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Optimization of river water quality surveys by multivariate analysis of physicochemical, bacteriological and ecotoxicological data

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Abstract

This study aims to optimize the water quality monitoring of a polluted watercourse (Leça River, Portugal) through the principal component analysis (PCA) and cluster analysis (CA). These statistical methodologies were applied to physicochemical, bacteriological and ecotoxicological data (with the marine bacterium *Vibrio fischeri* and the green alga *Chlorella vulgaris*) obtained with the analysis of water samples monthly collected at seven monitoring sites and during five campaigns (February, May, June, August, and September 2006).

The results of some variables were assigned to water quality classes according to national guidelines. Chemical and bacteriological quality data led to classify Leça River water quality as “bad” or “very bad”. PCA and CA identified monitoring sites with similar pollution pattern, giving to site 1 (located in the upstream stretch of the river) a distinct feature from all other sampling sites downstream. Ecotoxicity results corroborated this classification thus revealing differences in space and time.

The present study includes not only physical, chemical and bacteriological but also ecotoxicological parameters, which broadens new perspectives in river water characterization. Moreover, the application of PCA and CA is very useful to optimize water quality monitoring networks, defining the minimum number of sites and their location. Thus, these tools can support appropriate management decisions.

Keywords: *Chlorella vulgaris; cluster analysis; ecotoxicology; principal component analysis; surface water quality; Vibrio fischeri.*

1. Introduction

Due to the complexity and variability of organic and inorganic compounds that may be found in natural waters, the results of physicochemical and bacteriological analyses are not sufficient to portray the impact caused by the contaminants, once they do not reveal the effects over the ecosystem (Abel 1996). Toxicity tests make possible to determine the toxic potential of a chemical agent or a complex mixture, through the evaluation of the response of living organisms (Tisler and Zagorc-Koncan 1999).

The use of different kinds of prokaryotic (e.g. *Vibrio fischeri*) and eukaryotic (e.g *Chlorella vulgaris*) organisms in inhibition tests provides a suitable evaluation of ecotoxicity. Simple multispecies laboratory studies not only could be beneficial in the risk assessment process, but are most appropriate when a substance impacts a known key species within a food chain (Boxall et al 2002; Selck et al 2002).

Microtox® is a method that allows the determination of toxicity of an aqueous solution by exposing it to the luminescent bacterium *Vibrio fischeri*, which was used in this study. The main advantages of this method are the short time required to obtain results (5, 15 and 30 minutes), the simplicity and high reproducibility (Munkittrick et al 1991; Argese et al 1998; Steevens et al 1998).

The utility of algae as a test-organism is based on its short life cycle, making it easy to study the exposure of several generations, its high growth rate, the facility to maintain cultures in the laboratory and the ability to grow in defined synthetic media (Lewis 1995). Since photoautotrophic microalgae are primary producers of essential nutrients in the ecosystem, toxicity against these organisms is considered to be of particular importance (Eguchi et al 2004). *Chlorella vulgaris*, which was selected for this study, has been widely used for toxicity bioassays (Eguchi et al 2004; Ma et al 2004; Santos et al 2010).

The application of different mathematical tools, such as principal component analysis (PCA) and cluster analysis (CA), allows the interpretation of complex data matrices to better understand the water quality and ecological status of the studied system (Kotti et al 2005; Koklu et al 2010; Ogleni and Topal 2011; Awadallah and Yousry 2012). These studies showed the ability of PCA and CA for the evaluation and interpretation of complex data sets to get better information about water quality and the design of the monitoring network for effective management of water resources.

The study here reported aims to evaluate the surface water quality of Leça River not only by means of a classical physical, chemical and bacteriological characterization but also by ecotoxicity tests to enhance the evaluation of water quality. Leça River was selected for this study because it is one of the most polluted rivers in Portugal. The application of the multivariate analysis (PCA and CA) to group sampling sites contributes to the optimization the water quality monitoring network in water courses, thus reducing analytical work and costs.

The objectives of the water framework Directive 2000/60/EC include prevention of degradation and improvement of surface and underground water bodies to achieve a good chemical and ecological status until 2015 and promote a sustainable water reuse based on a long-term protection of available water resources. Thus classification of water bodies status is mandatory to allow the definition of environmental objectives and the implementation of management programs. Within this aim, the findings of this work are not only of local interest, regarding that Leça is a very polluted river that has to be recovered, but may also be applied to other European rivers.

2. Materials and methods

2.1 Study area and water sampling

Leça River, located in northern Portugal, flows for approximately 48 km from Santo Tirso district to the Atlantic Ocean. The high industrial and urban densities in the downstream stretch of the river originate very high pollution levels and therefore ichthyofauna has no relevance. This contrasts with sparsely populated, agricultural and forested areas at the upstream stretch (Ministry of Environment 1994, 2000).

Most of the pollution load is originated by textile dyeing and printing, metallurgical and mechanical and agro-food plants, some of them discharging untreated effluents into the river (Ministry of Environment 1994, 2000).

Leça River receives also the treated effluents of several wastewater treatment plants (WWTP). The most important are located in Maia: one of them treats around 21,900 m^3/d and the other receives around 760 m^3/d of urban wastewaters, both receiving domestic and industrial effluents. Sampling points located downstream from these WWTP were selected, respectively sites 2 and 4 (Figure 1).

Sampling locations were selected to depict the water quality evolution along the river, including an unpolluted upstream reach, a critical area affected by effluent discharges and a downstream stretch.

Figure 1 shows the location of the major industrial activities, as well as the seven sampling sites selected. The respective coordinates and some details useful for a further analysis of experimental data are provided in Table 1.

To characterize extreme weather conditions, water samples were monthly collected in five different periods, within one day in each month: February, May, June, August and September of 2006. Winter and autumn high rainfall periods are represented by February and September, respectively, which are usually associated to high turbidity,

suspended solids concentration and flow rate, leading to diluted concentration of other pollutants. The hot season (from May to August) has usually low rainfall which causes a reduction in the flow rate and therefore high concentration of most pollutants together with low dissolved oxygen, due to high temperatures. The most critical situation is achieved at the end of this period.

Most of the samples were collected from bridges, to obtain samples from running water which were representative of the river water. Grab samples were manually collected using 5 L plastic bottles for physicochemical analyses, 1.5 L plastic bottles for algal inhibition growth bioassays, 0.25 L borosilicate glass bottles for Microtox® toxicity bioassays and previously sterilized diving bottles for bacteriological analysis. The water samples were kept refrigerated during transportation to laboratory and were stored at 4 ºC (no chemical preservatives were added).

2.2 Physicochemical analysis

Temperature, pH and oxidation-reduction potential (HANNA Instruments model 991003), dissolved oxygen (HANNA Instruments model 9143) and conductivity (WTW, LF 330) were measured *in situ*. For dissolved organic carbon and metals the samples were filtered by 0.45 μm pore diameter membrane filters before storage.

Water samples were analyzed in duplicate within 24 hours, according to Standard Methods (APHA et al 2005) for turbidity (Turbiquant 3000 IR, Merck - Method 2130 B), dissolved organic carbon (DOC, Shimadzu 5000 A - Method 5310 B), biochemical oxygen demand (BOD, Crison OXI 45 - Method 5210 B), total nitrogen (spectrophotometer PYE Unicam PU 8600 UV/Vis. PHILIPS - Method 4500N C), total phosphorus (spectrophotometer PYE Unicam PU 8600 UV/Vis. PHILIPS - Method

4500 P), hardness (Method 2340 C) and dissolved metals – Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn (Atomic Absorption Spectrometer GBC 932 plus –Methods 3111 B and D), Hg and As (Atomic Absorption Spectrometer GBC 932 plus and GBC HG 3000 - Methods 3112 B and 3114 C). Color was measured according to the colorimetric-platinum-cobalt method 110.2 (USEPA 1983) using a PYE Unicam PU 8600 UV/Visible PHILIPS spectrophotometer. All reagents employed were analytic grade.

The physicochemical results were compared with the quality criteria for surface water provided in Table 2.

2.3 Bacteriological analysis

All samples were analyzed (within 6 hours after collection) in duplicate for three different concentrations, by diluting with saline medium, and filtrated by cellulose-nitrate membranes (Albeit 0.45 µm pore size).

Total coliforms concentration was determined by the membrane filtration method (ISO 9308-1). Fecal coliforms concentration (thermotolerant coliforms) was determined by the membrane filtration method (ISO 9308-1). Fecal Streptococcus concentration was determined by the membrane filtration method (ISO 7899-2). The bacteriological results were compared with the quality criteria for surface water (Table 2).

2.4 Ecotoxicological analysis

Bioassays were performed within 48 hours after sampling.

The bioluminescent inhibition toxicity tests (ISO 11348) were performed using the Microtox Toxicity Analyzer Model 2055, Microbics Corporation (at present time, AZUR Environmental), and the bacterium *Vibrio fischeri* (strain NRRL B 11177). All

166 samples were tested within 48 hours after sampling following the Basic Test protocol
167 (ISO 11348). Tested concentrations were 5.6%, 11.3%, 22.5% and 45% (v/v). The
168 values of EC_{50} and EC_{20} (effective concentration of the sample that causes 50 or 20%
169 inhibition to the test-organisms, respectively) and the corresponding 95% confidence
170 intervals were determined for 5 and 15 minutes of bacterial exposure.
171 The green algal inhibition growth tests were performed with the microalga *Chlorella*
172 *vulgaris* according to USEPA Guideline (2002). Three replicates of each sample were
173 tested for five different concentrations (10%, 20%, 40% 60% and 80%), plus the control
174 test. The test solutions were incubated for 72 hours, under continuous cool white
175 fluorescent light. Agitation was manually performed twice per day and bottles were
176 randomized. Initial and final absorbances were measured at 440 nm (Carvalho et al
177 1995), using a Shimadzu UV-Visible spectrometer, to evaluate the growth of the algal
178 population. The acceptability criterion considered was variability less than 20% among
179 replicates. Shapiro-Wilk's Normality Test and Bartlett's Test for Homogeneity of
180 Variance were performed to validate data, and Dunnett's procedure was followed
181 (USEPA 2002). Since these assumptions were met, EC_{50} and EC_{20} were calculated by
182 linear interpolation.
183 The validation of each test was performed using a reference toxicant, phenol and
184 potassium dichromate, respectively for *V. fischeri* and *C. vulgaris* bioassays.
185 To simplify the results expression, toxicity units were used ($TU_{xx} = 100/EC_{xx}$), as
186 suggested by Wisconsin Department of Natural Resources (WDNR 2004). Expressing
187 EC_{xx} in percentage, TU = 1 means that the sample has no toxicity. For practical reasons,
188 the biostimulation responses were also considered not toxic (TU = 1), especially since
189 Microtox® biostimulation present a negative gamma value. TU_{50} was used for
190 regression models.

2.5 Multivariate statistical methods

As indicated in previous studies (Brogueira and Cabeçadas 2006; Kannel et al 2007), Principal Component Analysis (PCA) and Cluster Analysis (CA) are multivariate statistical methods very useful to evaluate river water quality data. So, in this work, these mathematical tools were used to group sampling sites with similar water pollution patterns to select the most representative sampling sites. Both methodologies were applied using Matlab codes developed by the authors.

PCA is designed to transform the original variables into new and uncorrelated variables, called the principal components (PC), which are linear combinations of the original variables. In this study, only the PCs with eigenvalues greater than 1 (Kaiser criterion) were considered (Yidana et al 2008). To evaluate the influence of each variable in the PC, varimax rotation was applied obtaining the rotated factor loadings that represent the contribution of each variable for a specific principal component.

CA is a multivariate technique whose primary purpose is to assemble objects based on the characteristics they possess. Hierarchical agglomerative clustering is the most common approach, which provides intuitive similarity relationships between any sample and the entire data set, and is typically illustrated by a dendrogram (tree diagram) (McKenna 2003; Varol et al 2012). The dendrogram provides a visual summary of the clustering processes, presenting a picture of the groups and their proximity. The Euclidean distance was used as a measure of the similarity between two objects. The clustering procedure adopted was the average linkage method (Otto 1998; Pires et al 2008).

To perform PCA and CA, data were Z standardized to have zero mean and unit standard deviation.

3. Results and Discussion

3.1 Physicochemical parameters

Physicochemical characterization of the samples is provided in Table 3 and Figure 2 (Gomes 2007). Temperature and conductivity presented the highest values in August while dissolved oxygen (DO) presented the lowest results, due not only to higher temperatures in summer, but also to higher organics concentration associated to this dry season; following the OD variation, ORP presented the lowest values in August too; color intensity was also greater in summer; the most critical pH values, below 6, were obtained in summer, being the minimum 5.64 in June - acid pH values are typical in the rivers of north Portugal due to its granitic soil; the highest values of turbidity were obtained in February and September, when sampling was done under intense rain.

It can be observed that site 1 shows a different behavior when compared with the other sampling locations along the river. The lowest concentrations of DOC, BOD, total nitrogen, total phosphorus and hardness were detected at the most upstream sampling site (site 1).

As concerns dissolved metals (As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn) only zinc, manganese, mercury, arsenic and iron were detected (Figure 2). Zinc was detected in all sampling sites in February, presenting the lowest concentration at sites 1, 5 and 6 (0.08 mg/L) and the highest at site 2 (0.22 mg/L) possibly due to metal plating industrial discharges, while manganese was detected only in June at site 5 (0.13 mg/L). Due to the lower river flowrate, highest values were generally obtained in summer, and especially downstream from site 3, where the chemical, metallurgic and mechanical industries are more concentrated. Although water was not polluted (Table 2) by arsenic

and iron (all values are below the limits, 0.010 and 0.50 mg/L, respectively), it was extremely polluted by mercury, especially in August, when all values largely exceed the limit of reasonable water quality (0.50 μg/L).

According to the quality criteria for surface water (Table 2), Leça River presents levels of physicochemical contamination that led to a water quality classification between "very bad" and "bad" - BOD and phosphorus exceeded the limits for minimum river water quality, according to quality standards (SNIRH 2011). Sampling site 1, at the upstream river stretch, is the less polluted site however its water quality cannot be classified as "excellent" because it exceeded the maximum mercury concentration in September (Figure 2). The "pollution" load of industrial origin increases along the river downstream.

3.2 Bacteriological parameters

The bacteriological parameters (Figure 3) showed the lowest values in February (winter) and the highest in August (summer). The evolution of the bacteriological contamination along the river indicates: low concentrations at site 1 (upstream); very high levels of contamination at sites 2 and 3 (located downstream an urban WWTP treating urban wastewater and in a strongly industrialized and populated area, respectively); a decrease of contamination at site 4 (possibly due to the dilution of the river with the high-quality effluent of a WWTP upstream from site 4); a contamination increase at site 5; and a slight decrease at sites 6 and 7.

Considering the bacteriological analyses, most of the values largely exceed the limits of excellent water quality, 50, 20 and 20 C.F.U./100 mL for total coliforms, fecal

263 coliforms and fecal Streptococcus, respectively. Thus, Leça River water quality was
264 classified as “bad” once again, except for sampling site 1.
265

266 **3.3 Ecotoxicological parameters**

267 The ecotoxicological results from inhibition of the bioluminescent bacterium *Vibrio*
268 *fischeri* (Microtox®) and the growth of the green alga *Chlorella vulgaris* are provided
269 in Table 4. The bacterial inhibition results shows that February was the critical month,
270 followed by September and May, while in June and August the bioassays with *Vibrio*
271 *fischeri* showed biostimulation responses and therefore, toxicity was not detected. No
272 toxicity was detected at site 1 but it increases downstream, generally showing the
273 highest values at site 3 (strongly industrialized area) and then decreasing towards the
274 river mouth. Although not expected, in site 4 in May and in site 7 in February for TU_{20},
275 the results indicate a decrease of toxicity (acclimation), which was not confirmed by the
276 correspondent TU_{50} used for regression.
277 The bioassays using *Chlorella vulgaris* also showed February as the critical month with
278 inhibition results in almost all samples, followed by September; biostimulation was also
279 detected especially in May, followed by August and September. In June almost all
280 samples presented inhibition that decreased with the increase of tested concentration.
281 The ecotoxicological evaluation by means of mono specific bioassays, with *Vibrio*
282 *fischeri* and *Chlorella vulgaris*, integrates the effect of physicochemical and
283 bacteriological water quality. Nevertheless, since bioassays were carried out under
284 controlled experimental conditions, they represent a simplified situation (Hsu et al
285 2007). In the river, physical factors such as temperature, flowrate, interactions among
286 abiotic factors and biotic interrelations may also affect the toxicological response of
287 aquatic organisms. In addition, synergistic and antagonist effects can vary at different

288 dilutions, e.g., in concentrated samples, some substances can form micelles and some
289 of the toxicological effects can be inhibited, whereas in more diluted samples,
290 these substances can be more bioavailable, so their effects increase (Farré et al 2007).
291 Almost no toxic effect was detected in spring and summer bioassays (May, June and
292 August), except for sampling site 3. Oppositely, a stimulation response was observed
293 for both test organisms. One possible explanation for this effect is the high
294 concentration of both nitrogen and phosphorus, especially in summer, which would
295 imply a prevalence of the stimulating effect of nutrients over the inhibiting effect of
296 toxicants (Olguin et al 2004). Another potential reason may be the natural algal bloom
297 during spring and summer where a variety of photosynthetic products are excreted into
298 the water, being used as substrates to support the growth and metabolism of bacteria
299 (Hsu et al 2007).
300

301 **3.4 Multivariate analysis**

302 The mathematical tools PCA and CA were applied to group sampling sites with similar
303 water pollution pattern. Concerning the analyzed metals, only the ones presenting
304 concentrations above the detection limit (Zn, Mn, Hg, As and Fe) were used in
305 these statistical analyses. For the measurements below the detection limit, the used
306 values were half of the correspondent limit value.
307 Figure 4 shows the graphical representation of PCA results according to
308 physicochemical, bacteriological, ecotoxicological and all parameters. For
309 physicochemical data, the first two principal components (PC_1 and PC_2) explain 93.3%
310 of total variance. PC_1 includes important contributions of sites 2, 3, 4, 5, 6 and 7, while
311 PC_2 is heavily loaded by site 1. Considering bacteriological data, three PCs explain
312 93.0% of the total variance. PC_1 shows important contributions of sites 2, 3 and 5; PC_2

313 is markedly related to sites 1 and 4; and sites 6 and 7 are significantly associated to PC_3.
314 Taking into consideration the ecotoxicological parameters, three PCs explain 73.3% of
315 the total variance. PC_1 has important contributions of sites 3, 4, 6 and 7; PC_2 was
316 strongly loaded by sites 2 and 5; and site 1 is significantly associated to PC3. Finally,
317 considering all parameters, only two PCs were obtained, explaining 76.6% of the total
318 variance. PC_2 is associated to site 1, while PC_1 includes important contributions of the
319 remaining sites. PCA groups the original variables (in this study, the sampling sites)
320 according similar variation of their values, i.e. correlated variables were grouped in the
321 same PC. The sampling sites corresponding to redundant measurements can be removed
322 from future water quality studies or relocated to other non-monitored regions to better
323 characterize the river water quality.
324 Figure 5 shows the dendrograms resulting from the application of CA to
325 physicochemical, bacteriological, ecotoxicological and all parameters. Analyzing the
326 different groups of parameters, the seven sampling sites can be divided in two clusters
327 (CL_1 and CL_2) with similar water pollution pattern. However, sites are grouped
328 in different ways while considering physicochemical (CL_1 – sites 2, 3, 4, 5, 6 and 7;
329 CL_2 - site 1), bacteriological (CL_1 – sites 1, 4, 6 and 7; CL_2 – sites 2, 3
330 and 5), ecotoxicological parameters (CL_1 – sites 1, 2, 4, 5, 6 and 7; CL_2 – site 3)
331 or all data (CL_1 – sites 2, 3, 4, 5, 6 and 7; CL_2 – site 1).
332 PCA and CA, based on the physicochemical data, divided the sampling sites in a similar
333 way: PC_1 and PC_2 correspond to CL_1 and CL_2, respectively (Figures 4a and
334 5a). Located at the upstream stretch of the river, site 1 revealed unique characteristics
335 and is different from all other sampling sites downstream.
336 With regard to the bacteriological data, once again PCA and CA results were consistent;
337 PC_1 corresponds to CL_2, while PC_2 and PC_3 correspond to CL_1 (Figures 4b and 5b).

This division is supported by the bacteriological results that showed high levels of contamination at site 2, followed by sites 3 and 5 (all located in the intermediate section of the river in a highly populated area), and relatively low levels of contamination at sites 1 (located upstream in a rural area), 4 (located after a sewage treatment plant), 6 and 7 (both located downstream, near the river mouth).

Concerning the ecotoxicological results, PCA and CA present a slightly different division: sampling sites 2 and 5 (PC_2) appear close to each other in the dendrogram while sites 4, 7 and 6 (PC_1) show proximity according CA (Figures 4c and 5c). Site 3 often presents positive ecotoxicity results and therefore it was included in CL_2.

PCA and CA, in the analysis to all parameters, equally divided the sampling sites, so that PC_1 and PC_2 corresponded to CL_1 and CL_2, respectively (Figures 4d and 5d). Once again site 1 appears to have distinct features from all other sites along the river. The classification scheme obtained by CA is confirmed by PCA. The same conclusion was verified by Papaioannou et al (2010). The application of these tools to water quality data showed that there are monitoring sites associated with the same pollution pattern, which corresponds to redundant measurements and should be moved to other locations, optimizing the water quality assessment in Leça river basin. For instance, one of the sampling sites 2 or 5, which presented redundant physicochemical, bacteriological and ecotoxicological measurements, should be eliminated or displaced. In this case, as shown in Figures 4c and 5c, the ecotoxicological parameters (Table 4) were determinant to distinguish site 3 from sites 2 and 5, meaning that the ecotoxicological analysis should be also considered if a complete characterization of water is demanded.

4. Conclusions

The water quality of Leça River was classified as “bad” or “very bad” due to contributions from numerous contamination sources that determine a sharp change in the physicochemical and bacteriological status in the downstream section.

Ecotoxicological tests were also performed to enhance the water quality evaluation and the results corroborated this classification, thus revealing differences in space and time. This new strategy of monitoring water quality includes physicochemical, bacteriological and ecotoxicological approaches. To group similar sampling sites, the application of PCA and CA showed that site 1, located upstream the river, presented unique characteristics, typical of “excellent” water quality, contrasting with the downstream sampling sites, where the water quality is highly affected by the intense demographic occupation and high industrialization. The results from multivariate analysis suggest redundant measurements in sampling sites 2 and 3, which should be removed or displaced to optimize the monitoring plan of this river. This integrated approach through multivariate analysis of physicochemical, bacteriological and ecotoxicological parameters may be applied to other rivers to compare their water quality. This study shows that ecotoxicological analysis must be taken into account for a complete characterization of water quality and application of PCA and CA are indispensable tools for optimizing water quality monitoring networks in any river.

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482

Captions of Figures

Fig. 1 Leça river basin: location of the major industrial activities and the seven selected sampling sites

Fig. 2 Dissolved metals in Leça river water samples (detection levels: Hg < 0.35 µg/L; As < 0.6 µg/L and Fe < 0.2 mg/L)

Fig. 3 Bacteriological characterization of Leça river water samples

Fig. 4 Graphical representation of PCA results according to: a) physicochemical parameters; b) bacteriological parameters; c) ecotoxicological parameters; d) all parameters

Fig. 5 Dendrograms showing clustering of sampling sites according to: a) physicochemical parameters; b) bacteriological parameters; c) ecotoxicological parameters; d) all parameters

Figure 1

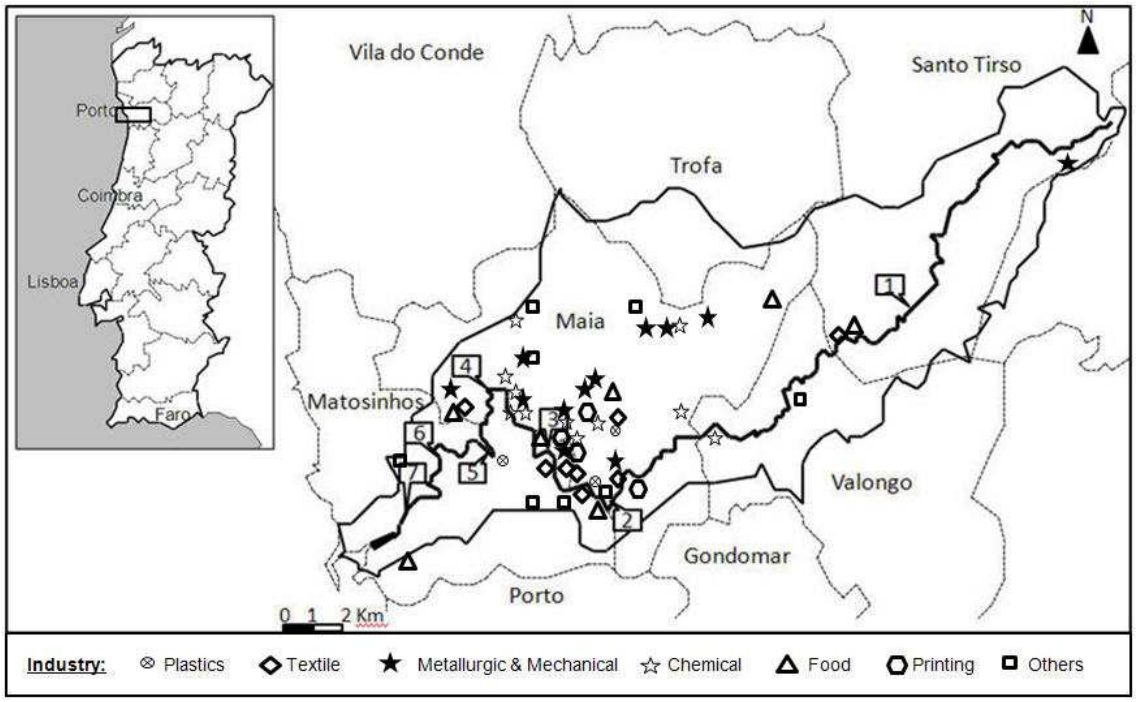

Porto
Coimbra
Lisboa
Faro
Vila do Conde
Santo Tirso
Trofa
Maia
Matosinhos
Valongo
Gondomar
Porto
N
0 1 2 Km
1
2
3
4
5
6
7
Industry:
Plastics
Textile
Metallurgic & Mechanical
Chemical
Food
Printing
Others

Figure 2

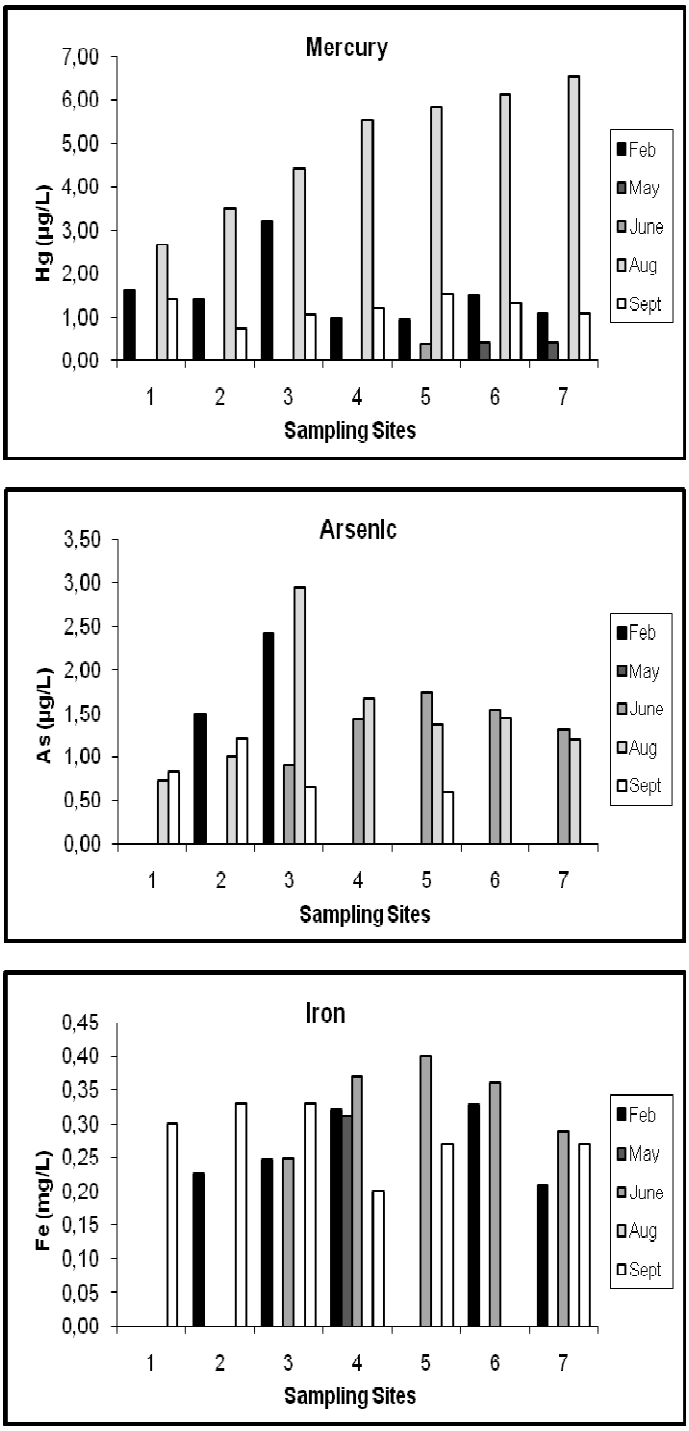
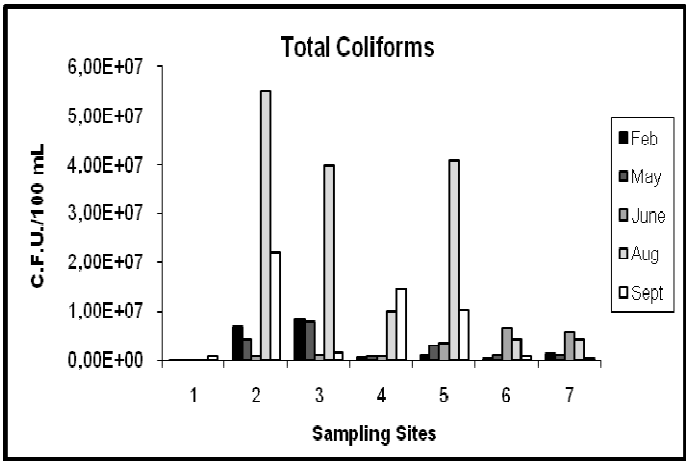
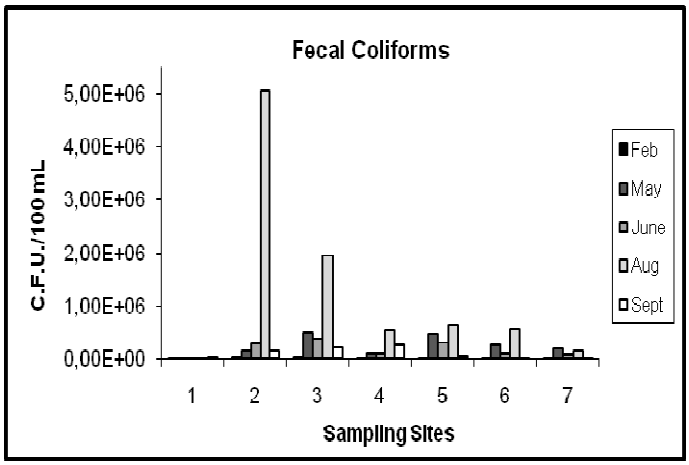

Mercury
Hg (µg/L)
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
1
2
3
4
5
6
7
Sampling Sites
Feb
May
June
Aug
Sept
Arsenic
As (µg/L)
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
1
2
3
4
5
6
7
Sampling Sites
Feb
May
June
Aug
Sept
Iron
Fe (mg/L)
0,45
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
1
2
3
4
5
6
7
Sampling Sites
Feb
May
June
Aug
Sept

Figure 3


Total Coliforms
6,00E+07
5,00E+07
4,00E+07
3,00E+07
2,00E+07
1,00E+07
0,00E+00
C.F.U./100 mL
1
2
3
4
5
6
7
Sampling Sites
Feb
May
June
Aug
Sept


Fecal Coliforms
5,00E+06
4,00E+06
3,00E+06
2,00E+06
1,00E+06
0,00E+00
C.F.U./100 mL
1
2
3
4
5
6
7
Sampling Sites
Feb
May
June
Aug
Sept

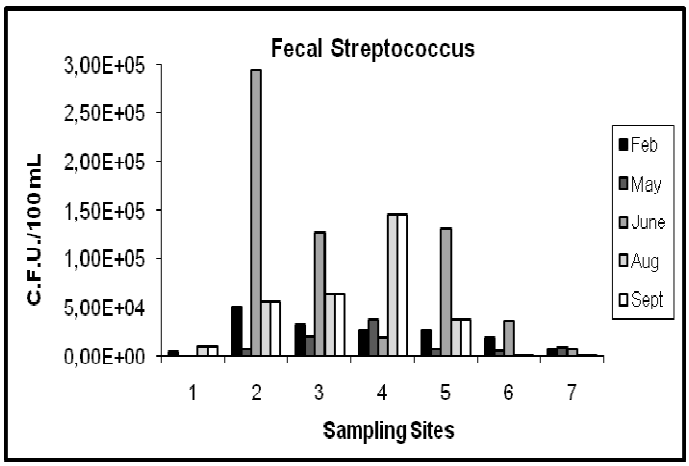

Fecal Streptococcus
3,00E+05
2,50E+05
2,00E+05
1,50E+05
1,00E+05
5,00E+04
0,00E+00
C.F.U./100 mL
1
2
3
4
5
6
7
Sampling Sites
Feb
May
June
Aug
Sept

Figure 4

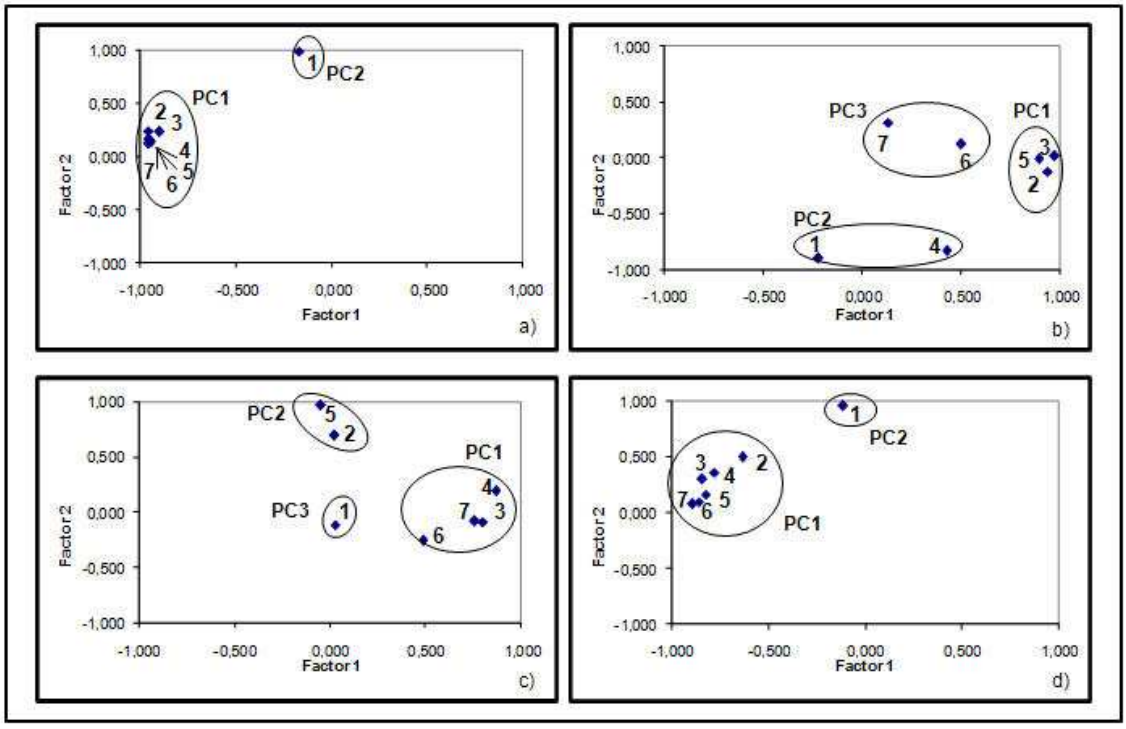

1,000
0,500
0,000
-0,500
-1,000
Factor 2
-1,000
-0,500
0,000
0,500
1,000
Factor 1
PC1
PC2
PC3
1
2
3
4
5
6
7
a)
b)
c)
d)

Figure 5

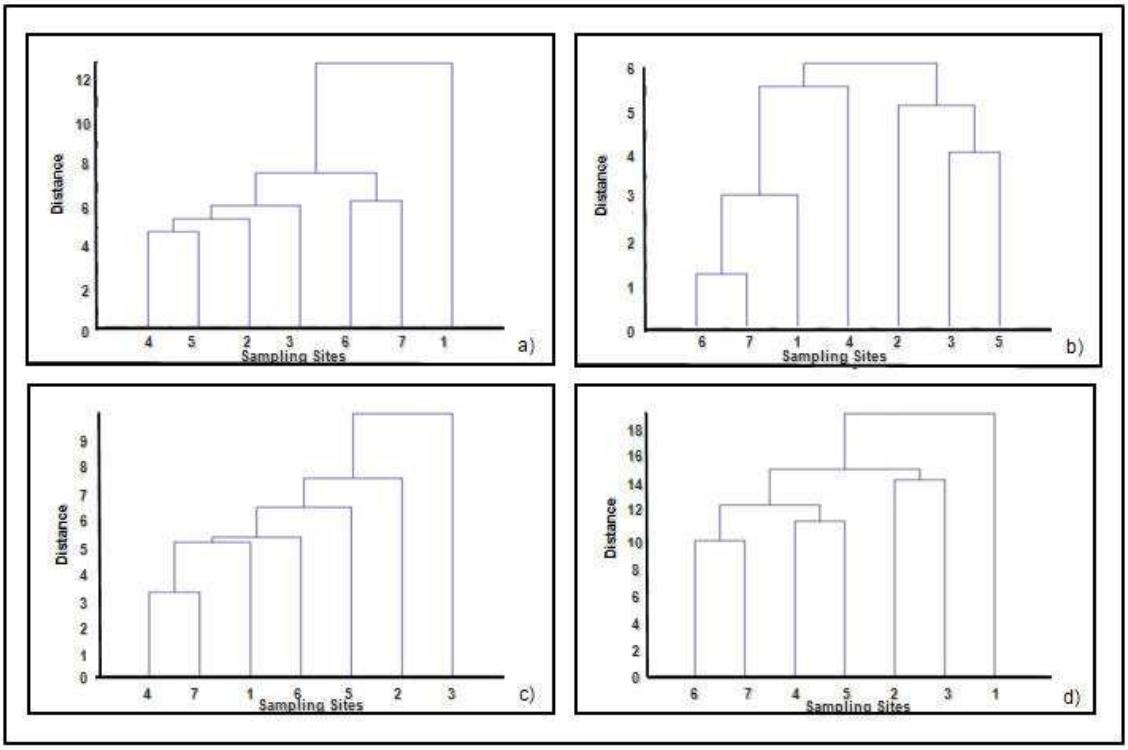

Distance
Sampling Sites
a)
4 5 2 3 6 7 1
b)
6 7 1 4 2 3 5
c)
4 7 1 6 5 2 3
d)
6 7 4 5 2 3 1

Table 1 Information about the selected sampling sites

Sampling sites	Coordinates [a]	Distance to river mouth (km)	Observations
1	41º15'41.11'' N 8º28'43.14'' W	36.5	Downstream a wine cellar; mainly rural.
2	41º12'8.41'' N 8º35'47.02'' W	20.5	Downstream the discharge of a WWTP (urban effluents)
3	41º13'5.16'' N 8º37'27.02'' W	15.5	Strongly industrialized area (Figure 1)
4	41º14'9.62'' N 8º38'49.27'' W	10.5	Downstream the discharge of a WWTP (urban effluents)
5	41º13'4.10'' N 8º38'47.68'' W	7.5	Strongly industrialized area (Figure 1)
6	41º12'54.92'' N 8º40'2.76'' W	4.5	Revitalized area with a recreational park
7	41º11'55.28'' N 8º40'52.23'' W	1	River mouth

[a] WGS 84 Geographical Coordinates

Table 2 Rating parameters for surface water quality, adapted from SNIRH (2011)

Parameter	Class	A (excellent)	B (good)	C (reasonable)	D (bad)	E (very bad)
pH [b]		6.5 – 8.5	-	6.0 – 9.0	5.5 – 9.5	-
Temperature	**(ºC)**	≤ 20	21 – 25	26 – 28	29 – 30	> 30
Conductivity	**(µS/cm, 20ºC)**	≤ 750	751 – 1 000	1 001 – 1 500	1 501 – 3 000	> 3 000
Dissolved Oxygen	**(%)**	≥ 90	89 – 70	69 – 50	49 – 30	< 30
BOD	**(mg O_2/L)**	≤ 3.0	3.1 – 5.0	5.1 – 8.0	8.1 – 20.0	> 20.0
COD	**(mg O_2/L)**	≤ 10.0	10.1 – 20.0	20.1 – 40.0	40.1 – 80.0	> 80.0
Total Coliforms	**(/100 mL)**	≤ 50	51 – 5 000	5 001 – 50 000	> 50 000	-
Fecal Coliforms	**(/100 mL)**	≤ 20	21 – 2 000	2 001 – 20 000	> 20 000	-
Fecal Streptococcus	**(/100 mL)**	≤ 20	21 – 2 000	2 001 – 20 000	> 20 000	-
Iron	**(mg/L)**	≤ 0.50	0.51 – 1.00	1.01 – 1.50	1.50 – 2.00	> 2.00
Manganese	**(mg/L)**	≤ 0.10	0.11 – 0.25	0.26 – 0.50	0.51 – 1.00	> 1.00
Zinc	**(mg/L)**	≤ 0.30	0.31 – 1.00	1.10 – 5.00	-	> 5.00
Copper	**(mg/L)**	≤ 0.020	0.021 – 0.05	0.051 – 1.00	-	> 1.00
Chromium	**(mg/L)**	≤ 0.05	-	-	-	>0.05
Selenium	**(mg/L)**	≤ 0.01	-	-	-	> 0.01
Cadmium	**(mg/L)**	≤ 0.0010	-	0.0011 – 0.0050	-	>0.0050
Lead	**(mg/L)**	≤ 0.050	-	0.051 – 0.100	-	>0.100
Mercury	**(mg/L)**	≤ 0.00050	-	0.00051 – 0.001	-	>0.001
Arsenic	**(mg/L)**	≤ 0.010	0.011 – 0.050	-	0.051 – 0.100	>0.100

[b]First verify if the value meets criteria A, then C and then D

Table 3 Physicochemical characterization of Leça river water samples (Gomes 2007)

Sampling Sites	Month	Physicochemical Parameters											
		Temp (ºC)	pH	ORP (mV)	Cond (µS/cm)	DO (mg/L)	DOC (mg/L)	BOD (mgO_2/L)	Turb (NTU)	Color (Pt-Co)	Total N (mgN/L)	Total P (mgP/L)	Hardness ($mgCaCO_3/L$)
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

---: not measured

Table 4 *V. fischeri* and *C. vulgaris* ecotoxicological results of Leça river water samples

Sampling sites	Month	*Vibrio fischeri*				*Chlorella vulgaris*	
		$TU_{50} = 100/EC_{50}$		$TU_{50} = 100/EC_{50}$		$TU_{20} = 100/EC_{20}$	$TU_{50} = 100/EC_{50}$
		5 min	15 min	5 min	15 min		
1	February	1	1 [c]	1.2	1[c]	1.9	1
	May	1	1	1	1	1 [c]	1 [c]
	June	1	1	1	1	1.1	1
	August	1 [c]	1 [c]	1 [c]	1 [c]	1 [c]	1 [c]
	September	1	1 [c]	2.7	1 [c]	27.8	10.3
2	February	1	1	1.3	2.0	2.6	1
	May	1.7	1.5	18.2	23.3	3.3	1.9
	June	1 [c]	1 [c]	1 [c]	1 [c]	1.2 [d]	1.6 [d]
	August	1 [c]	1 [c]	1 [c]	1 [c]	1 [c]	1 [c]
	September	6.2	5.3	10.2	8.1	27.8	11.1
3	February	6.3	5.7	18.2	16.9	29.4	11.9
	May	1	1.2	3.5	11.6	3.1	1.8
	June	1	1	4.1	5.9	1.2 [d]	3.7 [d]
	August	4.0	4.5	7.5	8.3	28.6	11.3
	September	5.8	6.2	10.8	10.0	22.9	8.8
4	February	6.3	5.7	18.2	15.9	16.7	2.4
	May	1	1	76.9	8.3	1 [c]	1 [c]
	June	1 [c]	1 [c]	1 [c]	1 [c]	1 [c]	1 [c]
	August	1 [c]	1 [c]	1 [c]	1 [c]	3.1	1
	September	1	1 [c]	1	1 [c]	1 [c]	1 [c]
5	February	1.4	2.1	4.7	4.3	3.3	1.2
	May	1	1	3.8	4.0	35.7	3.5
	June	1 [c]	1 [c]	1 [c]	1 [c]	1 [c]	1 [c]
	August	1 [c]	1 [c]	1 [c]	1 [c]	2.3	1.6
	September	3.4	3.4	6.9	6.5	1 [c]	1 [c]
6	February	1.5	1.7	4.7	4.7	2.5	1
	May	1	1	3.8	4.0	1 [c]	1 [c]
	June	1 [c]	1 [c]	1 [c]	1 [c]	1.2 [d]	2.5 [d]
	August	3.1	3.2	6.5	5.5	1 [c]	1 [c]
	September	1 [c]	1 [c]	1 [c]	1 [c]	1 [c]	1 [c]
7	February	6.3	1 [c]	18.2	1 [c]	13.9	1
	May	1	1	1.3	3.1	1 [c]	1 [c]
	June	1 [c]	1 [c]	1 [c]	1 [c]	1 [d]	1.6 [d]
	August	1	1	1.2	1.2	1 [d]	1.6 [d]
	September	1 [c]	1 [c]	1 [c]	1 [c]	1 [d]	1.6 [d]

[c] biostimulation for all concentrations tested
[d] toxicity decreased with the increase of tested concentration