6] It is difficult to extrapolate the potential damage on the aquatic ecosystem from the test results with specific species, particularly because not all species respond identically to the same pollution stresses.[10] It is also quite difficult to evaluate the actual exposure levels

and ecotoxicological effects of all coexisting chemicals on aquatic organisms by measuring the concentrations of individual chemicals (United States Environmental Protection Agency – USEPA).[11,12] It must also be kept in mind that there is an uncertainty factor when laboratory results are extrapolated to field conditions because of the simultaneous influence of a number of environmental and biological factors (bioavailability, toxicokinetics, sensitivity of organisms, etc.).[4] However, direct toxicity test of environmental water sample can provide an integral view on ecotoxicological effects of all chemicals coexisting in water as a mixture and has been widely used in safety assessment of water quality.[6,13,14]

The study of ecological properties of different organization levels may reveal changes of potential ecological signification that cannot be detected by other analyses.[1] The bacterium *Vibrio fischeri* (decomposer) and the alga *Chlorella vulgaris* (first producer) were selected for this study because they belong to different trophic levels and are widely used in ecotoxicity tests.[1,2] One of the advantages of these tests is the fast assessment of ecotoxicity.

The ecotoxicological response of the living organisms in an aquatic system depends on several variables, such as nutrient quantitative and qualitative profiles, temperature,

physicochemical properties of the water and grazing pressure.[15] An important challenge for scientists is to develop analytical tools that could be used to understand the interaction and behaviour of factors involved in a multidimensional process [16] such as the ecotoxicological response, and to provide the necessary tools for monitoring and management of resources. Modelling is regarded as an important analytical tool for biological and ecological studies.[17,18]

Multivariate statistical techniques are useful for the evaluation and interpretation of large and complex water quality data sets.[19] Multiple linear regression (MLR) is one of the most widely used methodologies for expressing the dependence of a response variable on several explanatory (predictor) variables.[16,20–22] Principal component analysis (PCA) is useful in pre-processing methodology for mitigating the problem of multicollinearity (when the explanatory variables are correlated with each other) and for exploring the relations among the input variables, particularly if it is not obvious which of the variables should be the predictors. PCA creates new variables, the principal components (PCs), by linear combination of the original variables. PCs are uncorrelated to each other, removing the multicollinearity problem. They are interpreted by the association with original variables through the corresponding factor loadings. Principal component regression (PCR) is the linear model that relates the dependent variable with these PCs. Both MLR [20,23] and PCR [16] approaches have been applied in studies of water quality.

The utility of *C. vulgaris* as test organism is based on its short life cycle, making it easy to study the exposure of several generations.[24] It is easily cultured in laboratory and is widespread in nature, being found in freshwater, saltwater and soil.[25]

Vibrio fischeri is a Gram-negative bioluminescent marine bacterium that forms mutually symbiotic

relationships with various species of fish and squids or can be found living freely in the oceans.[26] The main advantages of using this species in ecotoxicity is the short time required to obtain results, the simplicity of the test and high reproducibility of results.[27]

The present study aims to model *C. vulgaris* and *V. fischeri* bioassays toxic response in concern to the Leça river water characterization by MLR and PCR. The achieved models lead to infer possible influences of physicochemical and microbiological variables of river water in bioassay results.

2. Materials and methods

2.1. Area description – sampling sites

The Leça river flows through a highly populated and industrialized area in the north of Portugal and receives a complex mixture of pollutants from poorly treated or untreated domestic, agricultural and industrial effluents, and other contaminated waters both from point and diff use sources.

Figure 1 presents the location of Leça river in the north of Portugal. It rises in the Mountain of Santa Luzia at Santo Tirso and flows for approximately 48 km until the Atlantic Ocean. Water samples were collected at seven sampling sites along the river: site 1 is located in the upstream part of the river in a main rural area; both sites 2 and 4 are located downstream from wastewater treatment plants in a highly populated area; sites 3 and 5 are situated in a strongly populated and industrialized area; site 6 is in a revitalized area with a recreational park; and site 7 is some metres upstream from the river mouth, before a waterfall, and therefore it does not receive any marine influence. Water samples were collected in five different periods – February, May, June, August and September of 2006, one day in each month (not always the same). Most of the samples were collected from bridges, in order to obtain samples from running water,

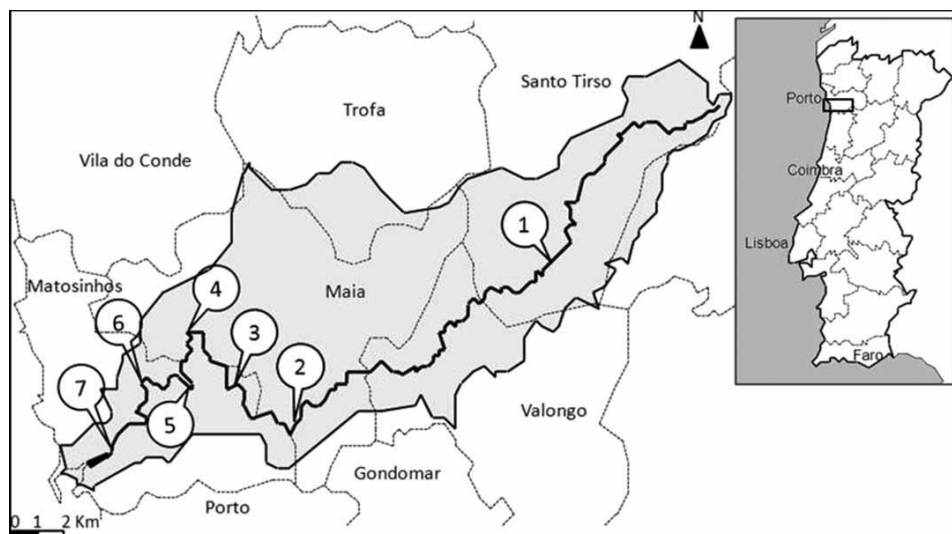


Figure 1. Leça river basin showing the geographical location of the sampling sites.

Table 1. Analytical procedures.

Parameter	Method	Equipment
<i>Physicochemical parameters</i>		
Temperature	Thermometry	Multiparameter analyser HANNA Instruments model 991003
pH	Electrometry	
ORP	Electrometry	
Dissolved oxygen (DO)	Membrane electrode	DO meter HANNA Instruments model 9143
Conductivity	Conductimetry	Conductivity meter WTW model LF 330
Turbidity	Nephelometry Method 2130 B [28]	Turbiquant 3000 IR, Merck
Colour	Spectrophotometry (platinum-cobalt) Method 110.2 [29]	UV/Vis Spectrometer PYE Unicam PU 8600
Dissolved organic carbon (DOC)	High-temperature combustion Method 5310 B [28]	Shimadzu analyser 5000 A -
Biochemical oxygen demand (BOD)	5-Day BOD Test Method 5210 B [28]	DO meter Crison OXI 45
Total nitrogen	Persulfate digestion method 4500N C [28]	UV/Vis Spectrometer PYE Unicam PU 8600
Total phosphorus	Persulfate digestion+ascorbic acid method 4500P E [28]	UV/Vis Spectrometer PYE Unicam PU 8600
Hardness	EDTA titrimetry method 2340 C [28]	
Dissolved Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn	Atomic absorption spectrometry – flame methods 3111 B and D [28]	AAS GBC 932 plus
Dissolved As and Hg	Hydride generation/cold-vapour atomic absorption spectrometry Methods 3112 B and 3114 C [28]	AAS GBC 932 plus and GBC HG 3000
<i>Bacteriological parameters</i>		
Total coliforms	Membrane filtration ISO Standard [30]	
Faecal coliforms	Membrane filtration ISO Standard [30]	
Faecal streptococcus	Membrane filtration ISO Standard [31]	
<i>Ecotoxicological parameters</i>		
Microtox [®] inhibition	Bioluminescent inhibition test of bacteria <i>Vibrio fischeri</i> (15 min) ISO Standard [32]	Microtox Analyser 2055, Microbics Corporation (at present time, AZUR) Environmental
Green algae inhibition	Inhibition growth test of microalgae <i>Chlorella vulgaris</i> USEPA Guideline [14]	Shimadzu UV/Vis spectrometer

which were representative of the river water. Grab samples were manually collected by immersing the plastic bottles into the river.

2.2. Analysis of the water samples

The analytical procedures used to characterize the water samples are presented in Table 1. All used reagents were of analytical grade.

Temperature, pH and oxidation—reduction potential, dissolved oxygen and conductivity were measured *in situ*. Water samples were stored at 4°C (no chemical preservatives were added) and analysed in duplicate within 24 h. For dissolved organic carbon (DOC) and metals, a filtration by 0.45- µm pore diameter membrane filter was performed. Bioassays were performed within (the maximum) 48 h after sampling.

The bioluminescent inhibition toxicity tests (ISO 11348) were performed using the bacteria *V. fischeri* (NRRL B 11177). Tested concentrations were 5.6%, 11.3%, 22.5% and 45% (v/v). The values of EC₅₀ (effective concentration of the sample that causes 50 inhibition to the

test organisms) and the corresponding 95% confidence intervals were determined for 5 and 15 min of bacterial exposure.

The green algae inhibition growth tests were performed with the microalgae *C. vulgaris* according to USEPA Guideline [14]. Three replicates of each sample were tested for five different concentrations (10%, 20%, 40%, 60% and 80%). The test solutions were incubated for 72 h, under continuous cool white fluorescent light. Agitation was performed manually twice per day. Initial and final absorbance was measured at 440 nm [33] to evaluate the growth of the algal population. A calibration curve was used to convert the absorbance in cell concentration (cells/mL) using the experimental linear relation obtained: (cell density) = $6.42 \times 10^4 + 8.00 \times 10^7 \times (\text{absorbance at 440 nm})$, with a square correlation factor of 0.995.

The acceptability criterion considered was variability < 20% among replicates. Shapiro-Wilk's normality test and Bartlett's test for homogeneity of variance were performed to validate data, and Dunnett's procedure was followed [14]. Since these assumptions were met, EC₅₀ was calculated by linear interpolation.

The reference toxicants used to validate tests were phenol and potassium dichromate, respectively, for *V. fischeri* and *C. vulgaris* bioassays.

The toxic response was evaluated through the calculation of EC₅₀, effective concentration that causes 50% of inhibition to test organism. For regression models purpose, EC₅₀ was converted in toxicity units, TU₅₀ (TU₅₀ = 100/EC₅₀), as suggested by Wisconsin Department of Natural Resources.[34] Because EC₅₀ was expressed in percentage, the sample is considered 'not toxic' when TU₅₀ = 1 and biostimulated when TU₅₀ < 1.

2.3. Regression models

The data considered for this analysis were the mean of replicates. Before the determination of the models, the data were Z standardized to have zero mean and unit standard deviation. MLR attempts to model the relationship between two or more explanatory variables and a response variable, by fitting a linear equation to the observed data.[35,36] The dependent variable (y) is given by

$$y = \hat{\beta}_0 + \sum_{i=1}^k \hat{\beta}_i x_i + \varepsilon \quad (1)$$

where x_i ($i = 1, \dots, k$) are the explanatory variables, $\hat{\beta}_i$ ($i = 0, \dots, k$) are the regression coefficients, and ε is the error associated with the regression and assumed to be normally distributed with both expectation value zero and constant variance.[37]

The predicted value given by the regression model (\hat{y}) is calculated by

$$\hat{y} = \hat{\beta}_0 + \sum_{i=1}^k \hat{\beta}_i x_i \quad (2)$$

To estimate the regression coefficients $\hat{\beta}_i$ the minimization of the sum of squared errors (SSE) method is used, as follows:

$$\hat{\beta}_i = \arg \min \sum_{i=0}^k (y_i - \hat{y}_i)^2 \quad (3)$$

PCR is a method that combines linear regression and PCA.[36] Essentially, PCA maximizes the correlation between the original variables to form new variables, the PCs that are orthogonal and uncorrelated. These variables are linear combinations of the original variables. The PCs are ordered in such a way that the first component has the largest fraction of the original data variability.[16,38] To evaluate the influence of each variable in the PCs, varimax rotation is generally used to obtain the rotated factor loadings that represent the contribution of each variable in a specific PC. PCR establishes a relationship between the output variable (y) and the selected PC obtained from the explanatory variables (x_i).[36]

The significance of the regression coefficients in the MLR and PCR models was evaluated through the calculation of their confidence intervals.[36,39] The regression coefficient $\hat{\beta}_i$ is statistically significant if:

$$\hat{\beta}_i = \arg \min \sum_{i=0}^k (y_i - \hat{y}_i)^2 \quad (3)$$

where t is the Student t distribution, n is the number of points, k is the number of parameters, α is the significance level, $\hat{\sigma}$ is the standard deviation given by and $SSE/(n-k-1)$ S_{xx_i} is the sum of the squares related to x_i given by $\sum_{j=1}^n (x_{ij} - \bar{x}_i)^2$.

Hence, several MLR and PCR models were determined by testing all combinations of the explanatory variables, selecting the ones that presented the lowest SSE and all statistically significant regression coefficients.[36]

The PCs were calculated using Matlab, whereas MLR and PCR models were evaluated by developed subroutines in Microsoft Visual Basic for Applications (Microsoft Excel).

2.4. Performance indexes

The performances of MLR and PCR models in the prediction of *C. vulgaris* and *V. fischeri* toxic response were evaluated through calculation of the coefficient of determination (R^2), mean absolute error (MAE), root mean squared error (RMSE) and index of agreement (d_2).[40,41] The MAE and the RMSE measures residual errors, which gives a global idea of the difference between the observed and modelled values. The values d_2 indicate the degree to which the predictions are error free, because it compares the difference between the mean, the predicted and the observed concentrations.

3. Results

The physicochemical, bacteriological and ecotoxicological results, presented in a previous study,[42] are shown in Table 2 for physicochemical data and in Figures 2 and 3 for ecotoxicological data. For bacteriological parameters, the maximum concentrations of total coliforms, faecal coliforms and faecal streptococcus were, respectively, 5.5×10^7 , 5.0×10^6 and 3.0×10^5 CFU/100 mL, all obtained at site 2. From the dissolved metals evaluated (As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn) only zinc (0.08–0.22 mg/L), manganese (< 0.13 mg/L), mercury (0.5–6.5 µg/L), arsenic (0.5–3.0 µg/L) and iron (0.20–0.40 mg/L) were detected.

The models were determined to model *C. vulgaris* and *V. fischeri* toxic response using physicochemical and bacteriological variables as predictors. Regarding *V. fischeri* results, only the 15 min-toxic responses were used in the regression models. From the 25 monitored variables, only 15 were applied for models development. Variables

Table 2. Physicochemical characterization of Leça river water samples.

Sampling sites	Physicochemical parameters												
	Month	Temp. (° C)	pH	ORP (mV)	Cond. (µS/cm)	DO (mg/L)	DOC (mg/L)	BOD (mg O ₂ /L)	Turb. (NTU)	Colour (Pt-Co)	Total N (mg N/L)	Total P (mg P/L)	Hardness (mg CaCO ₃ /L)
1	February	9.8	7.14	235	121	10.5	3.6	–	28	10	27.4	0.8	37.1
	May	12.1	6.75	263	73	8.5	1.3	2.6	0.60	0	2.3	< 0.1	28.7
	June	17.0	6.11	176	89	8.3	2.2	0.6	0.06	14	7.9	0.1	40.7
	August	21.0	6.61	79	179	6.1	3.8	1.5	3.5	1	5.4	0.2	44.3
	September	18.0	5.88	153	123	6.8	11.3	5.6	240	43	3.3	0.2	35.9
2	February	9.8	7.07	187	150	10.3	5.7	–	110	21	35.5	0.8	46.7
	May	14.4	6.04	244	226	8.2	3.0	5.1	3.5	2	11.7	0.8	67.0
	June	20.0	5.64	222	483	7.9	12.0	10.2	7.8	43	30.7	2.4	89.7
	August	22.2	6.00	71	1050	5.4	24.6	21.2	12	44	70.7	3.7	140.0
	September	18.7	5.85	133	160	5.0	11.1	9.6	130	32	4.7	1.8	75.2
3	February	10.6	6.94	161	179	9.5	5.4	–	60	13	28.3	0.9	51.4
	May	15.0	6.03	236	251	7.8	4.3	6.0	3.5	1	17.4	0.9	71.8
	June	20.2	5.96	197	496	7.5	13.6	15.0	9.3	46	38.2	2.8	101.7
	August	22.5	5.96	109	857	5.2	23.1	10.6	8.5	35	57.8	4.3	140.0
	September	18.6	6.55	80	174	5.3	11.0	12.0	170	33	3.5	2.3	83.6
4	February	10.9	7.01	187	180	9.8	4.7	–	65	16	30.9	0.6	89.7
	May	15.4	6.28	204	287	7.8	4.8	7.7	4.3	3	22.2	1.0	82.5
	June	20.5	6.12	206	577	8.1	16.6	15.7	17	62	33.7	3.0	99.3
	August	23.0	6.07	94	935	5.8	21.7	31.3	10	38	54.7	2.7	130.4
	September	18.6	5.91	105	178	5.2	10.5	15.6	260	28	12.8	2.8	90.5
5	February	10.4	6.65	183	176	9.8	4.4	–	65	15	26.4	0.8	69.4
	May	15.0	6.07	230	265	8.0	3.8	12.0	6.7	1	14.4	0.9	76.6
	June	20.4	6.05	203	556	7.9	15.3	12.6	12	57	30.7	2.6	100.5
	August	23.3	5.97	72	952	5.5	21.8	20.2	13	32	57.0	3.0	140.0
	September	18.7	6.07	98	194	4.5	10.9	13.2	180	30	5.4	1.8	82.5
6	February	10.7	6.50	158	192	11.7	4.8	–	100	16	25.0	0.7	62.2
	May	16.0	6.34	197	318	7.7	5.6	8.7	10	3	19.8	1.2	82.5
	June	21.8	6.07	241	560	7.9	15.5	18.9	11	61	30.4	2.7	102.9
	August	22.4	6.23	109	932	5.3	20.7	22.7	13	39	56.4	8.2	131.6
	September	18.3	6.41	149	305	6.3	12.7	3.8	200	29	11.1	2.5	89.7
7	February	10.9	6.98	145	187	10.3	5.0	–	120	18	27.7	0.7	58.6
	May	16.0	6.25	204	343	8.3	5.7	9.3	8.1	3	27.9	1.1	88.5
	June	22.9	6.05	253	578	7.6	14.8	19.2	12	58	34.0	2.8	117.2
	August	23.2	5.98	113	1769	5.2	19.1	24.7	8.2	38	26.0	12.2	226.0
	September	18.2	6.74	90	298	5.6	12.7	13.6	180	28	117.0	2.8	100.4

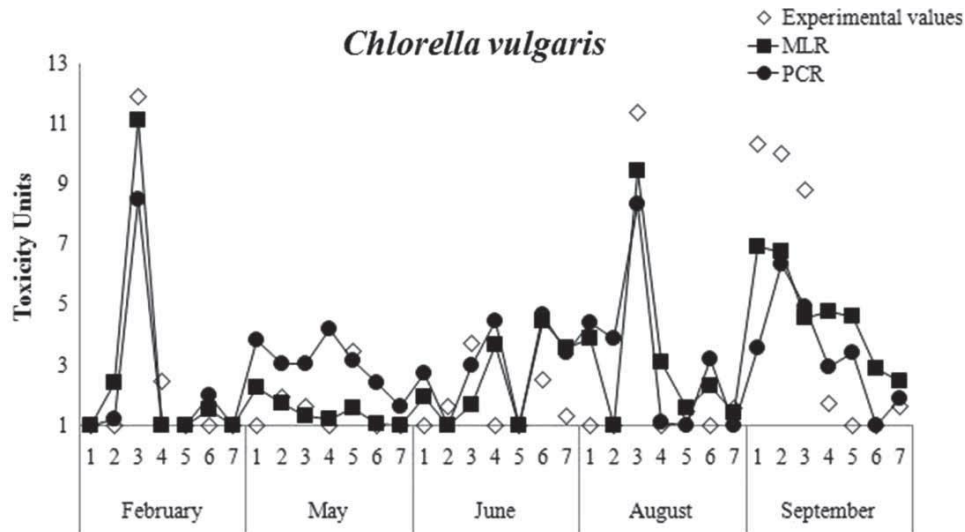


Figure 2. Comparison between experimental values and values given by MLR and PCR models for *Chlorella vulgaris* toxic response.

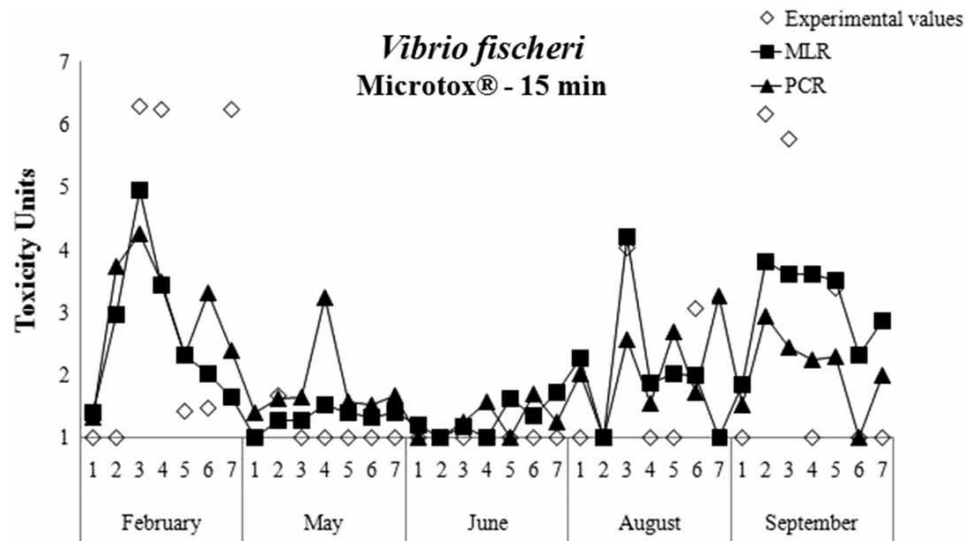


Figure 3. Comparison between experimental values and values given by MLR and PCR models for *Vibrio fischeri* toxic response.

that were measured *in situ* and that presented always values below the detection limit were not considered. Both MLR and PCR models were determined by statistically significant regression coefficients with a significance level of 0.05.

The MLR led to the following results: (i) *C. vulgaris* toxic response was negatively affected by DOC, Zn and Mn, and positively affected by turbidity and As; and (ii) *V. fischeri* toxic response was negatively affected by conductivity and turbidity, and positively affected by phosphorus, hardness, Fe, Hg, As and faecal coliforms. The regression models obtained by MLR were as follows:

$$\begin{aligned}
 C.vulgaris = & 2.719 - 2.193 (\text{DOC}) - 1.399 (\text{Zn}) \\
 & - 0.782 (\text{Mn}) + 1.651 (\text{turbidity}) \\
 & + 3.643 (\text{As})
 \end{aligned}
 \quad (5)$$

$$\begin{aligned}
 V.fischeri = & 1.849 - 5.845 (\text{conductivity}) \\
 & - 0.860 (\text{turbidity}) \\
 & + 0.971 (\text{phosphorus}) + 2.951 (\text{hardness}) \\
 & + 0.551 (\text{Fe}) + 1.624 (\text{Hg}) \\
 & + 0.595 (\text{As}) + 0.657 (\text{faecal coliforms})
 \end{aligned}
 \quad (6)$$

PCA was performed to obtain in the PCs all variance contained in the original data. Thus, 15 PCs were determined. Table 3 presents the results from PCA showing the rotated factor loadings for all 15 PCs. Values in bold correspond to the greatest contributions of the original variables on the PCs. PC1 had important contributions from conductivity, DOC, total nitrogen, total phosphorus, hardness and Hg. PC3 was heavily loaded by all bacteriological

Table 3. Rotated factor loadings for all principal components (PC) of the physical, chemical and bacteriological variables.

Variables	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12	PC13	PC14	PC15
Conductivity ($\mu\text{S}/\text{cm}$)	-0.896	0.034	0.223	-0.130	0.213	0.137	-0.088	-0.194	-0.035	-0.072	0.068	0.029	0.003	-0.007	0.085
DOC (mg/L)	-0.615	0.052	0.415	-0.217	-0.064	0.299	0.030	-0.501	-0.019	-0.107	0.048	0.030	0.199	-0.018	-0.001
Turbidity (NTU)	0.169	-0.067	-0.011	0.062	-0.963	-0.082	0.159	-0.064	0.009	-0.013	-0.003	-0.001	0.001	-0.001	-0.001
Colour (Pt-Co)	-0.352	0.148	0.139	-0.109	-0.091	0.218	0.332	-0.809	-0.002	0.040	-0.015	-0.005	-0.030	0.004	0.001
Total nitrogen (mgN/L)	-0.884	0.005	0.235	0.188	0.254	0.101	-0.090	-0.075	-0.028	-0.056	0.097	-0.024	-0.029	0.159	-0.007
Total phosphorus (mgP/L)	-0.938	0.007	0.028	-0.126	-0.043	0.101	-0.037	-0.143	-0.001	0.017	-0.257	-0.053	-0.025	-0.017	-0.003
Hardness (mgCaCO ₃ /L)	-0.940	0.013	0.178	-0.177	0.058	0.055	0.040	-0.110	0.034	0.075	0.094	0.059	0.032	-0.094	-0.061
Zn (mg/L)	0.131	-0.037	-0.056	0.980	-0.059	0.063	0.049	0.084	0.003	-0.009	0.003	-0.003	-0.003	0.004	0.000
Fe (mg/L)	0.146	0.184	-0.111	0.064	-0.186	0.092	0.917	-0.204	0.008	0.043	0.001	-0.004	0.002	-0.002	-0.001
Mn (mg/L)	-0.009	0.978	-0.025	-0.037	0.064	0.082	0.153	-0.089	-0.001	0.014	0.000	-0.002	0.001	0.000	0.000
Hg ($\mu\text{g}/\text{L}$)	-0.725	-0.083	0.181	0.051	-0.070	0.323	-0.259	0.062	0.006	-0.502	0.004	0.012	0.008	0.004	0.002
As ($\mu\text{g}/\text{L}$)	-0.296	0.120	0.206	0.094	0.120	0.877	0.119	-0.213	0.000	-0.052	-0.003	0.007	0.002	0.002	0.001
Total coliforms (CFU/100mL)	-0.207	-0.046	0.884	-0.055	0.007	0.266	-0.094	-0.045	0.102	-0.067	0.046	0.268	0.014	-0.009	0.001
Faecal coliforms (CFU/100mL)	-0.190	0.009	0.905	-0.065	0.144	0.007	-0.127	-0.079	-0.305	0.019	-0.001	-0.068	0.006	0.007	0.002
Faecal streptococcus (CFU/100mL)	-0.161	-0.006	0.960	0.016	-0.083	0.044	0.047	-0.080	0.138	-0.021	-0.024	-0.120	-0.008	0.005	-0.001

Note: Values in bold correspond to the greatest contributions of the original variables on the PCs.

Table 4. Transformation matrix used to calculate the PCs from the physical, chemical and bacteriological variables.

Variables	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12	PC13	PC14	PC15
Conductivity	0.367	0.020	-0.212	0.048	-0.011	0.072	-0.087	0.002	-0.138	0.041	-0.130	0.015	-0.113	-0.215	-0.841
DOC	0.354	-0.177	0.083	0.067	0.132	-0.164	0.052	0.277	-0.231	0.091	-0.117	0.009	0.601	0.519	-0.040
Turbidity	-0.103	-0.233	0.225	-0.274	0.654	0.229	0.379	0.078	0.101	-0.100	-0.285	0.180	-0.187	0.020	-0.105
Colour	0.225	-0.484	0.075	0.060	0.094	-0.159	-0.249	0.548	-0.132	0.030	0.225	-0.074	-0.306	-0.323	0.193
Total nitrogen	0.338	0.094	-0.205	-0.192	-0.128	0.236	-0.211	-0.010	-0.106	0.073	-0.103	0.432	-0.460	0.453	0.224
Total phosphorus	0.318	-0.067	-0.303	-0.031	0.205	0.178	-0.003	-0.050	0.579	-0.184	0.346	-0.429	-0.010	0.231	0.002
Hardness	0.344	-0.039	-0.222	0.061	0.143	0.199	-0.136	-0.258	-0.117	-0.370	-0.306	0.104	0.320	-0.444	0.358
Zn	-0.098	0.018	0.066	-0.785	-0.273	0.266	-0.199	0.249	-0.057	-0.118	-0.021	-0.202	0.232	-0.075	-0.065
Fe	-0.067	-0.592	0.144	-0.094	0.007	0.043	-0.336	-0.598	-0.052	0.319	-0.050	-0.151	0.003	0.102	-0.045
Mn	0.029	-0.384	0.012	0.246	-0.504	0.518	0.509	0.072	-0.046	-0.040	0.009	-0.018	0.003	0.005	0.016
Hg	0.299	0.153	-0.163	-0.303	0.090	-0.082	0.457	-0.147	-0.204	0.577	0.188	-0.141	0.001	-0.237	0.193
As	0.240	-0.226	0.077	-0.266	-0.343	-0.576	0.240	-0.122	0.401	-0.194	-0.152	0.255	-0.031	-0.067	-0.031
Total coliforms	0.265	0.169	0.446	-0.008	-0.039	-0.103	0.119	-0.206	-0.380	-0.403	-0.029	-0.460	-0.306	0.142	0.029
Faecal coliforms	0.244	0.225	0.424	0.156	-0.095	0.172	-0.152	0.153	0.423	0.389	-0.462	-0.184	-0.021	-0.115	0.097
Faecal streptococcus	0.231	0.112	0.523	-0.005	0.042	0.210	-0.078	-0.142	0.081	-0.030	0.582	0.431	0.184	-0.119	-0.095

Table 5. Performance indexes for MLR and PCR in the fitting of the *Chlorella vulgaris* and *Vibrio fischeri* toxic responses.

	MLR				PCR			
	MAE	RMSE	d_2	R^2	MAE	RMSE	d_2	R^2
<i>Chlorella vulgaris</i>	1.532	1.945	0.884	0.643	1.901	2.364	0.797	0.473
<i>Vibrio fischeri</i> (15 min)	0.613	0.860	0.911	0.711	0.817	1.008	0.864	0.603

parameters. PC2, PC4, PC5, PC6, PC7 and PC8 had important contributions from Mn, Zn, turbidity, As, Fe and colour, respectively. PC9 to PC15 did not present any significant contribution of the original variables; however, they were used in PCR to analyse if these minor contributions are statistically significant in the ecotoxicological response of living organisms. The regression models using PCs as input variables (PCR) were the following:

$$C.vulgaris = 2.719 + 0.683 (PC3) - 1.899 (PC6) - 1.677 (PC8) + 2.841 (PC9) \quad (7)$$

$$V.fischeri = 1.849 - 0.442 (PC4) - 1.304 (PC8) + 1.087 (PC9) + 8.596 (PC15) \quad (8)$$

Table 4 presents the matrix that multiplied by the original variables matrix gives the values of PCs. These values show how a PC was influenced by each original variable. For instance, negative values showed that the original value and the PC are negatively correlated. Taking values in Table 4 corresponding to high factor loadings (in Table 3) and the regression coefficients for each PC, it is possible to infer the relationship between the original variables and the output variable. If both values have the same signal, the influence is positive; otherwise, the influence is negative. According to this transformation and the regression coefficients given by the models, PCR showed that: (i) *C. vulgaris* toxic response was negatively influenced by colour and DOC, and positively by As, Hg and all bacteriological parameters, especially faecal coliforms; and (ii) *V. fischeri* toxic response was negatively correlated with colour and DOC, and positively with Zn and faecal coliforms.

Figures 2 and 3 present the comparison between toxicity experimental and calculated values (TU_{50}) from MLR and PCR, respectively. Table 5 shows the performance indexes for MLR and PCR. MLR is the regression model that best fit the *C. vulgaris* and *V. fischeri* toxic response with respect to the Leça river water characterization.

4. Discussion

4.1. Multiple linear regression

The MLR results for *C. vulgaris* showed a negative correlation between the toxic response and the DOC, Zn and Mn parameters. DOC is extremely important in the transport of metals in aquatic systems, forming strong complexes

with metals, enhancing metal solubility while also reducing metal bioavailability. Studies using multispecies laboratory bioassays proved *C. vulgaris* resistance to toxicants like Zn.[43,44].

Turbidity is considered an important variable relative to transport and bioavailability of contaminants in natural waters.[45] In addition, turbidity affects the results of tests based on photometric measurements, produces light losses and leads to toxicity overestimation.[46] In the present study, turbidity was positively related to *C. vulgaris* toxic response results due to the scattering of incident light by colloidal and particulate matter in water.

The *V. fischeri* toxic response, according to MLR, presented a negative relation with conductivity and turbidity. Conductivity is related to ionic concentrations and pH. The Microtox® test procedure, based on the inhibition of *V. fischeri* marine bacteria, involves the addition of sodium chloride, therefore, possibly changing sample ionic concentration and, consequently, metals toxic potential. This effect may be due to competition between toxic ions and chloride ions in the cellular membrane.[47] Some studies showed silver toxicity diminishing with the raise of salinity up to 25%; however, for salinity above 25‰ an increase in the metal toxicity was observed, which was attributed to osmotic imbalance caused by chloride ions.[48–50]

The hardness, the metals Fe, Hg and As and the faecal coliforms presented a positive correlation with the toxic response of *V. fischeri*. Concerning the effect of hardness on metals toxicity, it is known that the presence of calcium and magnesium carbonates in water can cause the precipitation of metals, making them insoluble and therefore not available to penetrate into the membranes of living organisms. This effect was observed for manganese chronic toxicity in aquatic species *Salmo trutta*, and also for other metals, such as copper, zinc and cadmium.[51–53] The hardness values obtained for Leça river were normal for surface water and, therefore, the metals Fe, Hg and As contributed to global toxic effect. Nevertheless, Microtox® test is especially sensitive to several metals, such as Hg, Pb, Zn and Cu [54,55]; the toxicity of heavy metals is highly influenced by matrix effects, conditions and concentration.[56,57] The faecal coliforms in Leça river presented extremely high concentrations showing positive correlation with the *V. fischeri* toxic response probably due to competition between the bacteria, both Gram-negative, heterotrophic and facultative anaerobes. This competition might be for oxygen,

which would influence the luminescence produced once its mechanism is intrinsically connected to the respiratory metabolism.[58]

4.2. Principal component regression

The PCR results for *C. vulgaris* toxic response showed a negative correlation with colour and DOC parameters. In the specific case of surface water samples in the natural environment, the colour is related to high concentrations of DOC, which could explain the inclusion in the same PC (PC8). As algae absorb light energy for photosynthesis, in coloured samples the light provided during the toxicity bioassay may be partially absorbed by the coloured compounds of the surface waters.[59]

Arsenic, mercury and all bacteriological parameters (especially faecal coliforms) showed a positive correlation with *C. vulgaris* toxic response. Algae are generally hyper-accumulators of heavy metals.[1,60–63] However, some studies showed that arsenic is toxic to algae but highly variable data have been reported due to different experimental conditions (e.g. ‘no effect’ concentrations ranged from 0.16 to 1000 mg/L).[57] With regard to the bacteriological parameters, a positive correlation was found. It might be related to the fact that bacteria respiration releases carbon dioxide, essential for algae photosynthesis.

According to PCR, the *V. fischeri* toxic response presented a negative correlation with colour and DOC. A coloured sample may potentially absorb a portion of the light produced by the *V. fischeri* before it reaches the photomultiplier, and the sample may appear more toxic than it really is.[64] In this manner, colour should present a positive and not a negative correlation. The DOC biodegradable fraction consists of organic molecules that can be used by heterotrophic bacteria, such as *V. fischeri*, as a source of energy and carbon, thus contributing to bacterial metabolism. Zn and faecal coliforms presented positive correlation with *V. fischeri* toxic response, which agrees with the result obtained by MLR, confirming the idea of competition between *V. fischeri* and coliforms.

5. Conclusions

To better understand the interaction of physical, chemical and bacteriological factors involved in a multidimensional process such as the ecotoxicological response, MLR and PCR were applied to the results of *C. vulgaris* and *V. fischeri* toxic response to the Leça river water characterization, both physicochemical and microbiological. According to the results obtained, the first seems to be more sensitive, which is in accordance with most studies presented in literature.

In a general way, and supported by the performance indexes, the MLR seems to be the most appropriate model to the Leça river data, presenting: (i) a negative correlation with DOC, Zn and Mn, and a positive one with turbidity

and As for *C. vulgaris* toxic response and (ii) a negative correlation with conductivity and turbidity, and a positive one with phosphorus, hardness, Fe, Hg, As and faecal coliforms for *V. fischeri* toxic response.

The results obtained may be useful in the future to evaluate the effect of pollution abatement measures over the water quality of Leça River. This approach will be helpful for the strategy dictated by the water framework Directive 2000/60/EC, which include the classification of water bodies to allow the definition of environmental objectives and the implementation of management programs.

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