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The nematode *Caenorhabditis elegans* as an integrated toxicological tool to assess water quality and pollution

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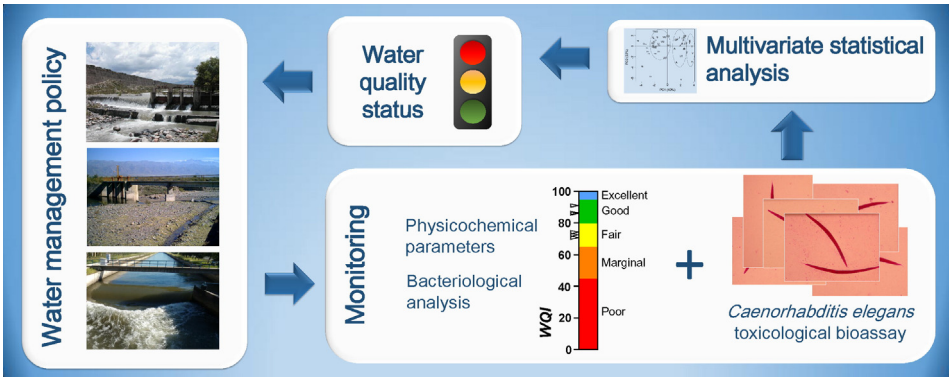
HIGHLIGHTS

- *C. elegans* bioassay was performed to assess water quality in Tunuyán River Basin.
- *C. elegans* bioassay should complement Water Quality Index calculation.
- Physicochemical analysis explain 62% of *C. elegans* growth variability.
- Tunuyán Upper Basin water quality is significantly better than lower basin.

GRAPHICAL ABSTRACT



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ABSTRACT

Determination of water quality status in rivers is critical to establish a sustainable water management policy. For this reason, over the last decades it has been recommended to perform integrated water assessments that include water quantities and physicochemical, ecological and toxicological tests. However, sometimes resources are limited and it is not possible to perform large-scale chemical determinations of pollutants or conduct numerous ecotoxicological tests. To overcome this problem we use and measure the growth, as a response parameter, of the soil nematode *Caenorhabditis elegans* to assess water quality in rivers. The *C. elegans* is a ubiquitous organism that has emerged as an important model organism in aquatic and soil toxicology research. The Tunuyán River Basin (Province of Mendoza, Argentina) has been selected as a representative traditional water monitoring system to test the applicability of the *C. elegans* toxicological bioassay to generate an integrated water quality evaluation. Jointly with the *C. elegans* toxic assays, physicochemical and bacteriological parameters were determined for each monitoring site. *C. elegans* bioassays help to identify different water qualities in the river basin. Multivariate statistical analysis (PCA and linear regression models) has allowed us to confirm that traditional water quality

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studies do not predict potential toxic effects on living organisms. On the contrary, physicochemical and bacteriological analyzes explain <62% of the *C. elegans* growth response variability, showing that ecotoxicological bioassays are important to obtain a realistic scenario of water quality threats. Our results confirm that the *C. elegans* bioassay is a sensible and suitable tool to assess toxicity and should be implemented in routine water quality monitoring.

1. Introduction

Rivers are dynamic systems that exhibit a large, temporal and spatial variability in water quality. Although much of this variability is due to the intrinsic characteristics of the system, some variations could be caused by pollution events as well (Mophin-Kani and Murugesan, 2014). Human activities not only use water resources that reduce the flow available but also discharge chemical and bacteriological pollutants. Consequently, anthropogenic activities are one of the most important threats to water resources conservation. Ongoing water monitoring is a powerful tool to provide trustful information for decision-making authorities to manage the resource and ensure its sustainability.

Nowadays there is worldwide consensus that water resources planning and management have to be an integrated concept that includes not only water quantity and quality but also water distribution, use and ecological services (Oliveira et al., 2007). In this regard, the European Water Framework Directive has established a common framework for sustainable and integrated management of water resources, in which sensitive environmental information and biological and ecological data play a leading role (EC, European Commission, 2000). Other environmental organizations such as the Canadian Water Network and the Clean Water Act guidance from USEPA have developed legislations that assure water security and governance (Bakker and Cook, 2011; USEPA, 2015).

However, routine monitoring includes only standardized physicochemical and bacteriological parameters. The use of advanced analytical technologies such as high-performance liquid chromatography, gas chromatography–mass spectrometry and atomic absorption makes it possible to detect a wider range of pollutants (Ju et al., 2010; Ruan et al., 2009). Still, all those technologies fail to identify all of the pollutants and their interactions, which are critical to assess the aquatic ecological status. That is the reason why, during the past 20 years, several ecotoxicological bioassays (covering different organization levels as well as indigenous species) have been developed and added to water monitoring plans (De Castro-Català et al., 2015; Faria et al., 2007; Kuzmanovic et al., 2015; Wernersson et al., 2015; Zhang et al., 2015). Nonetheless, it has not always been possible to include ecotoxicological assays due to the lack of compulsory regulations (Worth et al., 2015).

Several criteria must be fulfilled for an organism to be adopted as a bioindicator. The organism should be sensitive for the testing toxicants, easy to manage in the laboratory and available throughout the year (Wah Chu and Chow, 2002). Many authors have demonstrated that the free-living soil nematode *Caenorhabditis elegans* (Rhabditidae, Nematoda) is a valuable bioindicator organism in ecological risk assessment in both aquatic and soil environments (Leung et al., 2008; Peredney and Williams, 2000; Leung et al., 2008; Xing et al., 2009). Moreover, *C. elegans* is a worldwide accepted model for Environmental Impact Assessment ratified by international standards (ASTM, American Society for Testing and Materials, 2014; Höss et al., 2012; ISO, International Organization for Standarization, 2010; Leung et al., 2008; Traunspurger et al., 1997). Many of the basic physiological processes and stress responses that are observed in higher organisms, including humans, are conserved in the nematode (Kaletta and Hengartner, 2006). Therefore, toxicological assays performed with *C. elegans* are useful not only to evaluate environmental pollution but also to predict a pollutant's mode of action such as heavy metal toxicity (Chen et al., 2013; Hägerbäumer et al., 2015; Hunt et al., 2012, 2014).

Traditionally, assessment of water quality can be defined as the analysis of the physical, chemical and biological characteristics of water. Water Quality Indices (WQI) synthesize complex information related to multi-parameter analysis (Abbasi, 2002; Ali Khan et al., 2004; Sutadian et al., 2016; Venkatesharaju et al., 2010). WQI is based on regulated parameters and summarizes a large number of water quality data that yield a single value for each site and facilitate its communication to stakeholders and help to establish management priorities (Bharti and Katyal, 2011; Bhutiani et al., 2014; Lumb et al., 2006). Several WQIs have been formulated all over the world such as the US National Sanitation Foundation WQI, the Canadian Council of Ministers of the Environment (CCME) WQI, the British Columbia WQI, among others (Abbasi, 2002; Debels et al., 2005; Kannel et al., 2007; Sharifi, 1990; Statistics Canada, 2007).

The aim of this study is to evaluate *C. elegans* bioassay as a toxicological tool to complement routine water monitoring. To this end, we used the nematode relative growth as an endpoint to assess the toxicological impact on the Tunuyán River Basin (Province of Mendoza, Argentina). These results were analyzed together with the physicochemical and bacteriological parameters as well as with its correspondent WQI. Our work proved that *C. elegans* growth was capable of identifying threats that traditional monitoring fails to detect. So we suggest that it should be included in future water management plans to ensure a more accurate, effective and sustainable river water assessment.

2. Materials and methods

2.1. Study area and sampling

The Province of Mendoza was selected because of its high vulnerability caused by water scarcity. Even though it is located in one of the driest regions in the western part of Argentina, it has one of the largest irrigated areas of the country. Water monitoring was carried out in the Tunuyán River, whose average water flow is 30.6 $m^3\ s^{-1}$.

Seven georeferenced points were selected: the Las Tunas River (LT); the Aguanda stream (A); the Yaucha stream (Y); the Tunuyán River in the Valle de Uco dam (VU); and the Costa Anzorena (CA), Tiburcio Benegas (TB) and San Martin (SM) canals (Fig. 1; for more details see Fig. S1 and Table S1). These sites were chosen from previous works considering their proximity to productive and urban development areas where water quality is threatened by agricultural activities, sewage and industrial effluents (Table S1) (Morábito et al., 2011; Salatino et al., 2014). Specifically, LT is the less developed area but in the last decades water demand increased due to the establishment of new vineyards. The upper basin (that includes LT, A, Y, and VU) comprises approximately 54,000 ha planted almost to vineyards and fruit trees that are under pest control treatments. By compulsory regulation only 17% of the river flow can be used in this area (Departamento General de Irrigación, DGI, 1996). The rest of the flow is used for productive purposes downstream of the El Carrizal dam (360 hm^3) which separates both sub-basins and was built for multipurpose uses such as energy generation, irrigation, recreational and aquatic sports. Moreover, intensive urban, cattle-raising, agricultural and industrial activities are developed downstream of the CA site. The TB canal is located downstream of the El Carrizal Dam and runs through the Department of San Martin where the SM monitoring canal is located. The site of the SM canal has been selected because of its great urban impact and large vineyard

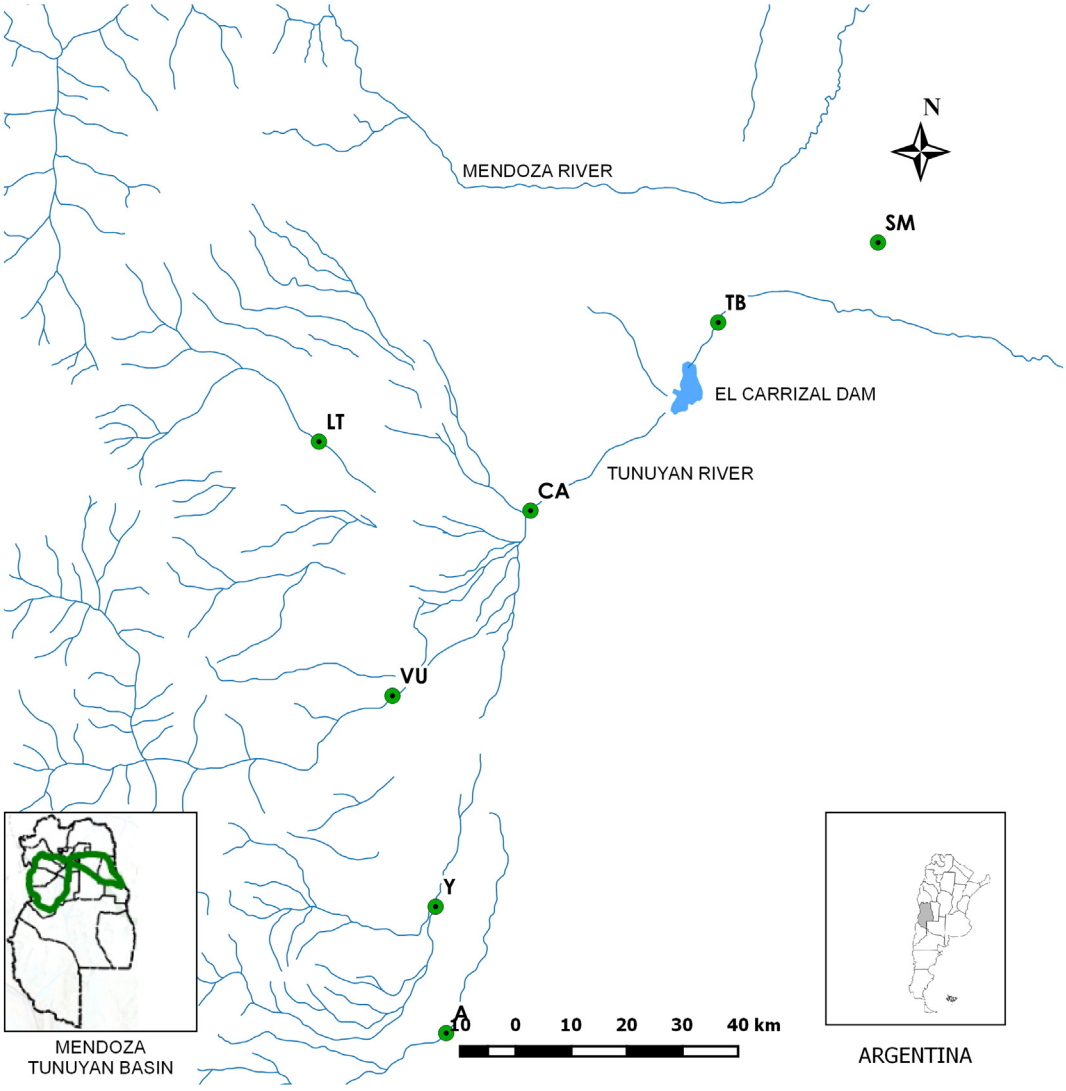

Fig. 1. Study area and location of monitoring sites in the Tunuyán River Basin.

development (that consumes a large amount of water from the Lower Tunuyán River Basin).

Monthly samplings were carried out in the Tunuyán River Basin from autumn 2014 to autumn 2015 to assess temporal variability. During winter months (June and July) monitoring was suspended due to the lack of water availability at the TB and SM sites. Because of operational difficulties, samples were not taken during January and February. River samples were obtained at 0.4 m from the surface water in sterile recipients at the middle of the river width using a wader equipment. The flow turbulence on the monitoring sites ensured the representativeness of the samples. All watercourses are shallow (<0.50 m), except VU and TB, which did not exceed 2 m in depth. Each sample was splitted into four aliquots; the first one was collected in a 1 L-plastic bottle to measure anions, cations and chemical oxygen demand (COD). The second one was placed into a 250 mL-plastic bottle for nitrates and phosphates determinations. The third one was collected into a 250 mL-clean amber glass bottle for dissolved oxygen (DO) analysis and the last one into a 250 mL-plastic bottle for bacteriological parameters quantification and *C. elegans* bioassay. Samples were stored, transported and preserved according to Standard Methods (SM 1060-C) (APHA, American Public Health Association, 2012). All analyzes were performed within 24 h after sample collection to avoid degradation.

2.2. Physicochemical and bacteriological parameters

Physicochemical and bacteriological parameters were measured at each monitoring site according to standardized methods (APHA, American Public Health Association, 2012; ASTM, American Society for Testing and Materials, 2014). Temperature (T), DO, pH and electrical conductivity (EC) were determined in situ using a multiparameter probe (Horiba U-10 Model). Sodium absorption rate (SAR) was determined by flame-ionization photometry (SM 3500-Na^{+}-B). Sulphate (SO_4^{2-}) and potassium concentration (K^{-}) were measured by the turbidimetric method (SM 4500–SO_4^{2-}.E) and the flame photometric method (SM 3500-K·B), respectively. Calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions were determined by EDTA complexometry (ASTM-D511-14). Carbonates (CO_3^{2-}) and bicarbonates (HCO_3^{-}) were measured by acid-basic titration (SM 2320-B). Chlorides (Cl^{-}) were determined by the argentometric method (SM 4500-Cl–B). Total phosphorus (P) was measured by the colorimetric analysis using ascorbic acid (SM 4500P·B). Soluble phosphate (PO_4^{3-}) levels were determined by the vanadomolibdophosphoric acid method (SM 4500P·C). Nitrates (NO_3^{-}) were measured using the cadmium reduction method (Hach Method 8171). COD was determined by the colorimetric method (SM 5220-D). Flow rates were either calculated or supplied by the General Department of Irrigation (Province of Mendoza).

Mesophilic aerobic bacteria (MAB) were quantified in accordance with the heterotrophic plate count (SM9215-B), thermotolerant coliforms (TTC) and total coliforms (TC) were determined by the multiple-tube fermentation technique (SM 9221-B and E).

2.3. Water Quality Index (WQI)

The CCME WQI calculation was based on the equation described in the Canadian Environmental Sustainability Indicators Report (Statistics Canada, 2007):

$$CCME\ WQI = 100 - \left(\frac{\sqrt{F_1^2 + F_2^2 + F_3^2}}{1.732}\right) \quad (1)$$

where F_1, F_2 and F_3 are dimensional vectors that represent respectively the scope, the amplitude and the frequency of parameters that did not comply with local current regulations (for WQI equation development see Supplementary data). Index value ranges from 0 to 100 (being 100 the best possible WQI score) and presents the following categories: excellent, good, fair, marginal and poor (Fig. 2S). To develop WQI data for the Tunuyán monitoring sites, the following parameters were selected: T, flow, pH, EC, SAR, Na^+, Cl^-, HCO_3^-, SO_4^{2-}, NO_3^-, PO_4^{3-}, P, COD, DO, MAB, TC and TTC. Parameters were determined in compliance with local current regulations which provides the maximum allowable limits for direct and indirect discharges into watercourses (Departamento General de Irrigación, DGI, 1996).

2.4. The nematode Caenorhabditis elegans toxicity test

2.4.1. Nematode cultures

C. elegans var. Bristol strain N2 was used throughout the experiment. Strain was obtained from the Caenorhabditis Genetics Center (CGC) and maintained as stocks on nematode growth medium agar plates (NGM) (per liter: 17 g bacto agar, 2.5 g bactopeptone and 3 g NaCl; with the addition after autoclaving of 1 mL 1 M $CaCl_2$, 1 mL 1 M $MgSO_4$, 25 mL 1 M KH_2PO_4, and 1 mL of a solution containing 5 g L^{-1} cholesterol, prepared in ethanol) seeded with *Escherichia coli* OP50-1 at 20 °C as described by Brenner (1974). Gravid *C. elegans* hermaphrodites were washed off the plates with M9 buffer (6 g L^{-1} Na_2HPO_4, 3 g L^{-1} KH_2PO_4, 5 g L^{-1} NaCl, 3 g L^{-1} $MgSO_4.7H_2O$), and synchronized by exposure to a bleaching mixture (0.45 N NaOH, 2% HOCl) following standard procedures (Stiernagle, 1999).

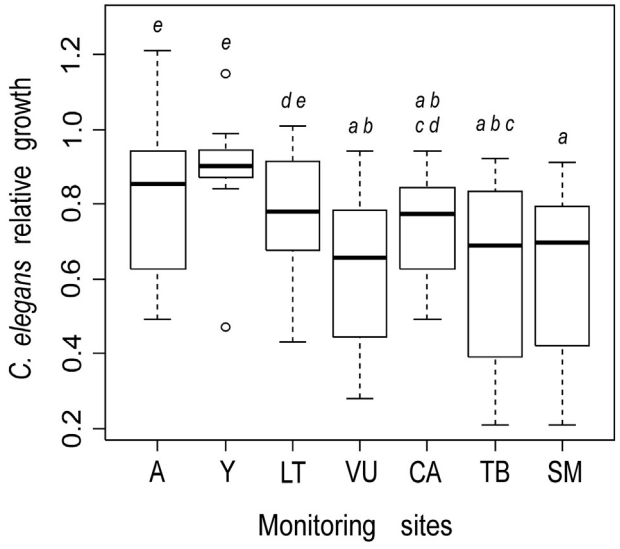

Fig. 2. Box and whiskers plots of *C. elegans* relative growth bioassay vs monitoring sites. Box plots with same letter are not different at *P*-value < 0.05 based on *t*-test.

2.4.2. C. elegans growth bioassay

The nematode bioassay with *C. elegans* was carried out, with a few modifications, according to standard methods (ISO, International Organization for Standarization, 2010) in which the endpoints for toxicity testing were body size measurements (Höss et al., 2012; Traunspurger et al., 1997). Exposures were performed in 24-well sterile tissue culture plates. In each well, 0.5 mL of the collected water sample was incubated with 15 μL M9 buffer containing ten L1 stage worms supplemented with *E. coli* OP-50.1. The final *E. coli* concentration was $OD_{600nm} = 1$. One growth control was carried out for each monthly sampling using M9 buffer instead of water samples. After 96 h of incubation at 20 °C, the bioassay was stopped by heat-killing the worms at 50 °C. The samples were stained with 0.5 mL of an aqueous solution of rose Bengal (0.5 g L^{-1}) for easier visualization. Four replicates with 10 L1-worms for each one of them were set up for the control and for the collected water samples.

2.4.3. C. elegans body length measurement

Rose Bengal stained nematodes samples were photographed using an optical microscope Nikon Eclipse 50i at 40× magnification (or 100× for L1 stage worms). Then body length along the body axis was measured using ImageJ software (Schneider et al., 2012). The average initial body length L1 stage worms was 221 ± 20 μm ($n = 30$). The body length in M9 controls at the end of the bioassay ranged between 1200 and 1300 μm. Body nematode growth was estimated as the difference between the average initial body length and the average body length after sample exposure. Results are expressed as nematode relative growth over monthly sampling control.

2.5. Statistical analysis

Data were analyzed using the R program (R Core Team, 2016). The Shapiro Wilks test were used to test normality in our variables. Normality was rejected in all variables except T, pH and nematode relative growth. To compare samples discriminated by locations and seasons, as well as by sub-basin groups non parametric Mann Whitney and Friedman tests were used (Conover, 1980). These rank-based tests, make no assumptions about the distribution of variables and maintain a reasonable asymptotic relative efficiency. The Student's *t*-test was performed a posteriori multiple comparisons.

Principal Component Analysis (PCA) was performed to relate physicochemical and bacteriological parameters as well as nematode relative growth. PCA is a useful technique to identify groups with similar or dissimilar properties within a large group of data characterized by many variables and experimental units (Li et al., 2007; Llario et al., 2006; Ouyang, 2005; Simeonov et al., 2002). The PCA purpose is to reduce data set dimensionality. The method is based on projections of initial multivariate samples onto a new coordinate axes (principal components) which are generated considering maximum samples variance and that are mutually orthogonal. The first principal component (PC1) of each multivariate observation was calculated using the followed equation:

$$PC1 = \sum_i w_i \cdot \log(1 + X_i) \quad (2)$$

where X_i is each variable (nematode relative growth, flow, Cl^-, Na^+, Ca^{2+}, HCO_3^-, SO_4^{2-}, MAB, TC, TTC) and w_i the coefficient of the lineal combination that generates the PC1 corresponding to each multivariate observation. The PC1 was a "shape component" that contrasted the

nematode relative growth variable against chemical and bacteriological variables according to:

$$w_0 \cdot growth > \sum_i w_i \cdot \log(1 + X_i); w_0, w_i > 0 \quad (3)$$

Also linear regression models were performed to relate physicochemical and bacteriological parameters with nematode relative growth as a dependent variable.

Three linear regression models were tested in order to find the best predictable model. The complete model used all available variables as regressors; the reduced model included only the variables present in WQI calculations and the optimal model considered the best regressors. All models followed the linear expression:

$$y_j = \beta_0 + \beta_1 X_{i1} + \beta_2 X_{i2} + \ldots + \beta_p X_{ip} + \varepsilon_i \quad (4)$$

where y_i is the corresponding response variable (nematode relative growth) to the i-th case, X_{ip} is the p-th regressor variable (physicochemical and bacteriological data) for the i-th case. The β_p is a statistical parameter and ε_i is a random normal variable (mean 0 and residual variance σ^2). Parameter estimation was performed by the least square method. The estimated model was used to produce predictions for the response variable given the known values of the regressor variables. For each model, R^2 and adjusted-R^2 were calculated. The R^2 is the proportion of variability in the response variable that is explained by the linear regression model with the regressors variables. The adjusted-R^2 responds to the above-mentioned description but is penalized by the number of parameters used in the model (Drapper and Smith, 1981).

For both the PCA and the linear model, logarithmic transformation [log (1 + X)] was applied to data in order to have more symmetrical distributions of physicochemical and biological variables (Conover, 1980; Drapper and Smith, 1981; Peña, 2002).

3. Results and discussion

3.1. Physicochemical and bacteriological parameters analysis

The minimum, maximum, mean and median for the 8 months data set of physicochemical and bacteriological parameters are shown in Tables 1 and 2. The difference between mean and median has long been used to evaluate the more frequent value from the average dispersion among series. Physicochemical and bacteriological water parameters exhibit a wide range of values among upper and lower basin sites. Mean and median values do not differ much among monitoring sites, but there are some differences. These results show that the selected monitoring sites have different physicochemical and bacteriological characteristics confirming that they are suitable to challenge the applicability of the *C. elegans* toxicological bioassay.

No significant variation in pH was measured along the river and through the seasons, with values ranging from 6.5 to 7.9 (P-value > 0.1). However, a sharp increase in salinity (expressed as EC) as well as in SO_4^{2-} concentration were detected in VU, CA, TB and in the SM group when compared to the A, Y and LT group (P-value < 0.01) (Table 1). The differences in salinity and SO_4^{2-} concentration were consistently observed along the annual monitoring (data not shown). In SM the values measured were three times higher than in the upper basin sites (A, Y and LT). The EC values exceed the maximum allowable

Table 1
Physicochemical parameters of water samples.

Site		T °C	Flow $m^3\ s^{-1}$	pH	EC μS cm^{-1}	SAR	HCO_3^- mg L^{-1}	SO_4^- mg L^{-1}	NO_3^- mg L^{-1}	PO_4^{3-} mg L^{-1}	P mg L^{-1}	COD mg L^{-1}	DO mg L^{-1}	Cl^- mg L^{-1}	Na^+ mg L^{-1}	K^+ mg L^{-1}	Ca^{2+} mg L^{-1}	Mg^{2+} mg L^{-1}
Aguanda (A)	Mean	14.8	0.83	7.29	403.8	0.59	90.00	126.00	1.55	0.43	0.14	26.00	7.30	27.96	19.18	3.46	57.00	13.80
	Median	16.3	0.77	7.31	400.0	0.63	103.73	129.60	1.33	0.38	0.12	18.00	7.40	26.63	20.70	2.93	56.00	12.00
	SD	3.9	0.15	0.37	37.3	0.15	29.49	25.24	0.53	0.18	0.06	29.59	0.38	4.00	4.73	2.67	4.90	5.70
	Min	7.0	0.72	6.60	350.0	0.26	36.61	91.20	0.89	0.24	0.08	2.00	6.80	24.85	9.20	1.56	50.00	7.20
	Max	19.0	1.14	7.83	481.0	0.78	115.94	158.40	2.66	0.73	0.24	76.00	7.60	35.50	25.30	9.75	64.00	24.00
Yaucha (Y)	Mean	13.8	1.83	7.32	245.5	0.63	110.60	25.20	1.94	1.03	0.33	15.67	7.66	25.29	15.61	1.80	37.00	6.00
	Median	14.8	1.73	7.50	245.0	0.73	122.04	21.60	1.77	0.82	0.22	6.00	7.80	24.85	18.40	1.56	36.00	4.80
	SD	3.6	0.50	0.48	26.0	0.23	29.07	23.48	0.88	0.84	0.28	18.50	0.48	5.51	5.79	1.43	4.00	4.21
	Min	6.0	1.41	6.50	190.0	0.26	61.02	0.00	0.89	0.29	0.10	4.00	6.90	17.75	6.90	0.78	32.00	1.20
	Max	18.0	3.03	7.86	270.0	0.86	134.24	72.00	3.54	2.42	0.79	37.0	8.10	31.95	20.70	5.07	42.00	12.00
Las Tunas (LT)	Mean	12.2	1.23	7.26	532.6	0.45	55.68	210.00	2.44	0.42	0.13	6.25	7.96	31.95	17.11	2.58	62.50	27.90
	Median	12.0	1.19	7.29	520.5	0.44	42.71	213.60	1.77	0.22	0.07	7.00	7.60	31.95	17.25	2.54	64.00	25.80
	SD	3.3	0.50	0.28	48.8	0.12	25.98	33.53	1.34	0.48	0.16	3.10	1.29	5.02	4.82	0.69	6.74	6.53
	Min	7.0	0.31	6.80	480.0	0.23	30.51	139.20	1.33	0.13	0.04	2.00	6.10	24.85	9.20	1.95	50.00	20.40
	Max	17.0	2.10	7.72	600.0	0.61	97.63	254.40	5.32	1.47	0.48	9.00	10.10	39.05	25.30	3.90	70.00	37.20
Valle de Uco (VU)	Mean	10.9	6.81	7.21	1231.3	1.22	125.85	327.60	2.23	0.64	0.21	20.00	8.53	158.86	65.55	4.83	176.00	22.95
	Median	12.0	6.73	7.17	1285.0	1.13	109.84	319.20	2.22	0.58	0.19	25.50	9.30	143.78	60.95	3.51	176.00	18.00
	SD	2.1	1.84	0.34	177.6	0.41	71.23	104.59	0.84	0.51	0.17	12.03	1.05	48.96	24.65	2.59	17.27	11.25
	Min	6.0	4.23	6.70	980.0	0.61	42.71	134.40	0.89	0.00	0.00	2.00	6.90	110.05	32.20	2.73	156.00	12.00
	Max	12.0	9.33	7.78	1470.0	1.86	280.69	513.60	3.54	1.74	0.57	27.0	9.40	230.75	101.20	9.75	196.00	39.60
Costa Anzorena (CA)	Mean	13.6	15.30	7.22	1371.3	1.24	183.82	393.00	1.61	0.65	0.22	26.25	7.64	144.22	69.98	8.53	190.75	31.05
	Median	14.0	13.89	7.16	1395.0	1.23	186.11	357.60	1.55	0.36	0.12	16.50	7.60	142.00	67.85	7.80	184.00	29.40
	SD	2.6	3.10	0.32	149.7	0.40	68.37	82.04	0.62	0.69	0.23	21.90	1.13	18.87	22.05	2.07	25.45	11.48
	Min	8.0	12.84	6.90	1160.0	0.61	54.92	321.60	0.89	0.15	0.05	13.00	5.90	117.15	34.50	5.85	156.00	15.60
	Max	16.0	22.13	7.74	1560.0	1.90	280.69	532.80	2.66	2.29	0.75	59.0	9.00	170.40	98.90	11.70	232.00	45.60
Tiburcio Benegas (TB)	Mean	14.9	39.49	7.26	1380.1	1.23	157.89	429.00	1.44	0.37	0.12	12.75	7.18	147.33	69.26	7.85	192.00	36.00
	Median	15.5	40.25	7.27	1385.0	1.14	164.75	408.00	1.55	0.35	0.11	13.00	7.15	152.65	62.33	7.80	190.00	25.20
	SD	4.2	11.88	0.33	143.9	0.40	46.52	74.18	1.18	0.23	0.07	7.85	1.09	17.70	20.55	3.99	11.86	28.52
	Min	8.0	24.50	6.70	1230.0	0.56	67.12	355.20	0	0	0	5.00	6.00	120.70	39.10	3.90	180.00	14.40
	Max	20.0	52.00	7.69	1660.0	1.80	231.88	585.60	3.54	0.76	0.25	20.0	8.30	166.85	98.90	16.77	216.00	103.20
San Martín (SM)	Mean	15.9	1.56	7.21	1341.7	1.24	169.98	417.60	1.84	0.56	0.15	14.80	7.46	139.46	69.99	7.41	199.14	30.17
	Median	16.5	1.38	7.28	1350.0	1.23	170.86	412.80	1.77	0.40	0.13	10.00	7.10	156.20	64.40	7.80	200.00	22.80
	SD	3.6	0.27	0.32	157.5	0.42	31.59	53.31	0.65	0.39	0.07	14.18	1.02	34.52	22.20	2.53	14.69	24.46
	Min	8.5	1.33	6.80	1140.0	0.58	140.35	355.20	0.89	0.21	0.07	4.00	6.70	71.00	39.10	3.90	180.00	12.00
	Max	19.0	2.00	7.69	1550.0	1.80	231.88	513.60	2.66	1.33	0.26	38.00	9.20	166.85	98.21	11.70	222.00	84.00

For each site, statistical parameters were calculated from $n = 8$. EC = electric conductibility; SAR = sodium absorption rate; COD = Chemical oxygen demand; DO = dissolved oxygen.

Table 2
Bacteriological parameters of water samples.

Site		MAB (CFU mL^{-1})	TC (MPN mL^{-1})	TTC (MPN mL^{-1})
Aguanda (A)	Mean	1.58E + 04	7.75E + 02	1.15E + 02
	Median	3.35E + 03	2.30E + 02	4.00E + 01
	SD	3.42E + 04	1.55E + 03	1.44E + 02
	Min	4.50E + 02	4.00E + 01	3.00E + 01
	Max	1.00E + 05	4.60E + 03	4.30E + 02
Yaucha (Y)	Mean	2.47E + 04	6.31E + 02	6.18E + 02
	Median	1.85E + 03	5.50E + 01	4.00E + 01
	SD	6.28E + 04	1.60E + 03	1.61E + 03
	Min	1.30E + 02	3.00E + 01	3.00E + 01
	Max	1.80E + 05	4.60E + 03	4.60E + 03
Las Tunas (LT)	Mean	2.34E + 03	7.63E + 01	3.88E + 01
	Median	1.26E + 02	5.50E + 01	3.50E + 01
	SD	5.56E + 03	6.55E + 01	1.36E + 01
	Min	2.00E + 01	3.00E + 01	3.00E + 01
	Max	1.60E + 04	2.30E + 02	7.00E + 01
Valle de Uco (VU)	Mean	3.52E + 03	8.75E + 01	4.88E + 01
	Median	1.65E + 03	4.00E + 01	3.50E + 01
	SD	3.89E + 03	7.80E + 01	4.12E + 01
	Min	2.00E + 01	3.00E + 01	3.00E + 01
	Max	9.30E + 03	2.30E + 02	1.50E + 02
Costa Anzorena (CA)	Mean	7.99E + 04	5.67E + 03	3.77E + 03
	Median	1.20E + 04	2.40E + 03	6.80E + 02
	SD	1.12E + 05	7.94E + 03	8.22E + 03
	Min	1.00E + 03	2.10E + 02	7.00E + 01
	Max	3.00E + 05	2.40E + 04	2.40E + 04
Tiburcio Benegas (TB)	Mean	1.53E + 04	3.15E + 03	3.03E + 03
	Median	1.60E + 03	4.00E + 01	3.00E + 01
	SD	2.84E + 04	8.43E + 03	8.47E + 03
	Min	3.10E + 02	3.00E + 01	3.00E + 01
	Max	8.00E + 04	2.40E + 04	2.40E + 04
San Martín (SM)	Mean	6.58E + 03	1.21E + 03	3.84E + 02
	Median	1.85E + 03	4.00E + 02	3.00E + 02
	SD	1.20E + 04	1.52E + 03	3.11E + 02
	Min	7.80E + 02	2.30E + 02	7.00E + 01
	Max	3.10E + 04	4.30E + 03	9.00E + 02

For each site, statistical parameters were calculated from $n = 8$. MAB = mesophilic aerobic bacteria; TC = total coliform; TTC = thermotolerant coliform.

limit (900 μS cm^{-1}) in the VU, CA, TB and SM sites, although all sites were below the tolerable upper limit (1800 μS cm^{-1}). Several reports show that the increased use of groundwater (with high EC) for irrigation in the Upper Tunuyán area and its consequent runoff could be responsible for salinization of the Lower Tunuyán River, while the high SO_4^{2-} concentration in the lower basin could be due to the natural soil composition in the area (Chambouleyron et al., 1993; Laviè et al., 2008). In the case of PO_4^{3-}, concentration values higher than current regulation limits (0.4 mg L^{-1}) were detected during May in all the monitoring sites (Departamento General de Irrigación, DGI, 1996). This high PO_4^{3-} concentration could be explained by a reduced flow during the autumn/winter monitoring sampling.

Other tested ionic parameters like Cl^-, Ca^{2+}, Mg^{2+}, HCO_3^-, K^+, NO_3^- did not show any significant difference among monitoring sites and seasonal periods (P-value > 0.1) (Table 1). When these ions concentrations were compared between VU, CA, TB and SM group and A, Y and LT group, significant difference were observed (P-value < 0.01), with the exception of NO_3^- (P-value > 0.1). NO_3^- and Cl^- values were below the regulation limits for the Mendoza region (maximum tolerable concentration of 45 mg L^{-1} and 400 mg L^{-1}, respectively).

Higher values of COD were found in A, VU and CA, especially during the spring season (data not shown), which may point to organic pollution due to an increase in recreational and livestock activities (Salatino et al., 2014). COD values decreased down the river showing a small recovery in TB that was sustained in SM.

Bacteriological parameters analysis revealed fluctuating values along the year with a sharp increase during November and December (data not shown). In this regard, the mean and median values of MAB differ by one order of magnitude in A, Y, LT and TB (Table 2). A moderate difference was detected when the MAB of these grouped sites was compared to the VU, CA and SM group (P-value < 0.01). The highest concentrations of bacteriological parameters were detected in CA and TB (considering MAB, TC and TTC) while the lowest ones were found in A, Y and LT, where water is supposed to be more pristine. The increase in bacterial concentration could be explained not only by domestic effluents but also by runoff of livestock wastes from snowmelt and rainfall.

The data generated in this section showed a spatio-temporal variation in water quality in the Tunuyán River highly dependent on anthropogenic activities. Therefore, the implementation of an integrated tool that can synthesize all these physicochemical and bacteriological results would be useful to better understand the water quality condition.

3.2. Water Quality Index

In order to integrate all the physicochemical and bacteriological parameters, an annual WQI was calculated. The highest WQI annual value was calculated for LT (91) followed by A and Y with values of 86.4 and 86 respectively. According to the CCME, all three values belong to the "good" water quality category (Fig. S2). VU WQI value was 74.2, which falls in the "fair" category. These results were consistent with the fact that the Upper Tunuyán Basin had a better water quality with the exception of the VU site. Even though VU belongs geographically to the upper basin, there were five parameters that exceeded the DGI resolution limits (three more than LT, A and Y). In the lower basin, WQIs were 70.7, 74.2 and 72.5 for CA, TB and SM respectively (fair category). This little fluctuation in water quality from CA to TB could be due to a temporary quality improvement after the water leaves the El Carrizal reservoir.

Even though all the WQI data describe acceptable water quality conditions, there is a clear deterioration of water quality downstream.

This situation may require further studies to evaluate the real impact on the ecosystem.

3.3. C. elegans growth response

In order to obtain an integrated Tunuyán River water quality assessment a *C. elegans* mean relative growth bioassay was performed to complement the standardized water quality analysis. *C. elegans* median relative growth showed a little decrease compared to the control in A (0.86), Y (0.90) and LT (0.74). Smaller relative growth was also measured in the other monitoring sites (0.65, 0.74, 0.63 and 0.70 for VU, CA, TB and SM respectively).

The box plot analysis shows the median values of relative growth for each monitoring site as a crossbar inside each box (Fig. 2). Each box begins near the first quartile and ends near the third quartile, showing the interquartile range as a measure of the spread of the data. Beyond the quartiles a line from each end of the box to the most remote point shows that it is not an outlier. Outliers are shown as isolated points.

Significant differences were revealed through a non-parametric ANOVA using a Friedman test in water samples from the monitoring sites (P-value = 0.0003) (Fig. 2). A posteriori, each of them was compared in pairs by a t-test. These comparisons indicated that relative growths (response) in A and Y were greater than in VU, TB and SM, whereas relative growth in Y was greater than in CA, TB, SM and VU, with a significance level of 0.01. When a significance level of 0.05 was tested, the relative growths in A and Y were greater than in CA, TB, SM and VU; while in LT the median value was greater than in VU, TB and SM. This statistical analysis of *C. elegans* bioassay data also reinforced the conclusion that water quality in the Upper Tunuyán Basin was better than in the lower basin.

The *C. elegans* growth bioassay showed that VU water quality was more similar to conditions in the lower basin monitoring sites than to the upper basin ones. This result is consistent with the WQI analysis. Surprisingly, even if the LT site presented the highest WQI value (91), the *C. elegans* relative growth assay was 0.74, indicating that there was a toxic effect. This suggests that occasionally the WQI does not suffice to evaluate water quality or to protect aquatic organisms. So additional toxicological assays must be performed to obtain an integrated and more realistic water assessment.

Our results underlined that the *C. elegans* growth bioassay, along with traditional water quality analysis, is a valuable tool to assess toxicological impact in order to estimate environmental threats. So far, *C. elegans* seems to be a sensitive organism to evaluate multi-stressor effects on ecosystems (Cesnaitis et al., 2014; Hägerbäumer et al., 2015; Höss and Weltje, 2007; Höss et al., 2013).

Moreover, using *C. elegans* growth as the endpoint can be suitable to detect pollutant interaction in environmental water samples that otherwise will not be detectable with the traditional methods, especially in countries with limited economic resources to perform analytical analyzes to detect a wide range of pollutants and their interactions.

3.4. Principal component analysis

In order to investigate further the relationship between *C. elegans* relative growth and physicochemical and bacteriological parameters, a PCA, multivariate statistical method was performed. In Fig. 3 it can be observed that the first two PCA components explain >62% of the total variance and showed two distinct groups in the PC1: one with positive values in the A, Y and LT sites, and the other with negative values in VU, CA, TB and SM. In the upper graphic area, the Y site presented the highest values followed by the A and LT sites. Conversely, VU exhibited mostly negative values with similar characteristics to the lower basin sites (CA, TB, SM).

According to Eq. 3 (Section 2.5), the extreme positive group (Y) reflects three possible combinations: higher *C. elegans* relative growth, lower physicochemical and bacteriological values, or a combination of both. In contrast, groups with small PC1 values express a low *C. elegans* growth, high physicochemical and bacteriological values, or a combination of both.

The PCA results provided the evidence to confirm the differences in water quality due to physicochemical composition and *C. elegans* growth between the upper and the lower Tunuyán river basins. The A, LT and Y sites are narrow valley rivers without large urban settlements and with moderate agricultural activity that have a mild effect on water quality. On the contrary, VU, CA, TB and SM are bigger flow with a greater number of industries, agricultural and recreational activities that have a stronger impact on water quality. VU is similar to CA and TB due to its natural intrinsic characteristics such as Na^+ and SO_4^-.

3.5. Multivariate linear model and simple regression of relative growth against WQI

In order to find which combination of physicochemical and bacteriological data could explain the variation in *C. elegans* relative growth, three linear models were calculated considering relative growth as a dependent variable. In the first one, the complete model, all available variables were used as regressors (T, flow, pH, EC, SAR, Cl^-, Na^{2+}, K^+, Ca^{2+}, Mg^{2+}, HCO_3^-, SO_4^{2-}, NO_3^-, PO_4^{3-}, P, MAB, TC, TTC). The second one, the reduced model, included only the variables selected for the WQI calculations (T, pH, SAR, Na^{2+}, Cl^-, HCO_3^-, SO_4^{2-}, NO_3^-, PO_4^{3-}, TC, TTC). The third one had the highest value of adjusted-R^2 considering the following best regressors: T, pH, EC, K^+, Ca^{2+}, Mg^{2+}, HCO_3^-, PO_4^{3-}, MAB, TTC. This last model is the optimal one, which best fitted the selected physicochemical parameters with *C. elegans* relative growth.

The reduced model had the lowest R^2 and adjusted-R^2 with 40.5 and 24.9 value respectively (Table 3). In the Complete model, even though it had the highest R^2 at 61.8%, the adjusted-R^2 was 42.1%. The optimal model has a 60.4% R^2 and the highest adjusted-R^2 at 51.2%. None of these models showed an R^2 higher than 62%, meaning that there was a 38% variability in *C. elegans* growth not explained by the

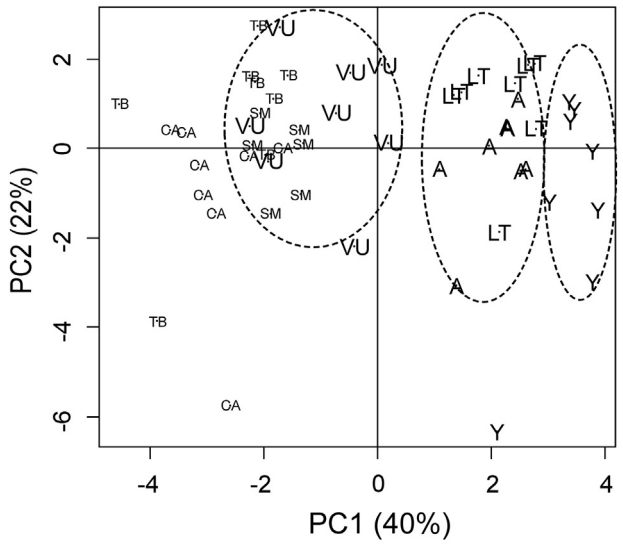

Fig. 3. PCA plot of monitoring sites according to *C. elegans* relative growth and physicochemical and bacteriological parameters.

Table 3
Linear models considering *C. elegans* relative growth as a dependent variable.

Model	R^2 (%)	R^2_{adj} (%)
Complete	61.8	42.1
Reduce	40.5	24.9
Optimal	60.4	51.2

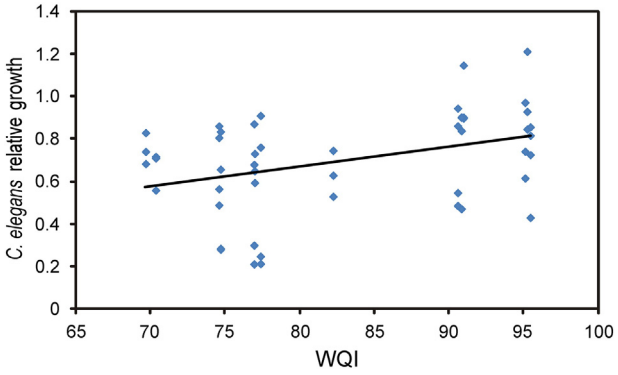

Fig. 4. Regression analysis between *C. elegans* relative growth vs WQI values.

physicochemical and bacteriological parameters. Regarding the adjusted-R^2, the optimal model presents the highest value at only 51.2% and the other two have much lower values indicating that all three models show a poor goodness of fit.

One of the most important observations in this work is that *C. elegans* growth was affected by changes in water quality, even when water samples met regulatory requirements and the quality seemed to be adequate. This aspect reinforces the concept that there must be other substances or chemicals in the water samples that were not detected a priori but even so exert weighty toxicological effects. Several reports showed that *C. elegans* was sensitive to different substances such as pesticides, heavy metals and toxins (Höss et al., 2013; Jiang et al., 2016; Lewis et al., 2013; Negga et al., 2011; Ruan et al., 2009; Yunhui et al., 2009). So it is possible that *C. elegans* toxicity could be explained by pollutants derived from the anthropogenic activities developed in the area (like agriculture, livestock and recreations). Unfortunately, there is not information about the presence of any contaminants in the Tunuyán River with the exception of Salatino et al. (2009). In this report the authors measured arsenic and heavy metals (such as zinc, chromium, cadmium, lead, copper) but did not find any differences along the River and values never exceeded the limits set by EPAS or the DGI (Departamento General de Irrigación, DGI, 1996; EPAS, Ente Provincial del Agua y del Saneamiento, 2001). Further studies need to be performed in the Tunuyán River to identify toxicity agents and understand their interactions.

Additionally, to assess linear dependence between *C. elegans* relative growth (as response) and WQI data (as explanatory variable), a linear regression analysis was performed (Fig. 4). To this end, every single WQI value was correlated with the monthly *C. elegans* relative growth set of data. As expected, a poor linear association ($R^2 = 0.1409$) was found between the WQI and *C. elegans* relative growth. Fig. 4 shows a big dispersion of the *C. elegans* relative growth value for each WQI value. One explanation could be that the physicochemical and bacteriological parameters used in the WQI only explained 24.9% of the variation of nematode growth (adjusted-R^2 linear model) (Table 3). Another possibility is that there is a great dispersion and this could be so because for each of the seven annual WQIs (one for each monitoring site) there were eight monthly values of *C. elegans* relative growth. Furthermore, *C. elegans* growth values exhibit significant seasonal differences that generated dispersion in the data (data not shown).

This work highlights the idea that good water quality (as reflected in the WQI) is not necessarily related to its toxicological condition as it is reflected by the *C. elegans* response. These results stress the need of including ecotoxicological bioassays, like *C. elegans* growth, into routine monitoring to assess multi-stressor effects on ecosystems (UNFCCC, United Nations Framework Convention on Climate Change, 2014).

4. Conclusions

The effects of chemical substances released into the water environment far exceed those that are specifically monitored or researched. This work reinforces the idea that water quality needs to be assessed with an integrated approach that includes toxicological evaluation. Thus, this kind of comprehensive studies will improve the understanding of how multiple stressors contribute to the degradation and alteration of the ecological water status.

In this sense, *C. elegans* could determine the biological toxicity impact of a low pollutants dose or a complex mixture present in water samples that otherwise could be overlooked. In the present report, the Tunuyán River Basin was selected as a representative traditional water monitoring area to test the applicability of *C. elegans* toxicological bioassay to generate an integrated water quality evaluation. *C. elegans* growth bioassay allowed us to identify different spatio-temporal water qualities. The bioassay confirms that the Tunuyán River has different water qualities in its upper and lower basins, the latter one being more contaminated. Multivariate statistical analyzes confirm that traditional water quality studies do not predict potential toxic effects on living organisms. These results underline the necessity to perform ecotoxicological assays in order to obtain a complete overview of water quality threats. Moreover, even though the WQI is a well-adopted index to assess water quality, it exhibits serious limitations to understanding the toxicological status of water. This may be due to the fact that the WQI is biased towards standardized parameters and the manner they exceed the regulatory limits, and that it does not consider other unregulated compounds. Currently, in order to determine the real water quality status, we are working on developing a more trustful WQI that would combine both routine parameters with *C. elegans* assays.

In conclusion, we demonstrate that *C. elegans* bioassay is an excellent tool for the assessment of multi-stressor effects on water environments. We also strongly support the idea that in Argentina, as well as worldwide, water quality indices should include toxicological bioassay data that will provide appropriate information to decision-makers to implement effective and sustainable water resources management plans and policies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi: http://dx.doi.org/10.1016/j.scitotenv.2016.06.057. These data include the Google map of the most important areas described in this article.

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