Selection of a bioassay battery to assess toxicity in the affluents and effluents of three water-treatment plants

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Abstract

Objective. The assessment of water quality includes the analysis of both physical-chemical and microbiological parameters. However, none of these evaluates the biological effect that can be generated in ecosystems or humans. In order to define the most suitable organisms to evaluate the toxicity in the affluent and effluent of three drinking-water treatment plants, five acute toxicity bioassays were used, incorporating three taxonomic groups of the food chain. **Materials and methods**. The bioassays used were *Daphnia magna* and *Hydra attenuata* as animal models, *Lactuca sativa* and *Pseudokirchneriella subcapitata* as plant models, and *Photobacterium leioghnathi* as bacterial model. To meet this objective, selection criteria of the organisms evaluated and cluster analysis were used to identify the most sensitive in the affluent and effluent of each plant. **Results.** All organisms are potentially useful in the assessment of water quality by meeting four essential requirements and 17 desirable requirements equivalent to 100% acceptability, except *P. leioghnathi* which does not meet two essential requirements that are the IC_{50} for the toxic reference and the confidence interval. The animal, plant and bacterial models showed different levels of sensitivity at the entrance and exit of the water treatment systems. **Conclusions**. *H. attenuata, P. subcapitata* and *P. leioghnathi* were the most effective organisms in detecting toxicity levels in the affluents and *D. magna, P. subcapitata* and *P. leioghnathi* in the effluents.

Key words: bioassays, cluster analysis, drinking water, raw water, toxicity.

Resumen

Selección de una batería de bioensayos para evaluar toxicidad en los afluentes y efluentes de tres plantas potabilizadoras.
Objetivo. La evaluación de la calidad del agua incluye el análisis de parámetros tanto físico-químicos como microbiológicos. Sin embargo, ninguno de estos evalúa el efecto biológico que se puede generar en los ecosistemas o en el hombre. Con el objetivo de definir los organismos más indicados para evaluar la toxicidad en el afluente y efluente de tres plantas potabilizadoras, se utilizaron cinco bioensayos de toxicidad aguda, incorporando tres grupos taxonómicos de la cadena trófica. **Materiales y métodos**. Los bioensayos empleados fueron *Daphnia magna* e *Hydra attenuata* como modelos animales, *Lactuca sativa* y *Pseudokirchneriella subcapitata* como modelos vegetales y *Photobacterium leioghnathi* como modelo bacteriano. Para cumplir con este objetivo, se utilizaron criterios de selección de los organismos a evaluar y análisis de conglomerados (AC) para identificar los más sensibles en los afluentes y efluentes de cada una de las plantas. **Resultados**. Todos los bioensayos son pruebas potencialmente útiles para evaluar la calidad del agua, al presentar cuatro requisitos esenciales y 17 requisitos deseables, salvo *P. leioghnathi* que no cumple con dos de los requisitos esenciales que son la CI_{50} para los tóxicos de referencia y el intervalo de confianza. En los modelos animales, vegetales y bacteriano se observaron

diferentes niveles de sensibilidad a la entrada y salida de los sistemas de potabilización. **Conclusiones**. *H. attenuata, P. subcapitata* y *P. leioghnathi* fueron los organismos más eficaces para detectar la toxicidad en los afluentes y *D. magna*, *P. subcapitata* y *P. leioghnathi* en los efluentes.

Palabras clave: bioensayos, análisis de conglomerados, agua potable, agua cruda, toxicidad.

Resumo

Seleção de uma bateria de bioensaios para avaliar a toxicidade em afluente e efluente de três estações potabilizadoras. Objetivo. A avaliação da qualidade da água inclui a análise dos parâmetros físico-químicos e microbiológicos. No entanto, nenhum destes avalia o efeito a nível biológico que pode ser gerado nos ecossistemas ou no homem. Com o objetivo de definir os organismos mais adequados para avaliar a toxicidade no afluente e efluente de três estações potabilizadoras, foram utilizados cinco bioensaios de toxicidade aguda, incorporando três grupos taxonômicos da cadeia alimentar. **Materiais e métodos**. Os bioensaios utilizados foram *Daphnia magna* e *Hydra attenuata* como modelos animais, *Lactuca sativa* e *Pseudokirchneriella subcapitata* como modelos vegetais e *Photobacterium leioghnathi* como modelo bacteriano. Para cumprir com este objetivo foram utilizados critérios de seleção dos organismos a avaliar e análise de agrupamento (AC), para definir os mais sensíveis nos afluentes e efluentes de cada estação de tratamento. **Resultados.** Todos os bioensaios são testes potencialmente úteis para avaliar a qualidade da água ao apresentar 4 requisitos essenciais e 17 requisitos desejáveis; exceto *P. leioghnathi* que não cumpre com dois dos requisitos essenciais: com o CI_{50} para substâncias tóxicas de referência e com o intervalo de confiança. Nos modelos animais, vegetais e bacteriano, foram observados níveis diferentes de sensibilidade à entrada e à saída dos sistemas de potabilização. **Conclusões.** *H. attenuata*, *P. subcapitata* e *P. leioghnathi* foram os organismos mais eficazes na detecção de toxicidade em afluentes e *D. magna*, *P. subcapitata* e *P. leioghnathi* nos efluentes.

Palavras-chave: bioensaios, análise de agrupamento, água potável, água sem tratamento, toxicidade.

Introduction

The discharge of wastewater into a water body involves a large number and diversity of chemicals, many of which are unknown. These substances can be mixed among them, increasing or decreasing the toxic effect and generating a negative impact on the structure and functioning of the natural ecosystem.

The tools commonly used to assess pollution in wastewater are based on physicochemical analyses such as pH, dissolved oxygen, Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total Organic Carbon (TOC), Total Dissolved Solids (TDS) and Total Suspended Solids (TSS), (1-3), which do not reflect the biological effects that pollution can cause in animals, plants and humans. A good alternative to assess such effects are bioassays (4, 5).

To assess the toxicity of wastewater and drinking water, different types of bioassays have been used with fish, protozoa, bacteria, algae and others (6) Organisms to assess toxicity are diverse in their composition and their sensitivity to toxicants; therefore, a battery of bioassays is often used instead of a single species to cover a wide range of sensitivities (1, 7, 8). The test organisms included in a battery include representatives of the food chain at the level of consumers, producers and decomposers (9). The criteria for selection of the battery include autochthonous populations, in particular those that are environmentally attractive, with broad distribution and easy to maintain in the laboratory (10-12).

Keddy *et al.* (9) proposed a decision-making approach that consists in assessing whether organisms meet some essential criteria such as easy access to publications, standard test methods, acceptability, confidence intervals of 95%, and other desirable criteria like organisms identified by species, measurable endpoint, frequency of observation, environmental test conditions and statistical analysis, among others. Criteria are assigned a weight; if they are over 80% of acceptability they can be recommended as candidates to make part of a battery. Once organisms are selected, their sensitivity to polluted water is evaluated, and then those organisms that are most useful are chosen to make part of the battery of bioassays.

The selection of organisms that are part of the battery of bioassays can be performed by using multivariate analysis and/or by combining some of them such as non-linear mapping, principal component analysis, cluster analysis (CA) or matching factors analysis (13-14). The cluster analysis is a mathematical tool used to classify objects or variables into groups based on their similarities. The clustering procedure is often initiated by the conversion of raw data into a similarity matrix. Pandard *et al.* (15) mentioned that this mathematical technique can lead to various structures in the dendrogram given small errors in the distances calculated from the matrix of similarities.

Bioassays to assess toxicity in Colombia were adopted after Decree 1575 of 2007, which states that any drinking water supply must have at the entrance to the treatment plant, and if possible in the water collection, an early warning system to detect the possible early toxic contamination in the water and to take precautionary measures and strategies for environmental management. Additionally, a risk map should be established for inspection, monitoring and control of risks associated with the conditions of the quality of the sources supplying water for human consumption.

To meet these requirements, the Bogota Water and Sewerage Company considered necessary to implement a battery of bioassays for analysing affluents and effluents of three drinking water treatment plants that supply the city of Bogota. To select this battery, we evaluated two animal models: *Daphnia magna* and *Hydra attenuata*; three model plants: *Lactuca sativa, Allium cepa* and *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*); and a bacterial model: *Photobacterium leioghnathi* (16-24).

Materials and methods

Test organisms

Animal models

Daphnia magna (25).

It is a static acute toxicity bioassay (48 h of exposure), in which 30 ml plastic containers are used with 25 ml of volume solution. As a positive control we used 0.13 mg Cr^{+6}/L with confidence intervals between 0.05 and 0.21 mg Cr^{+6}/L, and reconstituted hard water as a negative control. Three replicates were performed for each control and dilution. In each container 10 neonates 24 h-old were transferred. The neonates were observed after 24 h and 48 h of incubation at $21\pm1°C$, with a photoperiod of 16 h light/8h dark, and a light intensity of 800 lux, and the number of dead organisms was recorded. Based upon the dead counts, we calculated the lethal concentration 50 (LC_{50}) at 48 h using the Probit method with a significance level of $P<0.05$.

Hydra attenuata (26).

It is a static test of acute toxicity (96 h of exposure), in which culture plates from 12 wells are used. As a positive control we used 0.78 mg Cr^{+6}/L with confidence intervals between 0.73 and 0.83 mg Cr^{+6}/L and reconstituted hard water as a negative control. Three replicates were performed for

each control and dilution: in each well three hydras were transferred to a volume of 4 ml of the solution and incubated at a temperature of $20 \pm 2°C$, a light intensity of 800 lux and a photoperiod of 16 h light/8 h dark. The morphological changes of the test organisms were recorded at 24, 48, 72 and 96 h of exposure. Morphology includes a normal stage, two of sublethality (organisms with rounded and shortened tentacles), and two of lethality (tentacles tulip-shaped and disintegrated organisms). With this assay we determined the average concentration that produces an effect in the exposed population (sublethal EC_{50} or lethal LC_{50}) using the Probit method with a significance level of $P<0.05$.

Vegetable model

Lactuca sativa (27).

It is a static acute toxicity test (120 h of exposure) with *Lactuca sativa* variety *Great Lake Batavia*. In the test, 25 seeds of similar size, shape, and colour are placed on a Whatman No. 3 filter paper impregnated with 4 ml of sample in a Petri Dish and incubated at $22 \pm 2°C$ in darkness for 5 days. As a positive control 18 mg Zn^{+2}/L were used with confidence intervals between 6.8 and 30 mg Zn^{+2}/L, and reconstituted hard water as a negative control. After incubation, the average length of roots per sample concentration is recorded and five outliers are discarded to reduce the coefficient of variation in the results. Finally, the concentration that produces 50% inhibition in root elongation (IC_{50}) is estimated using the Probit method with a significance level of $P<0.05$.

Pseudokirchneriella subcapitata (28).

It is a static acute toxicity test with *P. subcapitata* (96 h of exposure). In the test, 18 25-ml Erlenmeyer flasks are used with a 10 ml solution volume. As a positive control 0.25 mg Cr^{+6}/L with confidence intervals between 0.05 and 0.46 mg Cr^{+6}/L was used and culture medium as a negative control. For each control and dilution three replicates were performed. The volume calculated from the culture is inoculated in each Erlenmeyer flask to set an initial cell density of 10^4 cell/ml. Subsequently, the cultures are incubated at $23 \pm 2°C$, light intensity of 4.300 ± 10 lux and at continuous agitation of 100 revolutions per minute. After the incubation period of 96 h the percentage of inhibition is determined for each concentration compared to the control turbidity at 750 nanometres and the concentration that produces 50% of inhibition in the growth of algal cells (IC_{50}) is calculated with the Probit method with a significance level of $P<0.05$.

Bacterial model

Photobacterium leioghnathi (29).

Bioluminescence test is used to determine the toxicity of compounds that interfere with the enzymatic system of bacteria causing a reduction in light output. Variations in light output are measured with a high sensitivity luminometer (1 femtomole) at a wavelength of 490 nanometers. ToxScreen II test (CheckLight ® Ltda.) includes the use of two buffers, one that favours the detection of heavy metals (Pro-Metal Buffer) and another one (Pro-Organic Buffer) that favours the detection of organic pollution. Toxicity is determined by the average effective or inhibitory concentration (IC_{50} (15-30 minutes) 30 ° C) in a given time and under controlled temperature. The CI_{50} is calculated when the inhibitory effect is greater than or equal to 50%, otherwise it is reported as a percentage of volume/volume effect.

Selection criteria for organisms

The first step in selecting the organisms of the battery in the affluent and effluent from three treatment plants was to apply the approach of Keddy *et al.* (9) which states that the following requirements must be met:

Essential requirements

1. To have easy access to the publications reported as standard test methods.

2. To have toxic reference values and their actual or median lethal concentration.

3. To have acceptability criteria, ideally associated to confidence intervals of 95%.

4. To have controls to ensure the health of test organisms to carry out the bioassays and the interpretation of results.

Desirable requirements

There were 12 inclusion criteria to be met by the test organisms and each criterion was assigned a score. The scores for each criterion were assigned as follows:

1. Test organisms identified by species (1)

2. Measurable endpoints (1)

3. Morphological characteristics of the test organism (1)

4. Number of organisms per replicate (1)

5. Frequency of observation (1)

6. Volume of test solution (1)

7. Volume of test containers (1)

8. Preparation of the test substance and its addition to the test container (2)

9. Continued cultivation of the organisms (1)

10. Environmental test conditions (3)

11. Definition of culture media and dilution (2)

12. Statistical analysis (2)

When methods meet the four essential requirements, tests are considered as 'potentially useful'; then they are analysed to find whether they meet all the desirable requirements to be regarded on the long term as 'prototype tests', that is both inclusion criteria mentioned above must be complemented to become 'useful tests'. When the analysed organism meets the 12 desirable criteria, it gets 17 points equivalent to 100% of acceptability for desirable requirements. In this case all the indicators to be evaluated obtained 17 points, which are equivalent to 100% of acceptability for desirable requirements. In addition to the selection of organisms, relevant information was considered for the application of the tests, such as representing the trophic level, sensitivity, reproducibility (coefficient of variation in control charts $\leq$ 30%) and ecological relevance, all criteria that complement the tests and make them more robust to be recommended in a battery of bioassays (9).

Water samples

Ten samples of raw water (affluent) and 10 samples of treated water (effluent) were taken from three drinking-water treatment plants that supply the city of Bogotá, Colombia. The water samples from the three treatment plants comply with national legislation. Given the physicochemical characteristics of the three affluents, they were analysed as untreated wastewater.

The Tibitoc plant collects water from Bogotá River to be treated by a conventional system, which consists of a pre-sedimentation, coagulation, flocculation, sedimentation, downward flow filtration through a bed of anthracite, and gas chlorination. El Dorado plant collects water from La Regadera water reservoir and its treatment is a pre-treatment where the water is stabilized with hydrated lime, coagulation, flocculation, sedimentation, downflow filtration through a bed of anthracite and gas chlorination, and finally a dosing

with lime to stabilize the pH of the water. The Francisco Wiesner plant collects water from two sources: the Chingaza Paramo and the San Rafael reservoir in which water is stored from the Chingaza Paramo and the Teusacá River. Its treatment is a direct filtration with sand and anthracite, and gas chlorination. The average flow treated in plants is 8.50 m³/s for Tibitoc, 11.75 m³/s for El Dorado, and 0.35 m³/s for Francisco Wiesner.

Two litres of water were collected from each affluent and effluent at different days of the week to get a better assessment of variation of input water and the operation of each plant. Water samples were refrigerated at 4°C during transportation to the laboratory and were analysed within 48h after collection.

We used as test organisms two animal models: *D. magna and H. attenuate*; three vegetable models: *L. sativa, P. subcapitata and Allium cepa;* and a bacterial model: *Photobacterium leioghnathi*. The results of *A. cepa* are not included in this study because of the difficulty in obtaining homogeneous onion bulbs, so we obtained a coefficient of variation of 59% in the control card.

In the bioassay with *P. leioghnathi*, the effluent samples were processed with chlorine and chlorine neutralizing with sodium thiosulfate pentahydrate 3% (60μl/ 50 ml of treated water).

Data analysis

Calculation of LC/EC/IC$_{50}$

To calculate the LC/EC/IC$_{50}$ and their 95% confidence limits, the Probit method was used (EPA, V). This is a parametric method to estimate the effective concentration or lethality (EC$_{50}$ or LC$_{50}$) by adjusting mortality data with a technique or effect of probability. One of the restrictions of the method is that to calculate the EC$_{50}$ or LC$_{50}$ intermediate values should be obtained between 0 and 100% effect. When results in EC or LC$_{50}$ cannot be reported by the demands of the statistical program, they are reported as the percentage of effect in the lowest concentration at which the event is still present on the evaluated population. The effects may be inhibition, sub-lethality and lethality or volume/volume.

Cluster analysis

CA was used for the selection of the battery of bioassays (13, 15). Cluster analysis is a mathematical tool that classifies objects or variables into groups. The procedure begins with the conversion of raw data into a similarity matrix. We used the method of classification by hierarchical clustering (linkage Intra-Group), whose graphical representation is a dendrogram (15). To calculate the distance matrix between the values of each bioassay, the results were consolidated at 100% effect, using the measure of the Chi-2. CA as a mathematical tool can lead to various structures in the dendrogram, providing small errors in the distances calculated from the similarity matrix.

Results

Selection criteria for organisms

The selection of organisms used to evaluate the affluent and effluent water of the treatment plants was conducted according to the scheme proposed by Keddy *et al* (9). Bioassays to identify whether they met this proposal took into account the four key requirements and the 12 desirable qualifications to determine if they are considered useful tests (**Tables 1 and 2**). The analysis found that all organisms are potentially useful to meet four key requirements and 17 points for the desirable qualifications, equivalent to 100% acceptability. *P. leioghnathi* does not meet two essential requirements: the CI$_{50}$ for the toxic reference and the confidence interval.

Battery of Bioassays

Regarding the analysis of toxicity in animal, plant and bacterial models, there were different levels of sensitivity to input and output of water treatment systems.

In the affluent of Francisco Wiesner plant (**Table 3**), *H. attenuata* presented sublethal effects in most samples with EC$_{50}$ values between 49.6 and 107.42 and case lethality rates between 11.1 and 100%. *D. magna* showed low sensitivity in mortality rates between 4 and 57% to 100%. In the plant model, a similar sensitivity was observed in bioassays *P. subcapitata* and *L. sativa*. The algae growth presented an inhibition in 70% of the samples and the rest of the growth stimulation assays. In weeks 6 and 8, *L. sativa* showed growth-stimulating effects while other samples observed inhibition of root growth between 1 and 20%. In the case of plant models, when the volume/volume percentage is greater than 100% effect, it indicates that there has been an overgrowth of algal cells and/or root elongation compared to the negative control, so it is also seen as a sign of toxicity. In the bacterial model, *P. leioghnathi*; showed sensitivity only to organic in week 1, exceeding a 50% inhibition as suggested by the protocol.

Table 1. Results of the essential requirements application for the organism selection.

Test organism	Test Method	Toxic reference endpoint IC_{50} / LC_{50}	Confidence Intervals	Controls
		Essential requirements		
H. attenuata	Trottier *et al.* 1997	0.80 mg Cr^{+6}/L LC_{50-96}h	95%	(+) Chrome (-) Reconstituted hard water
D. magna	McInnis 1989	0.15 mg Cr^{+6}/L LC_{50-48}h	95%	(+) Chrome (-) Reconstituted hard water
L. sativa	McInnis 1989	20 mg Zn^{+2}/L IC_{50-120}h	95%	(+) Zinc (-) Distilled water
P. subcapitata	EPA 1994	0.30 mg Cr^{+6}/L IC_{50-96}h	95%	(+) Chrome (-) Culture medium
P. leioghnathi	Standard methods 8050B	N.A	N.A	(+) Sodium chloroacetate (pro-organic Buffer) Cupric chloride (pro-metallic Buffer) (-) Deionized water

N.A: Not Applicable. (+): Positive control. (-): Negative control

Table 2. Results of the desirable requirements application for organism selection.

Test organism	Organism identified by species	Measurable endpoints	We know the characteristics of the organism	Number of organisms per replicate	Observation frequency	Test solution volume	Test container volume	Test substance preparation and addition to vessel	Cultures are maintained in the organism	Test Conditions	Medium definition	Statistical analysis	Total score
					Desirable requirements								
H. attenuata	Yes	Sublethal lethality	Yes	9	24, 48, 72, 96h	4 ml	5 ml	Yes	Yes	800 lux, 20±2 ºC 16 h light/8 h dark	Yes	EC_{50} y/o LC_{50}	17
D. magna	Yes	Mortality	Yes	10	24, 48 h	30 ml	35 ml	Yes	Yes	800 lux, 21±2 ºC 16 h light/8 h dark	Yes	LC_{50}	17
L. sativa	Yes	Inhibition	Yes	25	120 h	4 ml	55 ml	Yes	No	22±2 ºC dark	Yes	IC_{50}	17
P. subcapitata	Yes	Inhibition	Yes	3	24, 96h	10 ml	25 ml	Yes	Yes	4300 lux, 21-25 ºC	Yes	IC_{50}	17
P. leioghnathi	Yes	Inhibition	Yes	N.A.	0, 15, 30 minutes	1 ml	3 ml	Yes	No	30 ºC	Yes	IC_{50}	17

N.A: Not Applicable

Table 3. Bioassay results from the Francisco Wiesner plant affluent.

Week	*H. attenuata* EC$_{50-96 h}$ or % (v/v) Effect	*D. magna* LC$_{50-48 h}$ or % (v/v)Effect	*P. subcapitata* IC$_{50-96 h}$ or % (v/v) Effect	*L. sativa* IC$_{50-120 h}$ or % (v/v) Effect	*P. leioghnathi* IC$_{50-30 min}$ or % (v/v) Effect
1	Lethality 11.1% al 100%	Mortality 0% al 100%	Overgrowth 141% al 100%	Inhibition 11% al 100%	Inhibition Inorganic 0% al 100% Organic 52% al 100%
2	EC 49.06	Mortality 7% al 100%	Overgrowth 201% al 100%	Inhibition 8% al 100%	Inhibition Inorganic 20% al 100% Organic 22% al 100%
3	EC 107.42	Mortality 17% al 100%	Overgrowth 136% al 100%	Inhibition 5% al 100%	Inhibition Inorganic 0% al 100% Organic 22% al 100%
4	EC 55.82	Mortality 4% al 100%	Inhibition 18% al 100%	Inhibition 1% al 100%	Inhibition Inorganic 0% al 100% Organic 38.5% al 100%
5	Lethality 100% al 100%	Mortality 57% al 100%	Overgrowth 125% al 100%	Inhibition 17% al 100%	Inhibition Inorganic 27% al 100% Organic 21.9% al 100%
6	EC 86.06	Mortality 0% al 100%	Inhibition 6% al 100%	Overgrowth 115% al 100%	Inhibition Inorganic 0% al 100% Organic 0% al 100%
7	Lethality 100% al 100%	LC 77.79	Inhibition 14% al 100%	Inhibition 20% al 100%	Inhibition Inorganic 6% al 100% Organic 0% al 100%
8	EC 90.19	Mortality 0% al 100%	Inhibition 10% al 100%	Overgrowth 109% al 100%	Inhibition Inorganic 0% al 100% Organic 0% al 100%
9	EC 88.17	Mortality 0% al 100%	Inhibition 8% al 100%	Inhibition 8% al 100%	Inhibition Inorganic 0% al 100% Organic 28% al 100%
10	Sublethality 55.6% al 100%	Mortality 0% al 100%	Inhibition 20% al 100%	Inhibition 1% al 100%	Inhibition Inorganic 0% al 100% Organic 0% al 100%

At El Dorado plant (**Table 4**), *H. attenuata* showed sublethality rates in most trials with EC$_{50}$ values between 25.32 and 177.80. *D. magna* presented mortality rates in 70% of the processed samples, with values between 9 and 36%. The model plants (*L. sativa* and *P. subcapitata*) showed a similar behaviour, presenting percentages of inhibition and stimulation of growth. *P. leioghnathi* showed no toxicity in any sampling event.

In the affluent of the Tibitoc Plant (**Table 5**), the indicator *H. attenuata* presented sublethality effects of 22.2 and 55.6% in the undiluted sample (weeks 7 and 9), EC$_{50}$ values between 30.51 and 130.62 in two events and an EC$_{50}$ of 82.48 and 55.54. *D. magna* showed toxicity in 60% of the samples, with values

between 23% and 100% in the second week, and EC$_{50}$ of 151.73. *P. subcapitata* presented growth inhibition between 5 and 11% at week 4 and an EC$_{50}$ value of 56.10. Other results show a stimulating effect, overcoming a 100% effect with respect to the negative control. *L. sativa* presented, in the same proportion, stimulation and inhibition. *P. leioghnathi* did not exhibit this kind of sensitivity to water.

In the effluent of Francisco Wiesner Plant (**Table 6**), *Hydra attenuata* exhibited sensitivity in all the effluent samples except for week 3. The sample 10 yielded a value of 100% sub-lethality and lethality in weeks 5, 6, 7 and 9 with values between 33.3 and 66.7%. *Daphnia magna* showed toxicity in the 10 samples tested indicating a high

Table 4. Bioassay results from the El Dorado plant affluent.

Week	*H. attenuata* EC$_{50\text{-}96\,h}$ or % (v/v) Effect	*D. magna* LC$_{50\text{-}48\,h}$ or % (v/v) Effect	*P. subcapitata* IC$_{50\text{-}96\,h}$ or % (v/v) Effect	*L. sativa* IC$_{50\text{-}120\,h}$ or % (v/v) Effect	*P. leioghnathi* IC$_{50\text{-}30\,min}$ or % (v/v) Effect
1	Sublethality 22.2% al 100%	Mortality 20% al 100%	Overgrowth 138% al 100%	Inhibition 9% al 100%	Inhibition Inorganic 0% al 100% Organic 22% al 100%
2	Sublethality 0% al 100%	CL 76.44	Overgrowth 207% al 100%	Overgrowth 110% al 100%	Inhibition Inorganic 20% al 100% Organic 0% al 100%
3	EC 68.54	Mortality 36% al 100%	Inhibition 22% al 100%	Inhibition 3% al 100%	Inhibition Inorganic 0% al 100% Organic 30% al 100%
4	EC 73.80	Mortality 13% al 100%	Overgrowth 109% al 100%	Inhibition 4% al 100%	Inhibition Inorganic 43% al 100% Organic 6,2% al 100%
5	EC 177.80	Mortality 0% al 100%	Overgrowth 105% al 100%	Overgrowth 120% al 100%	Inhibition Inorganic 0% al 100% Organic 0% al 100%
6	EC 62.70	Mortality 9% al 100%	Inhibition 17% al 100%	Overgrowth 104% al 100%	Inhibition Inorganic 10% al 100% Organic 0% al 100%
7	EC 125.82	Mortality 14% al 100%	Inhibition 9% al 100%	Overgrowth 104% al 100%	Inhibition Inorganic 0% al 100% Organic 0% al 100%
8	EC 95.53	Mortality 0% al 100%	Inhibition 17% al 100%	Inhibition 1% al 100%	Inhibition Inorganic 0% al 100% Organic 0% al 100%
9	EC 25.32	Mortality 20% al 100%	Overgrowth 103% al 100%	Inhibition 7% al 100%	Inhibition Inorganic 0% al 100% Organic 41% al 100%
10	EC 130.62	Mortality 0% al 100%	Inhibition 6% al 100%	Overgrowth 112% al 100%	Inhibition Inorganic 0% al 100% Organic 0% al 100%

Table 5. Results of bioassays from the Tibitoc plant affluent.

Week	*H. attenuata* EC/LC$_{50\text{-}96\,h}$ or % (v/v) Effect	*D. magna* LC$_{50\text{-}48\,h}$ or % (v/v) Effect	*P. subcapitata* IC$_{50\text{-}96\,h}$ or % (v/v) Effect	*L. sativa* IC$_{50\text{-}120\,h}$ or % (v/v) Effect	*P. leioghnathi* IC$_{50\text{-}30\,min}$ or % (v/v) Effect
1	LC 82.48	Mortality 23% to100%	Overgrowth 140% to100%	Inhibition 8% to100%	Inhibition Inorganic 0% to100% Organic 22% to100%
2	EC 30.51	LC 151.73	Overgrowth 273% to100%	Inhibition 8% to100%	Inhibition Inorganic 20 to100% Organic 11% to100%
3	EC 64.38	Mortality 7% to100%	Overgrowth 121% to100%	Overgrowth 110% to100%	Inhibition Inorganic 0% to100% Organic 26% to100%

Week					
4	LC 55.54	Mortality 13% to100%	IC 56.10	Inhibition 4% to100%	Inhibition Inorganic 0% to100% Organic 28.6% to100%
5	EC 130.62	Mortality 3% to100%	Overgrowth 124% to100%	Overgrowth 101% to100%	Inhibition Inorganic 0% to100% Organic 6.5 % to100%
6	EC 55.88	Mortality 3% to100%	Inhibition 5% to100%	Overgrowth 110% to100%	Inhibition Inorganic 10% to100% Organic 0% to100%
7	Sublethality 22.2% to100%	Mortality 0% to100%	Overgrowth 105% to100%	Inhibition 1% to100%	Inhibition Inorganic 0% to100% Organic 0% to100%
8	EC 55.76	Mortality 0% to100%	Inhibition 7% to100%	Overgrowth 111% to100%	Inhibition Inorganic 0% to100% Organic 0% to100%
9	Sublethality 55.6% to100%	Mortality 0% to100%	Inhibition 6% to100%	Inhibition 9% to100%	Inhibition Inorganic 0% to100% Organic 32% to100%
10	EC 66.72	Mortality 0% to100%	Inhibition 11% to100%	Overgrowth 123% to100%	Inhibition Inorganic 0% to100% Organic 0% to100%

Table 6. Bioassay results from the Francisco Wiesner plant effluent.

Week	*H. attenuata* $EC_{50\text{-}96\,h}$ or % (v/v) Effect	*D. magna* $LC_{50\text{-}48\,h}$ or % (v/v) Effect	*P. subcapitata* $IC_{50\text{-}96\,h}$ or % (v/v) Effect	*L. sativa* $IC_{50\text{-}120\,h}$ or % (v/v) Effect	*P. leioghnathi* $IC_{50\text{-}30\,min}$ or % (v/v) Effect
1	EC 26.02	LC 2.08	Overgrowth 176% al 100%	Inhibition 9% al 100%	Inhibition Inorganic 0% al 100% Organic 42% al 100%
2	EC 84.10	LC 12.19	Overgrowth 215% al 100%	Inhibition 15% al 100%	Inhibition Inorganic 18% al 100% Organic 14% al 100%
3	Sublethality 0% al 100%	LC 22.29	Overgrowth 121% al 100%	Inhibition 13% al 100%	Inhibition Inorganic 0% al 100% Organic 32% al 100%
4	EC 14.64	LC 10.47	Inhibition 59% al 100%	Inhibition 8% al 100%	Inhibition Inorganic 10.6% al 100% Organic 43.4% al 100%
5	Lethality 66.7% al 100%	LC 10.32	Inhibition 81% al 100%	Inhibition 19% al 100%	Inhibition Inorganic 14% al 100% Organic 30.5% al 100%
6	Lethality 44.4% al 100%	LC 7.33	IC 70.30	Inhibition 6% al 100%	Inhibition Inorganic 0% al 100% Organic 0% al 100%
7	Lethality 33.3% al 100%	Mortality 100% al 12.5%	IC 80.32	Inhibition 2% al 100%	Inhibition Inorganic 0% al 100% Organic 19.4% al 100%
8	EC 31.53	Mortality 21% al 18%	IC 28.78	Inhibition 1% al 100%	Inhibition Inorganic 0% al 100% Organic 0% al 100%
9	Lethality 55.6% al 100%	Mortality 100% al 12.5%	IC 17.79	Inhibition 24% al 100%	Inhibition Inorganic 0% al 100% Organic 33% al 100%
10	Sublethality 100% al 100%	LC 17.48	Inhibition 98% al 100%	Inhibition 21% al 100%	Inhibition Inorganic 0% al 100% Organic 0% al 100%

sensitivity of this organism in this type of water. In *P. subcapitata* we observed inhibition of cell growth in 70% of the cases and growth was stimulated only in the first three weeks. *L. sativa* in all samples showed inhibitory effects on root elongation, with values between 1 and 24% to 100%. *P. leioghnathi* did not provide sensitivity to possible toxicity by organic or inorganic in 10 samples of water with chlorine neutralization. In water samples without neutralization of chlorine, chlorine concentration was between 2 and 2.8 mg/l.

Table 7 presents the results of toxicity bioassays in the effluent from El Dorado Plant. *H. attenuata* showed toxicity in all samples tested, with EC_{50} values between

19.89 and 66.15. *D. magna* showed high rates of mortality and LC_{50-48h} between 6.44 and 24.53. *P. subcapitata* showed growth inhibition in 80% of the cases and stimulation of growth in two samples. *L. sativa* showed both inhibition and stimulation of growth. Finally, *P. leioghnathi* presented an $IC_{50-15min}$ in the chlorine samples neutralized with sodium thiosulfate pentahydrate only in the first week, indicating toxicity of inorganic origin. The chlorine concentration in El Dorado was between 2.1 and 2.4 mg/l. In the remaining samples no effect of inhibition of bioluminescence was detected.

In the Tibitoc effluent (**Table 8**), *Hydra* and *Daphnia* were sensitive in 100% of the samples tested, but the *Daphnia*

Table 7. Results of bioassays from El Dorado plant effluent.

Week	*H. attenuata* EC_{50-96h} o % (v/v) Effect	*D. magna* LC_{50-48h} Or % (v/v) Effect	*P. subcapitata* IC_{50-96h} o % (v/v) Effect	*L. sativa* $IC_{50-120h}$ o % (v/v) Effect	*P. leioghnathi* $IC_{50-30min}$ o % (v/v) Effect
1	EC 38.01	Mortality 100% al 12.5%	Overgrowth 138% al 100%	Inhibition 9% al 100%	Inhibition Inorganic 54% al 100% $CI_{50-15min}$ 10 Organic 26% al 100%
2	Lethality 77.8 al 100%	LC 19.34	Overgrowth 194% al 100%	Overgrowth 116% al 100%	Inhibition Inorganic 0% al 100% Organic 29% al 100%
3	Lethality 100 al 100%	Mortality 100% al 12.5%	Inhibition 84.99% al 100%	Inhibition 53% al 100%	Inhibition Inorganic 0% al 100% Organic 42% al 100%
4	EC 66.15	LC 9.50	Inhibition 39% al 100%	Inhibition 2% al 100%	Inhibition Inorganic 46.5% al 100% Organic 43.9% al 100%
5	EC 64.54	Mortality 87% al 12.5%	IC 46.29	Inhibition 14% al 100%	Inhibition Inorganic 6% al 100% Organic 26% al 100%
6	Lethality 55.6% al 100%	LC 6.44	IC 31.21	Overgrowth 107% al 100%	Inhibition Inorganic 6% al 100% Organic 0% al 100%
7	Lethality 100% al 100%	LC 17.97	IC 33.03	Inhibition 16% al 100%	Inhibition Inorganic 8% al 100% Organic 26.2% al 100%
8	EC 19.89	LC 24.53	Inhibition 79% al 100%	Overgrowth 129% al 100%	Inhibition Inorganic 0% al 100% Organic 0% al 100%
9	Lethality 77.8% al 100%	Mortality 100% al 25%	Inhibition 74% al 100%	Inhibition 25% al 100%	Inhibition Inorganic 0% al 100% Organic 48% al 100%
10	Sublethality 44.4% al 100%	Mortality 100% al 18%	Inhibition 88% al 100%	Overgrowth 108% al 100%	Inhibition Inorganic 0% al 100% Organic 0% al 100%

Table 8. Results of bioassays from the Tibitoc plant effluent.

Week	*H. attenuata* EC/LC$_{50\text{-}96\,h}$ Or % (v/v) Effect	*D. magna* LC$_{50\text{-}48\,h}$ or % (v/v) Effect	*P. subcapitata* IC$_{50\text{-}96\,h}$ or % (v/v) Effect	*L. sativa* IC$_{50\text{-}120\,h}$ or % (v/v) Effect	*P. leioghnathi* IC$_{50\text{-}30\,min}$ or % (v/v) Effect
1	Sublethality 33.3 al 100%	LC 32.83	Overgrowth 108% al 100%	Inhibition 15% al 100%	Inhibition Inorganic 0% al 100% Organic 29% al 100%
2	EC 74.52	Mortality 100% al 12.5%	Overgrowth 243% al 100%	Inhibition 4% al 100%	Inhibition Inorganic 0% al 100% Organic 29% al 100%
3	EC 45.38	Mortality 100% al 6.25%	IC 71.34	Overgrowth 114% al 100%	Inhibition Inorganic 0% al 100% Organic 30% al 100%
4	EC24.68	Mortality 100% al 25%	IC 71.19	Inhibition 1% al 100%	%Inhibition Inorganic 0% al 100% Organic 43.8% al 100%
5	EC 67.87	Mortality 100% al 25%	IC 62.32	Inhibition 5% al 100%	Inhibition Inorganic 0% al 100% Organic 30.8% al 100%
6	EC 23.64	Mortality 100% al 3.12%	IC 35.74	Inhibition 105% al 100%	Inhibition Inorganic 0% al 100% Organic 30% al 100%
7	Sublethality 55.6 al 100%	Mortality 100% al 25%	IC 40.63	Inhibition 3% al 100%	%Inhibition Inorganic 0% al 100% Organic 35% al 100%
8	EC 26.00	LC10.48	Inhibition 62% al 100%	Inhibition 9% al 100%	Inhibition Inorganic 7% al 100% Organic 0% al 100%
9	EC 66.30	Mortality 100% al 12.5%	IC 28.93	Inhibition 12% al 100%	Inhibition Inorganic 0% al 100% Organic 41% al 100%
10	EC 32.46	LC 14.09	IC 28.89	Overgrowth 110% al 100%	Inhibition Inorganic 0% al 100% Organic 0% al 100%

had greater mortality rates in the higher dilutions of the sample. Plant models showed no significant difference in terms of response or inhibition of growth effect; however, *P. subcapitata* showed greater sensitivity to the present average IC$_{50}$ values of 48.43 in 70% of the samples. Only in the first two weeks showed a stimulating effect on cell growth. *L. sativa* showed inhibition values between 1 and 15%, and samples from week 3 and week 10 showed a stimulating effect on root elongation. The bacterial model *P. leioghnathi* did not show toxicity in chlorine neutralizing samples. The chlorine concentration in the effluents was between 3 and 7 mg/l.

Cluster analysis

For the CA we used data obtained at 100%, i.e. from the undiluted sample. Dendrograms are shown in Figure 1. Bacterial model results with *P. leioghnathi* were excluded

from the analysis due to failure to report positive results above 50% as suggested by the protocol.

The choice of battery for each affluent and effluent of the three treatment plants was based on the comparison of the sensitivity of the test organisms by the CA. We obtained homogeneous groups of organisms, with the same potential for toxicity detection and the same range of sensitivity, with distances below 5 standard units with the Chi-2 method. In the affluents of Francisco Wiesner and El Dorado, the animal model *H. attenuata* showed a greater homogeneity in the results. For Tibitoc, it was *D. magna* the organism with the greatest homogeneity. Regarding the plant model, *P. subcapitata* showed a greater homogeneity in the three treatment plants, although *L. sativa* was also highly homogeneous for El Dorado. In the three effluents it can be seen that *D. magna* and *P. subcapitata* represent greater homogeneity in their behavior, although *H. attenuata* is also
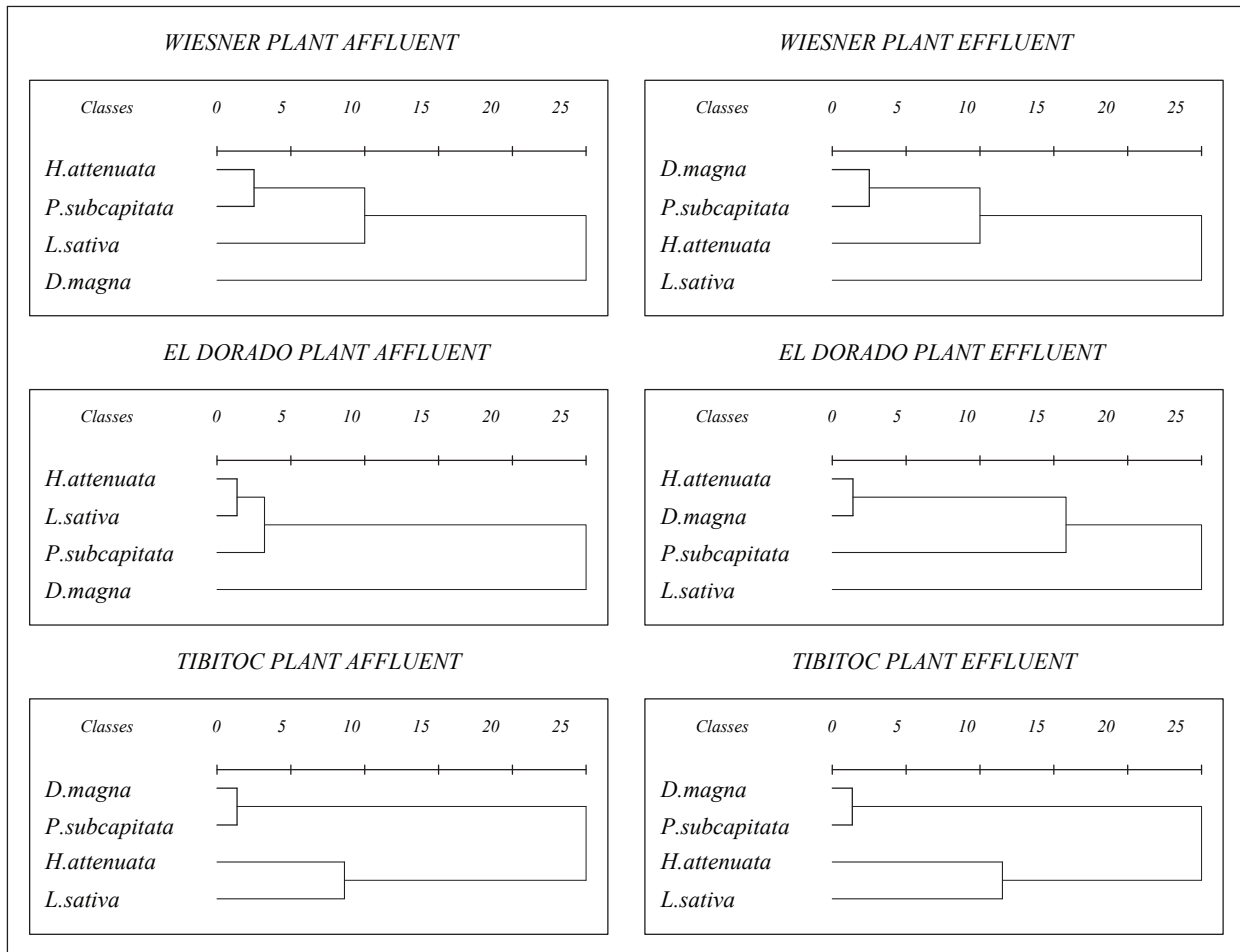
Figure 1. Dendrograms for the affluent and effluent of the water treatment plants Francisco Wiesner, El Dorado and Tibitoc with *H. attenuata, D magna, P. subcapitata* and *L. sativa.*

below 5% in El Dorado. Based on these results the battery to the affluents of the treatment plants includes the following organisms: Francisco Wiesner: *H. attenuata*, *P. subcapitata* and *P. leioghnathi*; El Dorado: *H. attenuata, L. sativa* and *P. leioghnathi;* and *Tibitoc: D. magna, P. subcapitata* and *P. leioghnathi.* In the case of the effluents, Francisco Wiesner*: D. magna, P. subcapitata* and *P. leioghnathi;* El Dorado: *H. attenuata, P. subcapitata* and *P. leioghnathi;* and Tibitoc: *D. magna, P. subcapitata* and *P. leioghnathi.* This selection included representatives of the food chain for animals, plants and bacteria.

Discussion

The results of bioassays with *H. attenuata* demonstrate an increased sensitivity of this organism for affluent or raw water from the three water treatment plants, a finding that

coincides with the results obtained by Castillo *et al.* (30) who assessed wastewater with *H. attenuata* and *D. magna* and found that *H. attenuata* shows a greater sensitivity to this type of water. On the other hand, Pardos (24) reported that in 35.7% of the wastewater studied, mortality was observed for *H. attenuata* and 71.4% sublethal responses. In subsequent studies, Pardos *et al.* (31) compared the sensitivity of *H. attenuata* and Microtox *(Vibrio fischeri)* in wastewater samples and higher sensitivity was observed by *H. attenuata*, attributing the observed toxicity for this organism to ammonia levels.

Slabbert and Venter (32) evaluated domestic sewage effluent and industrial wastewater with *D. magna* and *S. capricornutum* and toxic activity was detected between 20 and 100% for both indicators. In our study we observed in *D. magna* as in *P. subcapitata* toxicity levels above 20%

in a single sampling event in affluents of Wiesner and El Dorado, while in Tibitoc toxicity levels were lower. By contrast, Kontana *et al.* (33) found mortality rates of 50% of *D. magna* in most wastewater samples. The toxicity values found in the affluents of this study showed that *P. subcapitata* presents both inhibition and overgrowth in all the events analysed in the three treatment plants. By contrast *D. magna* has little sensitivity to this type of water. Similar results reported Ra *et al.* (34) in assessing wastewater with *S. capricornutum and D. magna*, who found that 33% of the samples showed acute toxicity to *D. magna* compared to 92% with *S. capricornutum*.

In assessing the effect of wastewater toxicity on *H. attenuata, Bacillus cereus, Panagrellus redivivus, D. magna, L. sativa, and Oncorhynchus mykiss*, Castillo *et al.* (30) found that *H. attenuata* showed the highest sensitivity in this type of water, while *L. sativa* had lower sensitivity even compared to *P. subcapitata*. Pica-Granados *et al.* (35) and Arkhipchuk *et al.* (36) reported inhibitory effects against organic substances, but Bohórquez and Campos (37) showed growth-stimulating effects of this alga.

In the case of effluent or potable water, *D. magna* showed higher sensitivity compared to other organisms evaluated. Cao *et al.* (18) reported similar results with *D. magna* when assessed town's secondary effluents before and after disinfection with chlorine, noting that this organism was more sensitive in samples treated with chlorine. Garzón (38) evaluated the toxicity of drinkable water from the Bogotá River and found the highest sensitivity with *H. attenuata*, showing EC$_{50}$ of 21.1 and LC$_{50}$ of 30.2. *L. sativa* and *S. capricornutum* showed moderate sensitivity, whereas in *D. magna* mortality was not observed, probably because chlorine was inactivated after purification.

In the vegetable models, although there was a similar inhibition effect between *Pseudokirchneriella* and *Lactuca*, microalgae showed signs of toxicity reflected in the cell overgrowth. On the other hand, the bacterial model *P. leioghnathi* showed sensitivity only against inorganic compounds in a sample of the effluent of El Dorado plant.

The results obtained in the CA do not coincide entirely with those obtained in bioassays of toxicity in relation to the animal model in the affluent of The Tibitoc plant, since the results suggest the use of *D. magna*, but in the affluent of the three water treatment plants *H. attenuata* appears to be more sensitive. In the effluent of El Dorado plant the cluster analysis suggests the use of *H. attenuata*, but in the results of the three water treatment plants *D. magna* shows greater sensitivity. This could be explained by the number of samples and / or the fact of using only the results in the

concentration of 100%. In many cases, positive results are obtained at lower concentrations, but these data are lost when entering into the analysis only the concentration of 100%. In these cases it is suggested to analyse a larger sample before making a decision and to take into account the initial results of the toxicity organisms tested. Such information is appropriate for decision making.

For the selection of organisms that are part of the battery of bioassays other tools can be used as suggested by Pandard *et al.* (15) who used CA as well as Principal Component Analysis (PCA) to select a battery of bioassays as part of the classification of hazardous waste of the Directive 91/689-CEE (39). In this case, they included *L. sativa* and *P. subcapitata* as vegetable models, *E. foetida, D. magna* and *C. dubia* as animal models and *V. fischeri* as a model for assessing bacterial toxicity in 40 residues. The authors note that the multivariate analysis can reduce the number of tests without changing the characteristics of the waste and that the combination of CA with PCA provides more robustness to the hierarchy of groups. Similarly, Rojíčková-Padrtová (14) used only PCA to select a battery of bioassays including 6 microarrays and three standard acute toxicity tests in environmental samples. The analysis showed three main components that explain 60% of the variance of the variables as follows: the first component (*P. subcapitata, T. platyurus, D. magna and B. calyciflorus*) explains 26%, the second component (*C. dubia, S. ambiguum*) explains 20.6% and the third component (*V. fischeri*) explains 13.5%. Results indicate that such selection is possible with this tool, allowing to conclude that the battery may contain *P. subcapitata, B. calyciflorus, T. platyurus* and *V. fischeri.*

Devillers (13) and Pandard (15) suggest a combination of multivariate analysis such as nonlinear mapping and principal component analysis among others, to provide more information about the analysed matrix for optimal selection. However, the structure and amount of data obtained in the three treatment plants did not meet the requirements of these tools, the reason why they were not implemented.

Conclusions

Based on the results obtained, we suggest the use of *H. attenuata, P. subcapitata* and *P. leioghnathi* to evaluate the affluents of the three water treatment plants and *D. magna, P. subcapitata* and *P. leioghnathi* for effluents. This decision takes into account the variability in the response of organisms, the type of water analysed, the taxonomic group within the food chain and the cost-benefit. Similarly, it would be more convenient for the laboratories responsible of the management of treatment plants to use the same battery

in all the three cases. Multivariate analysis and cluster analysis proved to be useful tools for selecting a battery of bioassays. The results for the effluents are useful as early warning systems for drinking-water treatment plants, but they do not determine by themselves the toxicity effects on the consumer. To rule out effects on human health other tests for an extended period of time are needed.

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Conflict of interest

The authors have no conflict of interest.

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