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European ring exercise on water toxicity using different bioluminescence inhibition tests based on *Vibrio fischeri*, in support to the implementation of the water framework directive

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Abstract

An inter-laboratory comparison exercise was conducted under the European Union funded project entitled: Screening Methods for Water Data Information in Support of the Implementation of the Water Framework Directive (SWIFT-WFD) and coordinated by the Consejo Superior de Investigaciones Científicas (CSIC), in order to evaluate the reproducibility of different toxicity tests based on the bioluminescence inhibition of *Vibrio fischeri*, for the rapid water toxicity assessment.

For the first time, this type of exercise has been organized in Europe, and using different tests based on the same principle. In this exercise, 10 laboratories from 8 countries (Austria, Cyprus, Germany, Greece, Italy, Portugal, Romania, and Spain) took place, and a total number of 360 samples were distributed.

During the exercise, six series of six samples were analyzed along 5 months. Every batch of samples was composed by three real samples and three standard solutions. The real samples were: a raw influent and the effluent of a wastewater treatment plant (WWTP), and a sample from a first settlement of the WWTP spiked with a mixture of toxicant standards.

A final number of 330 (91.7%) samples was analyzed, 3300 values in duplicate were collected, and the results for each sample were expressed as the 50% effective concentration (EC_{50}) values calculated through five points of dilution inhibition curves, after 5 and 15 min of incubation times.

A statistical study was initiated using 660 results. The mean values, standard deviations (σ), variances (σ^2), and upper and lower warning limits (UWL and LWL) were obtained, using the EC_{50} values calculated with the result from the participating laboratories.

The main objectives of this toxicity ring study were to evaluate the repeatability (r) and reproducibility (R) when different laboratories conduct the test, the influence of complex matrix samples, the variability between different tests based on the same principle, and to determine the rate at which participating laboratories successfully completed tests initiated.

In this exercise, the 3.93% toxicity values were outliers according with the Z-score values and the Dixon test. The samples with the greater number of outliers were those with the smallest variability coefficient, corresponding to the greater and the smaller toxicity level.

No relation was found through the cluster analysis, between the final results and the different commercial devices involved. Testing by multiple commercial devices did not appear to reduce the precision of the results, and the variability coefficient for the exercise was nearby to the average value for past editions carried out at national level, where the different participants used the same commercial device.

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Stability of samples was also followed during the exercise. While statistical significance differences were not found for the greater part of samples, for the sample from the WWTP influent, a significant decrease of the toxicity value was found along this study. Nevertheless, this was a type of sample with a high toxicity level during all the exercise.

On the other hand, in order to obtain the chemical characterization of real samples, those were analyzed by chromatographic techniques, using different sequential solid phase extraction (SSPE) procedures, followed by liquid chromatography coupled with mass spectrometry (LC–MS), and gas chromatography–mass spectrometry (GC–MS). Good agreement was found between the chemical analysis results and the toxicity level of the samples.

Keywords: Bioluminescence; Toxicity; Inter-laboratory; *Vibrio fischeri*

1. Introduction

Rapid biomonitoring tests have played a major role in aquatic hazard and risk assessments, especially at a screening level of evaluation. A number of alternative tests have been proposed and applied for the rapid screening of toxicity [1,2], genotoxicity [3,4], and estrogenicity [5]. These rapid screening tests are not intended to replace standard methods, but they are a useful tool in situations where their rapidity and relative low cost make it practical to screen large numbers of samples for preliminary indications.

In this sense, rapid biomonitoring tests can be an essential tool for the successful implementation of Water Framework Directive (WFD) [6], because its implementation requires measurement techniques, which will be able to deliver reliable data at an affordable cost.

Due to the presence of numerous pollutants originated from industrial wastewaters, wastewater treatment plant effluents and runoff from agricultural fields, a practical assessment of the chemical and ecological status of river basin should be based on the measurement of biological effects in combination with chemical analysis of target compounds.

The quantitative analysis of all the components of a complex sample is an utopia today. Due to this, one of the main advantages obtained from global biological measurements is the information about effects produced by the cocktail of pollutants which total composition is unknown, including synergistic and antagonistic effects between the different compounds.

After three decades of history, bioluminescence inhibition tests for toxicity assessment based on *Vibrio fischeri* [7–9] have a high level of acceptance. *V. fischeri* is a standardized toxicity test organism [10], and different commercial devices, preserved bacterial reagents, and formats of performance are available in the market.

Nevertheless, in spite of being a standard test, sources of variability are introduced when different laboratories carry out the measurements, namely the transport, small differences on bacterial reagent preservation, times of bacterial reagent reconstitution, sample preservation protocols, and sample manipulation. On the other hand, additional sources of variability may be introduced when the toxicity measurements are carried out using different commercial tests, and also the variability of results can be enhanced according to the complexity of matrix samples.

Since several years we have been working in the toxicity evaluation of wastewater and surface waters by different toxicity assays based on *V. fischeri* [11], or using the CellSense system, an electrochemical biosensor with different bacteria such as *Pseudomonas putida* [12], or *Escherechia coli* [13]. Recently, our group [14] coordinated an inter-laboratory study in Spain using *V. fischeri*, and it showed promising results of this toxicity assays to be used as a fast screening method for toxicity with comparable results among the different participating laboratories. The present work presents the results obtained during a ring exercise carried out under the frame of the SWIFT-WFD project, conducted among 10 laboratories, from 8 countries, using 5 different types of commercial devices.

This inter-laboratory comparison exercise was composed by six series of six samples analyzed along 5 months. Every batch of samples was composed by three standard solutions and three real samples, one of them fortified with phenol and Zn-sulphate.

This ring exercise aims:

- To evaluate the variability of results between different laboratories.
- To evaluate the rate at which participating laboratories successfully completed the exercise.
- To evaluate the capacity and variability in front of complex real samples.
- To evaluate the influence of different commercial tests based on the same principle.
- To compare the toxicity evaluation of real samples and the chemical analysis results obtained by chromatographic procedures.

2. Experimental

2.1. Toxicity tests devices

The inter-laboratory exercise involved different toxicity tests based on the bioluminescence inhibition of *V. fischeri* measurement. In Table 1 are summarized the commercial devices used and the number of laboratories that use each one.

2.2. Reagents

V. fischeri bacterial reagents were purchased by each participant from different commercial suppliers according to the commercial device used.

Phenol, 2-chlorophenol, 3-chlorophenol, 2,4-dichlorophenol, 3,5-dichlorophenol, nitrophenol, 1,3,5-trichlorophenol,

Table 1
Commercial devices used for the toxicity ring test

Commercial device	No. of participants
Microtox M500	4
ToxAlert	3
BioFix®Lumi	1
ToxTracer	1
LCK 480	1

pentachlorophenol, and zinc-sulphate were purchased from Merck (Darmstadt, Germany). The HPLC-grade solvents acetonitrile, methanol, and water were purchased from Merck.

The standards of alcohol ethoxylates (CnEOx) were individual pure C10 through C16 ethoxylates with an even number of carbon atoms and an average of four ethoxy units.

High purity standard (98% pure) of 4-*tert*-octylphenol (OP) and 4-nonylphenol (NP) were obtained from Aldrich (Milwaukee, USA).

Commercial linear alkyl benzene sulphonates (LAS) with a low dialkyltetralinsulphonates (DATS) content (<0.5%) were supplied by Petroquimica Española S.A. in a single standard mixture with the proportional composition of the different homologues as follows: C10, 3.9%; C11, 37.4%; C12, 35.4% and C13, 23.1%.

Pesticides such as aldrine, dieldrin, α- and β-endosulfan, endrin, isodrine dichlorodiphenyltrichloroethanes (2,4-DDT and 4,4-DDT), dichlorodiphenyldichloroethylenes (2,4-DDE and 4,4-DDE), and dichlorodiphenyldichloroethanes (2,4-DDD and 2,4-DDD), hexachlorocyclohexanes (α-, β-, γ-, and δ-HCH) and polyaromatic hydrocarbons (PAHs) were standards were of high purity (99.9%) and were purchased from Promochem (Wesel, Germany).

The reference substances in form of the free acids or their sodium salts were obtained form different suppliers (Aldrich, Fluka and Sigma).

2.3. Experimental design, sample collection and handling

Ten laboratories participated in the ring exercise.

Six series of samples were analyzed along 5 months and every batch was composed by three real samples and three standard solutions. Table 2 indicates the distribution dates, and deadlines for reporting the results of batch and samples with indication of codification (A–F) for every series.

The selection of real samples was according with their expected toxicity and matrix complexity. Real samples were from a WWTP located in an industrialized area near Barcelona (Spain): from the raw influent, the first settlement and the final effluent. The sample from the first settlement was fortified in order to obtain a sample with a medium level of toxicity, stable along the exercise and with a very complex matrix. The raw effluent was a very complex matrix with a high toxic content, and the final effluent from the WWTP was selected as a real non-toxic sample, but with a complex matrix.

Samples were collected in Pyrex borosilicate glass containers. Each bottle was rinsed with tap water and high-purity water prior to the sample addition. The samples were transported to the laboratory at 4 °C.

All water samples were filtered through 0.7 and 0.45 μm glass micro-fiber filters to remove suspended matter and they were homogenized in a polyethylene bucked.

On the other hand, three toxicant standard solutions were also distributed in each batch: phenol (60 mg/L), 3,5-dichlorophenol (50 mg/L) and zinc-sulphate (50 mg/L) solutions.

In order to minimize sources of variation not associated with the participant laboratories, wastewater collection, samples processing (centrifugation when necessary, filtration, ...), as well, the three toxicant standard solutions preparation were conducted by a central source, all samples were distributed in polyethylene containers at same time and, all tests were conducted on the same dates.

The preservation of water samples since the distribution till the end of the exercise was accomplished in each participant laboratory, and they were requested to store the samples at −20 °C.

2.4. Solid phase extractions and chromatographic analysis

Different chromatographic approaches were applied in order to identify the main sources of toxicity due to the organic pollutants in the real samples.

The samples from the first settlement were analyzed prior to the fortification with Zn-sulphate and phenol.

Solid phase extraction (SPE) procedures, as well the analytical methods by gas chromatography coupled to mass spectrometry (GC–MS) and liquid chromatography coupled to mass spectrometry (LC–MS), were according to the methodologies

Table 2
Distribution codes and analysis dates

	Dates	A	B	C	D	E	F
Batch 1	24 March	3,5-DCP	Phenol	Zn-sulphate	Influent	Effluent	Fortified
Batch 2	26 April	3,5-DCP	Phenol	Zn-sulphate	Influent	Effluent	Fortified
Batch 3	24 May	Phenol	Influent	Fortified	Zn-sulphate	Effluent	3,5-DCP
Batch 4	28 June	Effluent	Fortified	Zn-sulphate	3,5-DCP	Influent	Phenol
Batch 5	26 July	Influent	Fortified	Effluent	Zn-sulphate	Phenol	3,5-DCP
Batch 6	01 September	Fortified	Effluent	Influent	Phenol	3,5-DCP	Zn-sulphate

Where 3,5-DCP means a standard solution of 50 mg/L of 3,5-dichlorophenol, phenol means a standard solution of 60 mg/L of phenol, Zn-sulphate means a standard solution of 50 mg/L of Zn-Sulphate, influent means the raw influent of a WWTP, effluent means the final effluent of a WWTP, fortified means a sample from the first settlement of a WWTP fortified with phenol and Zn-sulphate.

described by Lacorte et al. [15] and Petrovic and Barceló [16], respectively.

Very briefly, the extraction procedures for the posterior GC–MS analysis were based on the use of Oasis disposable cartridges from Waters. Two hundred milliliters water sample was filtered through glass fiber filters of 0.7 and 0.45 mm (Whatman, England). SPE-cartridges were solvated with 10 mL of dichloromethane and 10 mL of acetonitrile at flow rate of 5 mL/min. Five milliliters of HPLC water were percolated at a flow rate of 5 mL/min. Then 200 mL of samples were loaded at a flow rate of 10 mL/min. In order to elute the main interferences, SPE cartridges were rinsed with 2 mL of HPLC water, and finally, analytes were eluted using 5 mL acetonitrile: dichloromethane (1:1) and 5 mL dichloromethane. The extracts were analyzed by gas chromatography coupled to mass spectrometry with electron impact ionization in selected ion monitoring (SIM) for tentative identification of target compounds, according to the protocol described by Lacorte et al. [15].

On the other hand, in order to identify and quantify anionic and nonionic surfactants and their degradation products an SPE-LC–MS were applied [13].

Target analytes were extracted using a two-step SPE procedure, using SPE cartridges packed with 500 mg of C18 sorbent, from Merck. Cartridges were conditioned by passing 7 mL methanol and 3 mL of HPLC water. Two hundred milliliters of each sample were loaded, and trapped compounds were desorbed using a two-step elution procedure. The eluants used in order to obtain each fraction were: fraction A hexane/dichloromethane (1:4, v/v) and fraction B methanol/dichloromethane (9:1, v/v). In fraction A were separated phtalate, ethoxilated nonylphenol (NPEOs), alcoholetoxilates (CnEOx), nonylphenol (NP) and octylphenol (OP), whereas in fraction B were collected the linear alkyl benzene sulphonates (LAS). The extracts were evaporated to dryness with a gentle stream of nitrogen and reconstituted with methanol to a final volume of 1 mL.

The extracts were analyzed using atmospheric pressure chemical ionization (APCI) interface in positive mode in order to determine less polar compounds and using the electrospray interface in negative ionization mode for the analysis of more polar compounds, such as NP and OP.

2.5. Toxicity test procedures

The concentrations tested for each sample were: 45.45; 22.73; 11.36; 5.68 and 2.84%.

Bacterial reagents were reconstituted just prior to the analysis, and the pre-incubations times were according to the device protocols.

In all measures, the percent of inhibition (%*I*) was determined by comparing the response given by a saline control solution to that corresponding to the diluted sample. After that, an inhibition curve was fitted and the 50% effective concentration (EC_{50}) was calculated.

Each laboratory that took part in the inter-laboratory exercise was requested to make all measurements in duplicate.

2.6. Statistical parameters

For each series, the mean value (X), the standard deviation (σ), variance (σ^2), upper warning limit value (UWL), lower warning limit value (LWL) number of outlier values, repeatability (r), reproducibility (R) and coefficient of variation (%CV) were calculated for the six samples.

The upper warning limits were calculated as: UWL = $(X + 2\sigma)$ and the lower warning limits as: LWL = $(X - 2\sigma)$.

As an acceptance criteria for each result were used the Z-score function according with the "Laboratory Accreditation and Audit Protocol: Food Inspection Directorate" [17,18], following the AOAC and ISO and IUPAC directives.

The Z-values were calculated according:

$$Z = \frac{X_{\text{lab}} - X}{\sigma}$$

where X_{lab} is the result for a laboratory; X the mean values between all laboratories; and σ is the standard deviation in the correspondent population (accounting the results obtained for the different laboratories in this series in front of this sample).

The results whose Z-value was over 3 was directly excluded and when the Z-score value was between 2 and 3 was applied the Dixon test with a 5% of significance level.

In order to follow the stability of samples along the test, it was necessary to study if mean values between series were significant different and it was applied the analysis of variance (ANOVA) to the results. First of all, it was necessary to prove if the groups of data followed Gaussian distributions and if the differences between standard deviations of the groups were significant to select a parametric or non-parametric procedure. The normality test applied was the Kolmogorov and Smirnov method and the Bartlett's test was applied to establish if the differences among the standard deviations were or not significant.

Finally, it was selected a parametric test, the one-way ANOVA.

The measurement of precision of each laboratory to repeat the measurements on a sample at different intervals: reproducibility (R) was calculated as:

$$R = \frac{\sum r_{\text{lab}}}{N}$$

where $r_{\text{lab}} = \sum(2 \times 2^{1/2})\sigma_{\text{lab}}$, N the number of samples (only results for stable samples were accounted) and σ_{lab} is the standard deviation between results from the same laboratory on a stable sample at different intervals.

The hieratichical cluster analysis was based on the similitude matrixes calculated using the squared Euclidean distances and the clustering method was the average linkage method between the different laboratories.

3. Results

Three humdred and sixty samples were distributed among 10 participants. A final number of 330 (91.7%) were analyzed. The results for each sample were expressed as EC_{50} after 5 min and after 15 min of exposure time, calculated through five points

of dilution inhibition curves, working on duplicate, that means that, 660 values were initially included for the statistical study.

Non-convergent values corresponding to non-toxic values were fixed as value 150.

The mean values, standard deviations (σ), variances (σ^2), and upper and lower warning limits (UWL and LWL) were calculated from the EC_{50} results previously calculated from the inhibition curves fitted with the results obtained for the participants at the different intervals of time.

Twenty-six results (3.9% of accounted values) were outliers according with the *Z*-score values and the Dixon test. The first step was to establish the upper and lower warning limit and *Z*-scores (see Table 4). The values out of these limits are outliers, whose *Z*-score values were over 3 S, the results whose *Z*-scores values were between 2 and 3 were considered questionable good results and then were applied the Dixon test formulas in order to identify outlier values. In Fig. 1, the *Z*-score graphs for the most toxic sample corresponding to the 3,5-dichlorophenol dilution are presented. On Table 3, the EC_{50} values excluding outliers, and the corrected statistical values (after outlier exclusion) are shown, as well the maximum and minimum values, total number of results and number of outliers for every series are presented (Table 4).

The samples with the maximum number of outliers were the most and the least toxic samples. Still the dispersion values for these samples were very low.

Three toxicant standard solutions were used: phenol (60 mg/mL) is an acute toxic organic substance (phenol exhibits toxicity after 5 min of exposure). It is a commonly used toxicity standard due to its stability. 3,5-dichlorophenol (50 mg/L) is as well an organic substance commonly used as toxicity standard with high toxic level and low dispersion values. Results for these samples were as expected, low dispersion and high toxicity.

The Zn-sulphate (50 mg/mL) is a heavy metal solution and high differences were noticed after different exposures time, whereas after 5 min almost no decreases on bioluminescence was produced, but contrary to that, after 15 min dramatic decreases was noticed. On the other hand, the toxicity of this substance is highly influenced by matrix effects, conditions and concentration. The results obtained were according with these facts, and the dispersions were higher than the values obtained for the other standard solutions.

In order to establish the stability of samples along this intercomparison test, differences between variances obtained for every type of sample at different intervals were evaluated.

Due to the results from the Kolmogorov–Smirnov normality test applied, the different group of results for every sample, in the six batch can be considered, and that its always followed a Gaussian distribution.

A parametric test (one-way ANOVA) with Turkey as a post-test was applied in order to evaluate the differences between the mean values of the EC_{50} obtained for the participants along the inter-laboratory exercise. Only in one case, corresponding to the sample from the WWTP influent, the *P*-value was 0.0487, considered significant, and the variation among the exercise should be considered greater than expected by chance. Table 5 summarized the ANOVA results for this sample, and Fig. 2 the variation of the mean value of EC_{50} along the exercise. Comparatively, in Fig. 3 is presented the mean value of the EC_{50} for the phenol standard solution sample along the exercise, which ANOVA results showed that it was stable. Nevertheless, this sample maintained a high level of toxicity during all the exercise. These results agree

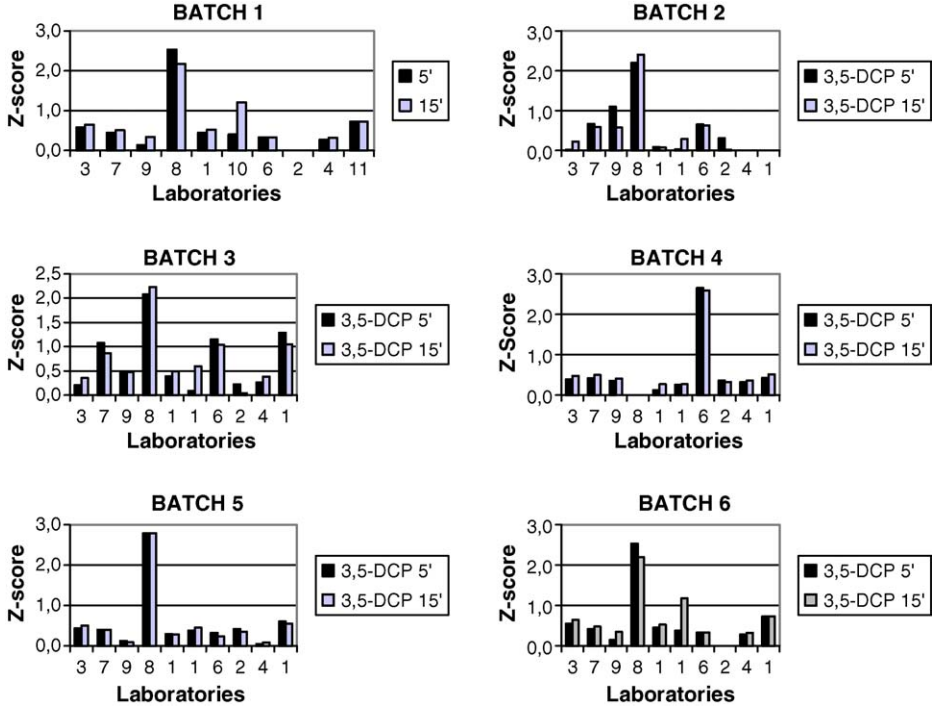

Fig. 1. Absolute values of *Z*-score for the sample of 3,5-dichlorophenol solution, obtained for the participating laboratories along the exercise.

Table 3
Mean values of the EC_{50} (% of dilution) obtained for each sample of each series by the participating laboratories after exclusion of outliers

	EC_{50}																			
	Lab 3		Lab 7		Lab 9		Lab 8		Lab 1		Lab 10		Lab 6		Lab 2		Lab 4		Lab 11	
	5′	15′	5′	15′	5′	15′	5′	15′	5′	15′	5′	15′	5′	15′	5′	15′	5′	15′	5′	15’
3,5-DCP																				
Batch 1	7.01	6.33	8.06	7.42	10.54	8.67		27.84	8.08	7.28	14.76	20.46	8.99	8.76			9.40	8.84	5.82	5.73
Batch 2	9.01	8.09	7.71	7.13	6.90	7.17			8.82	8.48	8.92	7.93	7.74	7.05	9.56	8.61				
Batch 3	8.17	7.26	6.77	6.35	7.73	7.05	11.83		9.12	8.76	8.36	6.83	10.35	9.72	8.15	7.81	8.08	7.21	6.44	6.03
Batch 4	7.64	6.73	6.92	6.48	8.56	7.47			14.25	14.66	10.92	8.96			8.37	8.37	9.11	7.95	6.49	6.35
Batch 5	7.57	6.54	7.87	7.33	12.17	11.24			8.78	8.25	8.02	6.95	8.48	8.64	7.75	7.71	10.73	9.86	6.24	6.20
Batch 6	7.25	6.34	8.33	7.62	10.48	8.62		27.91	8.03	7.26	14.57	20.17	8.99	8.73			9.34	8.77	5.82	5.72
Phenol																				
Batch 1	13.03	13.45	13.57	13.71	63.28	63.18	59.76	64.98	13.58	15.50	20.23	21.80	28.68	23.77			25.65	23.43	11.14	12.00
Batch 2	13.34	14.94	10.56	12.11	28.12	35.62	14.28	14.96	23.32	29.44	20.44	21.84	21.54	21.82	11.30	12.98				
Batch 3	12.80	14.87	13.06	13.88	40.49		27.24	30.14	23.32	27.13	13.60	13.89	12.68	14.06	8.50	12.10	29.81	28.70	7.85	8.93
Batch 4	11.73	13.67	10.58	10.90					15.16	16.15	15.53	19.25	9.72	10.22	12.39	13.77	22.15	21.67	13.45	14.96
Batch 5	10.26	11.67	10.86	11.77	61.66	59.88	49.68	57.24	17.62	20.01		19.13	23.93	19.64	9.01	9.87	17.62	16.47	11.64	12.41
Batch 6	11.99	13.37	13.10	13.32	60.73	62.58	57.76	63.06	13.56	15.40	19.92	21.43	27.81	23.33			25.17	23.00	11.18	12.00
Zn-sulphate																				
Batch 1	51.97	7.42	22.87	4.92	150.00	150.00	150.00	124.00	55.86	11.70	1.73	2.77	53.45	15.76			150.00	150.00	148.00	73.34
Batch 2	34.30	5.70	25.03	1.99	47.33	21.84	15.75	17.00	150.00	150.00	150.00	67.20	150.00	150.00	28.50	2.17				
Batch 3	46.36	5.74	15.24	1.55	89.71	20.06	150.00	150.00	150.00	150.00	150.00	55.87	83.96	39.08	28.79	3.02	150.00	150.00	118.20	46.33
Batch 4	74.70	7.95	26.50	2.12	78.46	15.16			52.22	11.34	44.93	22.08	77.86	83.82	78.50	11.27		150.00	82.19	34.36
Batch 5	33.64	5.02	33.74	1.18	63.59	0.00	150.00	150.00	39.92	9.42	91.65	38.42	37.33	8.03	36.69	3.69	150.00	150.00	150.00	66.20
Batch 6	51.97	7.42	22.87	4.92	150.00	150.00	150.00	150.00	55.86	11.70	1.73	2.77	53.45	15.76			150.00	150.00	150.00	81.39
Influent																				
Batch 1	19.93	9.71	13.62	12.58	12.60	12.59	24.50	17.95	21.64	19.61	14.36	15.77	23.90	24.43			10.72	9.91	9.13	9.23
Batch 2	10.58	9.78	5.84	5.14		24.00	15.01	14.81	13.41	12.61	9.55	9.54	10.30	9.65	9.27	7.64				
Batch 3	8.38	7.68	9.03	7.30	16.22	15.21	11.64	11.95	13.44	13.84	9.89	9.28	7.69	7.90	9.91	8.74	13.22	11.49	10.31	9.79
Batch 4	9.65	8.25	18.43	15.26	17.04	13.98			15.34	12.09	11.64	10.71			10.51	9.97	11.08	10.37	10.60	10.42
Batch 5	8.23	8.21	11.79	10.64	17.16	19.14	22.28	22.91	12.98	13.72	10.51	10.27	13.92	13.67	11.32	10.89	11.03	8.43	10.09	11.03
Batch 6	19.93	9.71	13.62	12.58	12.60	12.59	24.50	17.95	21.64	19.61	14.36	15.77	23.90	24.43			10.72	9.91	9.13	9.23
Dec-Dop																				
Batch 1	25.61	16.74	11.69	12.71	41.09	35.56	90.66	75.79	51.22	50.52	31.29	31.75	81.17	75.77			37.36	35.09	85.66	62.38
Batch 2	56.43	64.93	30.24	26.31	150.00	150.00	16.71	17.76	150.00	150.00	27.67	31.25	55.83	57.76	48.36	89.71				
Batch 3	53.59	25.72	30.75	33.03			38.42	15.87	50.12	54.34	29.25	24.12	77.86	83.81	32.29	35.50	50.82	47.42	46.87	50.37
Batch 4	43.20	41.62	34.15	28.32	93.98	58.75			75.58	76.36	26.86	36.18	7.88	7.10	44.37	43.35	45.12	39.76	97.98	94.43
Batch 5	41.74	47.79	28.30	23.95	150.00	150.00	102.00	80.55	67.89	67.74	28.28	29.59	150.00	150.00	46.18	54.86	45.05	41.20	52.21	37.58
Batch 6	25.61	16.74	11.69	12.71	41.09	35.56	90.66	75.79	51.22	50.52	31.29	31.75	81.17	75.77			37.36	35.09	85.66	62.38
Effluent																				
Batch 1	150.00	150.00	150.00	150.00			150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00			150.00	150.00	150.00	150.00
Batch 2	150.00	150.00			150.00	150.00			150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00				
Batch 3	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	102.70	150.00	150.00	150.00	150.00	150.00
Batch 4	150.00	150.00	150.00	150.00	150.00	150.00			150.00	150.00	150.00	150.00			150.00	150.00	150.00	150.00	150.00	150.00
Batch 5	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	143.90	150.00	150.00	150.00	150.00	150.00
Batch 6	150.00	150.00	150.00	150.00			150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00			150.00	150.00	150.00	150.00

Table 4
Corrected statistical values (after outlier exclusion) for each type of sample along the exercise

	Mean		S.D.		Var		Max		Min		UWL		LWL		No. outlier		*N*	Total outliers	Outliers		Mean		S.D.		Var	
	5′	15′	5′	15′	5′	15′	5′	15′	5′	15′	5′	15′	5′	15′	5′	15′			5′	15′	5′	15′	5′	15′	5′	15′
3,5-DCP																										
Batch 1	9.1	11.3	2.7	7.6	7.4	58.0	14.80	27.80	5.80	5.70	36.9	113.7	−22.2	2.3	1	0	18.0	1	5	3	8.8	9.1	2.0	3.7	4.5	21.5
Batch 2	8.4	7.8	0.9	0.7	0.9	0.4	9.60	8.60	6.90	7.10	12.8	13.9	5.2	3.5	1	1	18.0	2								
Batch 3	8.5	7.9	1.6	1.8	2.6	3.1	11.80	11.80	6.40	6.00	11.7	11.4	5.3	4.3	0	0	18.0	0								
Batch 4	9.0	8.4	2.5	2.7	6.3	7.3	14.30	14.70	6.50	6.40	67.5	32.9	−32.8	−9.3	1	1	18.0	2								
Batch 5	8.6	8.1	1.8	1.6	3.2	2.7	12.20	11.20	6.20	6.20	27.4	26.2	−5.1	−5.2	1	1	18.0	2								
Batch 6	9.1	11.2	2.6	7.6	6.8	57.5	14.60	27.90	5.80	5.70	36.0	113.4	−22.3	1.7	1	0	18.0	1								
Phenol																										
Batch 1	27.7	28.0	20.1	20.9	405.4	438.8	63.30	65.00	11.10	12.00	532.0	568.8	278.8	308.9	0	0	16.0	0	2	2	21.5	22.2	13.3	13.4	218.8	226.6
Batch 2	17.9	20.5	6.4	8.5	40.7	71.5	28.10	35.60	10.60	12.10	30.6	37.4	5.1	3.6	0	0	16.0	0								
Batch 3	18.9	18.2	10.8	8.1	115.8	65.1	40.50	30.10	7.90	8.90	40.5	44.5	−2.6	−2.5	0	1	16.0	1								
Batch 4	13.8	15.1	3.9	3.9	15.4	15.3	22.20	21.70	9.70	10.20	53.9	60.8	−15.0	−17.8	1	1	16.0	2								
Batch 5	23.6	23.8	19.0	18.7	362.1	349.2	61.70	59.90	9.00	9.90	113.5	61.2	−44.2	−13.6	1	0	16.0	1								
Batch 6	26.8	27.5	19.3	20.5	373.4	419.6	60.70	63.10	11.20	12.00	494.8	545.7	251.9	293.5	0	0	16.0	0								
Zn-sulphate																										
Batch 1	87.1	60.0	61.6	65.0	3791.7	4229.7	150.00	150.00	1.70	2.80	4091.7	4529.7	3491.7	3929.7	0	0	20.0	0	1	0	81.8	53.1	52.1	61.7	2928.3	3845.0
Batch 2	75.1	52.0	62.6	64.0	3923.8	4100.2	150.00	150.00	15.80	2.00	200.4	180.1	−50.2	−76.1	0	0	20.0	0								
Batch 3	98.2	62.2	53.5	63.3	2866.1	4010.8	150.00	150.00	15.20	1.60	205.3	188.8	−8.8	−64.5	0	0	20.0	0								
Batch 4	64.4	37.6	20.6	48.8	423.4	2380.7	82.20	150.00	26.50	2.10	142.8	135.2	5.1	−60.0	1	0	20.0	1								
Batch 5	78.7	43.2	52.4	60.0	2742.1	3599.2	150.00	150.00	33.60	0.00	183.4	163.2	−26.1	−76.8	0	0	20.0	0								
Batch 6	87.3	63.8	61.8	68.9	3822.6	4749.1	150.00	150.00	1.70	2.80	4122.6	5049.1	3522.6	4449.1	0	0	20.0	0								
Influent																										
Batch 1	16.7	14.6	5.8	5.2	34.0	27.1	24.50	24.40	9.10	9.20	83.0	76.0	−15.0	−21.7	0	0	18.0	0	2	1	13.5	12.6	4.1	4.3	18.6	20.4
Batch 2	10.6	11.6	3.0	5.8	8.8	33.4	15.00	24.00	5.80	5.10	23.3	23.2	1.2	0.1	1	0	18.0	1								
Batch 3	11.0	10.3	2.6	2.7	7.0	7.3	16.20	15.20	7.70	7.30	16.3	15.7	5.7	4.9	0	0	18.0	0								
Batch 4	13.0	11.4	3.4	2.3	11.4	5.2	18.40	15.30	9.70	8.30	119.8	119.3	−63.3	−65.7	1	1	18.0	2								
Batch 5	12.9	12.9	4.1	4.7	16.6	22.4	22.30	22.90	8.20	8.20	21.1	22.4	4.8	3.4	0	0	18.0	0								
Batch 6	16.7	14.6	5.8	5.2	34.0	27.1	24.50	24.40	9.10	9.20	83.0	76.0	−15.0	−21.7	0	0	18.0	0								
Dec-Dop																										
Batch 1	50.6	44.0	28.6	23.5	818.3	552.9	90.70	75.80	11.70	12.70	999.6	704.5	636.9	401.3	0	0	20.0	0	1	1	56.2	53.0	33.8	32.1	1302.4	1185.8
Batch 2	66.9	73.5	53.2	52.7	2827.0	2772.5	150.00	150.00	16.70	17.80	173.2	178.8	−39.4	−31.8	0	0	20.0	0								
Batch 3	45.6	41.1	15.3	20.6	233.8	422.9	77.90	83.80	29.30	15.90	111.8	125.4	−4.7	−23.1	1	1	20.0	2								
Batch 4	52.1	47.3	30.6	26.0	939.1	675.2	98.00	94.40	7.90	7.10	113.4	99.3	−9.2	−4.6	0	0	20.0	0								
Batch 5	71.2	68.3	46.7	46.2	2177.7	2138.3	150.00	150.00	28.30	24.00	164.5	160.8	−22.2	−24.2	0	0	20.0	0								
Batch 6	50.6	44.0	28.6	23.5	818.3	552.9	90.70	75.80	11.70	12.70	999.6	704.5	636.9	401.3	0	0	20.0	0								
Effluent																										
Batch 1							0.00	0.00	0.00	0.00					1	1	18.0	2	4	4	148.9	150.0	3.4	9.1	45.5	0.0
Batch 2	150.0	150.0	0.0	0.0	0.0	0.0	150.00	150.00	150.00	150.00	235.9	227.1	0.7	17.7	2	2	18.0	4								
Batch 3	145.3	150.0	15.0	0.0	223.7	0.0	150.00	150.00	102.70	150.00	175.2	150.0	115.4	150.0	0	0	18.0	0								
Batch 4	150.0	150.0	0.0	0.0	0.0	0.0	150.00	150.00	150.00	150.00	150.0	150.0	150.0	150.0	0	0	18.0	0								
Batch 5	149.4	150.0	1.9	45.7	3.7	0.0	150.00	150.00	143.90	150.00	153.2	150.0	145.5	150.0	0	0	18.0	0								
Batch 6	150.0	150.0	0.0	0.0	0.0	0.0	150.00	150.00	150.00	150.00	300	300	−300	−300	1	1	18.0	2								

S.D.: standard deviation; Var: variance; Max: maximum value obtained by the participants; Min: minimum value obtained by the participants; UWL: upper warning limit; LWL: lower warning limit; no outlier: number of outliers after 5 and 15 min of exposure; *N*: total number of collected values.

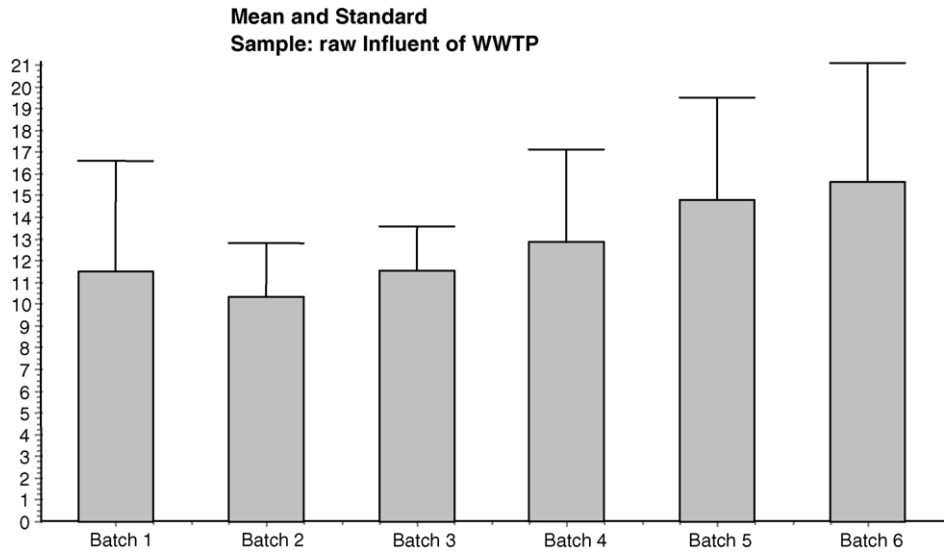

Fig. 2. Variation of mean value of EC_{50} for the influent of WWTP along the exercise. The slight increase of the mean EC_{50} value point out a decrease of toxicity.

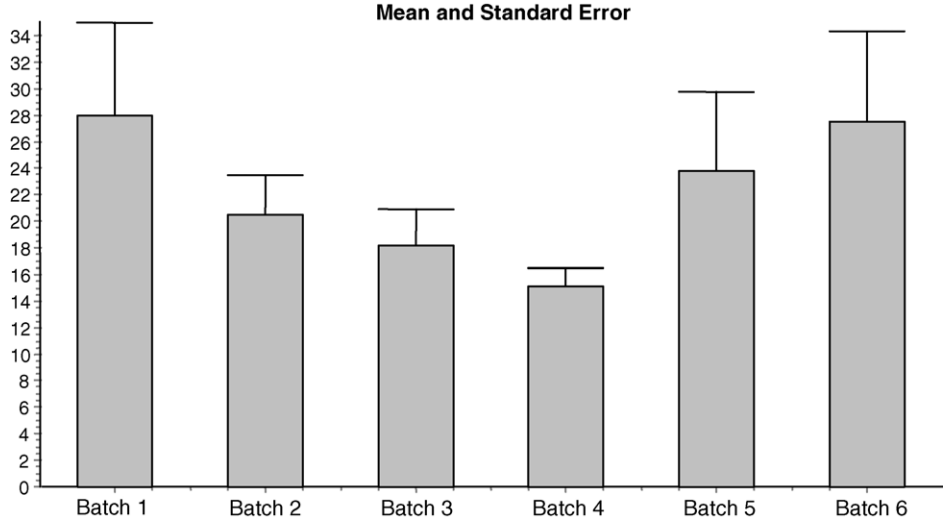

Fig. 3. Variation of mean value of EC_{50} for the phenol standard solution along the exercise.

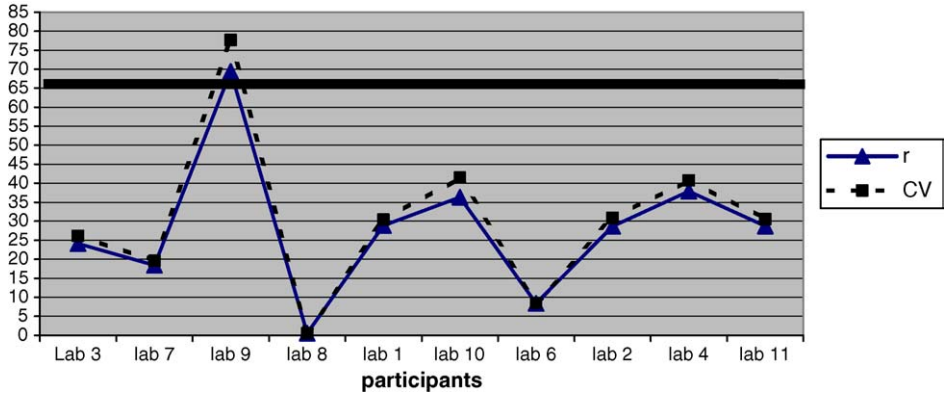

Fig. 4. Repeatability coefficients (r) and coefficients of variation (CV) intra-laboratories.

Table 5
One-way analysis of variance of the data from the WWTP influent

Comparison	Mean difference	q	P-value
Batch 1 vs. batch 2	1.164	0.9357	ns, >0.05
Batch 1 vs. batch 3	−0.008000	0.006159	ns, >0.05
Batch 1 vs. batch 4	−1.355	1.090	ns, >0.05
Batch 1 vs. batch 5	−3.288	2.591	ns, >0.05
Batch 1 vs. batch 6	−4.104	2.868	ns, >0.05
Batch 2 vs. batch 3	−1.172	0.9422	ns, >0.05
Batch 2 vs. batch 4	−2.519	2.125	ns, >0.05
Batch 2 vs. batch 5	−4.452	3.672	ns, >0.05
Batch 2 vs. batch 6	−5.268	3.814	ns, >0.05
Batch 3 vs. batch 4	−1.347	1.084	ns, >0.05
Batch 3 vs. batch 5	−3.280	2.585	ns, >0.05
Batch 3 vs. batch 6	−4.096	2.862	ns, >0.05
Batch 4 vs. batch 5	−1.933	1.594	ns, >0.05
Batch 4 vs. batch 6	−2.749	1.990	ns, >0.05
Batch 5 vs. batch 6	−0.8160	0.5811	ns, >0.05

Difference	Mean difference	95% Confidence interval	
		From	To
Batch 1 – batch 2	1.164	−4.031	6.358
Batch 1 – batch 3	−0.008000	−5.433	5.417
Batch 1 – batch 4	−1.355	−6.550	3.839
Batch 1 – batch 5	−3.288	−8.589	2.012
Batch 1 – batch 6	−4.104	−10.083	1.874
Batch 2 – batch 3	−1.172	−6.366	4.023
Batch 2 – batch 4	−2.519	−7.472	2.433
Batch 2 – batch 5	−4.452	−9.516	0.6117
Batch 2 – batch 6	−5.268	−11.038	0.5015
Batch 3 – batch 4	−1.347	−6.542	3.847
Batch 3 – batch 5	−3.280	−8.581	2.020
Batch 3 – batch 6	−4.096	−10.075	1.882
Batch 4 – batch 5	−1.933	−6.997	3.131
Batch 4 – batch 6	−2.749	−8.518	3.021
Batch 5 – batch 6	−0.8160	−6.681	5.049

Source of variation		Degrees of freedom	Sum of squares	Mean square
Intermediate calculations (ANOVA table)				
Treatments (between columns)		5	202.15	40.431
Residuals (within columns)		56	944.80	16.872
Total	61	1147.0		
$F = 2.396$ =(MStreatment/MSresidual)				

One-way analysis of variance (ANOVA), the P-value is 0.0487, considered significant. Variation among column means is significantly greater than expected by chance. Tukey–Kramer multiple comparisons test, if the value of q is greater than 4.177 then the P-value is less than 0.05.

to the fact that this was a sample with a very complex matrix, with high amounts of organic material.

The chromatographic results confirm that this was the more polluted real sample of the exercise, see the chromatographic characterization of real samples summarized in Table 6.

In Fig. 3, the repeatability coefficients (r) and coefficients of variation intra-laboratories are shown. Must be said that only one laboratory presented a superior variation coefficient and lower precision. The line corresponding to the warning limits for the

Table 6
Organic pollutants investigated in the real samples by SPE followed by GC–MS and LC–MS

Compounds	Raw influent WWTP (μg/L)	First settlement WWTP (μg/L)	Effluent WWTP (μg/L)
Acenaphthene	Nd	Nd	Nd
Acenachthylene	Nd	Nd	Nd
Aldrine	Nd	Nd	Nd
Anthracene	Nd	Nd	Nd
Benzo(*a*) anthracene	Nd	Nd	Nd
Benzo(*a*) pyrene	Nd	Nd	Nd
Benzo(*a*, *h*) anthracene	Nd	Nd	Nd
Benzo(*k*) fluoranthene	Nd	Nd	Nd
Benzo(*g*, *h*, *i*) perylene	Nd	Nd	Nd
Benzo(*b*) fluoranthene	Nd	Nd	Nd
C_{10}EOx	67	60	Nd
C_{10}LAS	82	53	Nd
C_{11}LAS	734	444	25
C_{12}EOx	205	192	Nd
C_{12}LAS	387	193	10
C_{13}LAS	57	21	Nd
C_{14}EOx	23	20	Nd
C_{16}EOx	Nd	Nd	Nd
C_{18}EOx	192	134	18
2-Chlorophenol	16	24	Nd
3-Chlorophenol	Nd	Nd	Nd
Chrysene	Nd	Nd	Nd
2,4-DDD	Nd	Nd	Nd
4,4-DDD	Nd	Nd	Nd
2,4-DDE	Nd	Nd	Nd
4,4-DDE	Nd	Nd	Nd
2,4-DDT	Nd	Nd	Nd
4,4-DDT	Nd	Nd	Nd
2,4-Dichlorophenol	Nd	Nd	Nd
3,5-Dichlorophenol	Nd	Nd	Nd
Dieldrin	Nd	Nd	Nd
α-Endosulfan	Nd	Nd	Nd
β-Endosulfan	Nd	Nd	Nd
Endosulfan-sulphate	Nd	Nd	Nd
Endrin	Nd	Nd	Nd
Endrin aldehid	Nd	Nd	Nd
Fluoranthene	Nd	Nd	Nd
Fluorene	Nd	Nd	Nd
α-HCH	Nd	Nd	Nd
β-HCH	Nd	Nd	Nd
δ-HCH	Nd	Nd	Nd
γ-HCH (lindane)	Nd	Nd	Nd
Heptachlor	Nd	Nd	Nd
Heptachlor epox endo	Nd	Nd	Nd
Heptachlor epox exo	Nd	Nd	Nd
Hexachlorbencene	Nd	Nd	Nd
Hexaclorbutadiene	Nd	Nd	Nd
Indeno(1,2,3-cd) pyrene	Nd	Nd	Nd
Isodrine	Nd	Nd	Nd
Naphthalene	Nd	Nd	Nd
Nitrophenol	25	28	Nd
NP	7.3	8	5
OP	0.13	0.24	0.3
PEGx	1330	2165	199
Pentachlorobencene	Nd	Nd	Nd
Pentachlorophenol	Nd	Nd	Nd
Phenanthrene	Nd	Nd	Nd
Pyrene	Nd	Nd	Nd
1,2,3-TCB	Nd	Nd	Nd
1,2,4-TCB	Nd	Nd	Nd
1,3,5-Trichlorophenol	Nd	Nd	Nd

Nd: not detected.

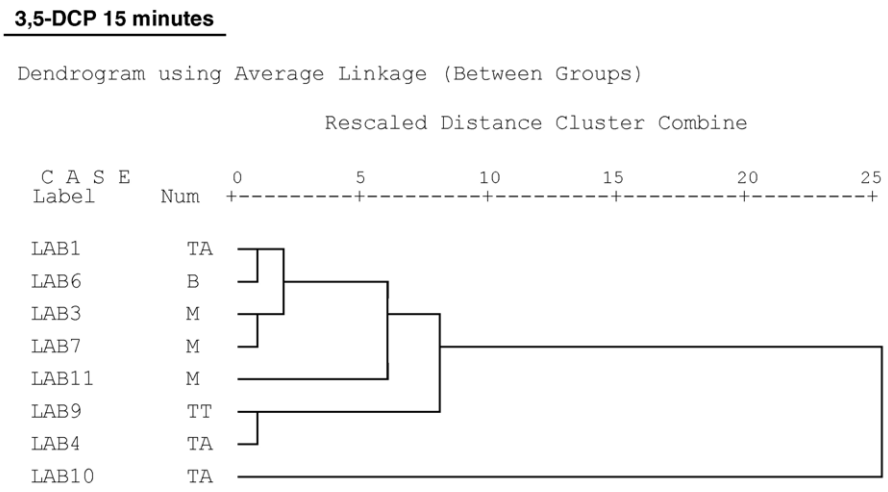

Fig. 5. Cluster analysis representation (dendogram) between the group of results for the 3,5 dichlorophenol, obtained along the inter-laboratory exercise by the participating laboratories. M: Microtox, B: Biotox, TA: ToxAlert, TT: ToxTracer, LCK.

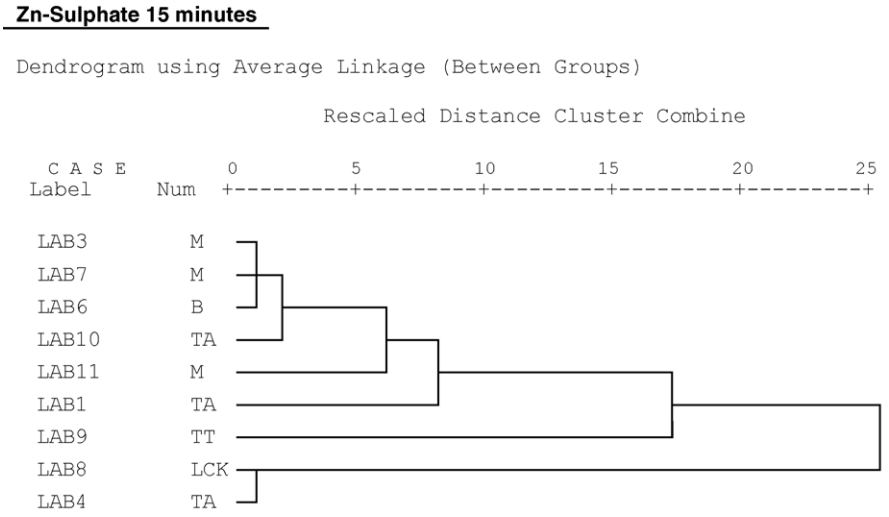

Fig. 6. Cluster analysis representation (dendogram) between the group of results for the Zn-sulphate, obtained along the inter-laboratory exercise by the participating laboratories. M: Microtox, B: Biotox, TA: ToxAlert, TT: ToxTracer, LCK.

repeatability show that laboratory number nine had a repeatability coefficient over than the upper warning limit. This coefficient is inversely proportional to the precision, which means a repeatability level for this laboratory significantly lower than the rest (Fig. 4).

The hieratichical cluster analysis between participants showed that the employed device did not influence the final results. In Figs. 5 and 6, the dendograms corresponding to the standard solutions 3,5-DCP and Zn-sulphate are presented. These diagrams show the distribution of the different laboratories in clusters using the average linkage method. This distribution was always different and the laboratories which results were carried out with different commercial devices followed different distribution.

The main sources of variation must be attributed to transport, storage, small variations on acclimatization and reconstitution times of bacterial reagents and variations due to the operators such as pipette operations and in general sample manipulations.

Finally, all participants completed the exercise, only 2 laboratories obtained the 72 results good, 2 laboratories obtained maximum 2 outliers and the rest obtained 3 or more outliers or missing values.

4. Conclusions

Toxicity tests using biological responses to measure toxic effects provide a total integrated information index about the significance of chemical contamination. In this sense, tests based on *V. fischeri* bioluminescence inhibition offer a rapid, easy handling and cost effectively responses for the toxicity assessment in real complex samples, but standard sample manipulation procedures still being needed. The precision of the test was proved and only one participant obtained a repeatability level significant lower than the rest.

These results were in accordince with previous toxicity interlaboratory exercise, carried out at national level and using only one or two different commercial toxicity tests.

The different commercial devices tested here showed comparatively good responses. The hieratichical cluster analysis between participants showed that the employed device and reagents did not influence the final results. In spite of being a standard test with a high reproducibility level, sources of variability are introduced when the measurements are carried out in different laboratories. Differences on sample handling may influence strongly on the final toxicity result when real complex samples are analyzed. In order to minimize the sources of variability handling samples standard protocols specifically designed for different matrix samples must be established.

The mean toxicity values for each real sample were correlated with the concentrations of main organic pollutants quantified by SPE–GC–MS and SSPE–LC–MS.

This exercise has demonstrated that aquatic toxicity evaluation using these tests can help the implementation of the Water Framework Directive. This toxic assay can be used in the regulations as a tools expressed as toxicity units to indicate if wastewater treatment was efficient or not, or if toxic discharges from industry have accrued.

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References

[1] J.M. Hemming, P.K. Turner, B.W. Brooks, W.T. Waller, T.W. La Point, Arch. Environ. Contam. Toxicol. 42 (2002) 9.
[2] M.D. Hernando, A.R. Fernandez-Alba, R. Tauler, D. Barcelo, Talanta 65 (2005) 358.
[3] H. Dizer, E. Wittekindt, B. Fisher, P.-D. Hansen, Chemosphere 46 (2002) 225.
[4] G. Chiti, G. Marrazza, M. Mascini, Anal. Chim. Acta 427 (2001) 155.
[5] N. García-Reyero, E. Grau, M. Castillo, M.J. López de Alda, D. Barceló, B. Piña, Environ. Toxicol. Chem. 20 (2001) 1152.
[6] The European Parliament and the Council, 2000, Directive of the European Parliament and of the Council concerning establishing a framework for community action in the field of water policy (2000/60/EC), October 23, 2000.
[7] E.G. Ruby, K.H. Nealson, Biol. Bull. 151 (1976) 574.
[8] R.N. Coleman, A.A. Quareshi, Bull. Environ. Contam. Toxicol. 35 (1985) 443.
[9] C. Curtis, A. Lima, S.J. Lozano, G.D. Veith, J.G. Pearson, et al., Aquatic Toxicology and Hazard Assessment: Fifth Conference, ASTM STP 766, vol. 6, American Society for Testing and Materials, 1982, pp. 170–178.
[10] ISO, Water quality: determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test), ISO 11348-1, 2 and 3, International Standardization Organization, Geneva 1998.
[11] M. Farré, M.-J. García, L.L. Tirapu, A. Ginebreda, D. Barceló, Anal. Chim. Acta 427 (2001) 181.
[12] M. Farré, D. Barceló, Fresenius J. Anal. Chem. 371 (2001) 467.
[13] M. Farré, O. Pasini, M.C. Alonso, M. Castillo, D. Barceló, Anal. Chim. Acta 426 (2001) 155.
[14] M. Farré, F. Arranz, J. Ribó, D. Barceló, Talanta 62 (2004) 549.
[15] S. Lacorte, I. Guiffard, D. Fraisse, D. Barceló, Anal. Chem. (2000) 1430.
[16] M. Petrovic, D. Barceló, Anal. Chem. (2000) 4560.
[17] AOAC & ISO & IUPAC "Z-Scores": Laboratory Accreditation & Audit Protocol, Food Inspection Directorate (Agriculture Canada, March 1987).
[18] R.B. Dean, W.J. Dixon, Anal. Chem. 23 (1951) 636.