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Toxicity of Anllóns River Sediment Extracts Using Microtox and the Zucconi Phytotoxicity Test

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Abstract Two methods of environmental toxicological tests were compared and applied to bed sediments of the Anllóns River: the standardized toxicological test based on inhibition of luminescence employing *Vibrio fischeri*, and a phytotoxicity test, using garden cress (*Lepidium sativum* L.), water cress (*Nasturtium officinalis* L.) and annual rye-grass (*Lolium multiflorum* L.) seeds. In addition, TCLP and HCl extracts were evaluated. The inhibition of luminescence test showed more sensitivity to toxicants than phytotoxicity assays, and no significant correlations were found between them. On the other hand, TCLP metal concentrations (Fe, Al, Zn, Pb, As) were lower than HCl concentrations, but seemed to represent more accurately the phytoavailability of metals to plants.

Keywords Sediments · *Vibrio fischeri* · Phytotoxicity · TCLP elutriate · Heavy metals

There are chemical compounds, bound to sediments, which are not determined in routine analyses. However, they are of great importance because they can be adsorbed onto organic matter and accumulate in benthic and other aquatic organisms. The diverse toxicological information available regarding sediments has revealed the need for the creation of standardized analyses which could be very useful to establish a relationship between pollution and biological effects (Párvez et al. 2006). The organisms mostly used for the determination of toxicity of industrial wastewaters are crustaceans, mainly *Ceriodaphnia dubia* (Keller 1993; Utz and Bohrer 2001), *Hyalella azteca* and *Daphnia magna* (Hosokawa et al. 1995) or bacteria as *Vibrio fischeri* (Guzzela 1998) (formerly *Photobacterium phosphoreum*), the most extensively used microbiological method of inhibition of luminescence. The bioassays based on a contact exposure and inhibition of luminescence have been regulated in Spain (MOPU 1989), and they are used throughout the world as a standard test for aquatic toxicity testing (Bennett and Cubbage 1992). This test is available commercially under several registered trademarks as Microtox®, LUMIStox® or Multitox® systems. Besides evaluating the toxicity of sediments to microorganisms, another important task is to know if sediments contain toxic components to plants. In toxicity studies, bed sediments are commonly treated as wastes and the phytotoxicity studies about them are mainly focused on dredged sediments and the potential toxicity of sediments over soils and crops (Chen et al. 2002; Bedell et al. 2003), whereas not many studies deal with the potential toxicity of sediments to the aquatic plants in the watercourse. One of the most common phytotoxic assay employed in the literature is the Zucconi test (Zucconi et al. 1985) which is applied to evaluate the phytotoxicity of plant growing media based on the germination index (GI) of seeds, and is also applied to identify contaminated soils and evaluate decontamination procedures. The GI combines measurements of relative seed germination and relative root elongation that are both sensitive to the presence of heavy metals and other phytotoxic compounds. Nevertheless this phytotoxic assay has been rarely employed with river bed sediments.

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In this study two methods of environmental toxicological tests were compared: the standardized toxicological test based on Microtox® system employing *Vibrio fischeri*, and a modified version of the Zucconi test, usually not employed for evaluating the toxicity of river bed sediments, using garden cress (*Lepidium sativum* L.*)*, water cress (*Nasturtium officinalis* L.*)* and annual rye-grass (*Lolium multiflorum* L.*)* seeds. In addition, two acid extractable fractions were obtained: on the one side, the weak acetic acid elutriate described in the TCLP method and, on the other side, the HCl-extractable elutriate. The heavy metals (Fe, Al, Zn, Pb and the metalloid As) obtained in the acid extracts were used as surrogates for phytoavailability and correlated with their respective biological assays, in order to determine which elements produces inhibitory effects in the biological tests assayed.

Materials and Methods

The Anllóns River is located in the NW of Spain and drains a rural catchment of 516 km^2 (Fig. 1). The Anllóns River was chosen as it is one of the few rivers in Galicia whose natural regime has not been altered by dams. This fact turns the Anllóns River into an appropriate system to study the sedimentation phenomena as well as the pollutant dispersion downstream.

In this way, the first sampling point was chosen at Carballo (the biggest settlement of the area with 25,000 inhabitants) and the samples were taken from Carballo to the mouth, in the depositional areas, covering a distance of approximately 30 km. The basin is affected by point and non-point sources of pollution. Sample 1 is located downstream the locality of Carballo, and affected by urban pollution, mainly the effluents of a wastewater treatment plant. Samples 10 and 11 are affected by mining activities,

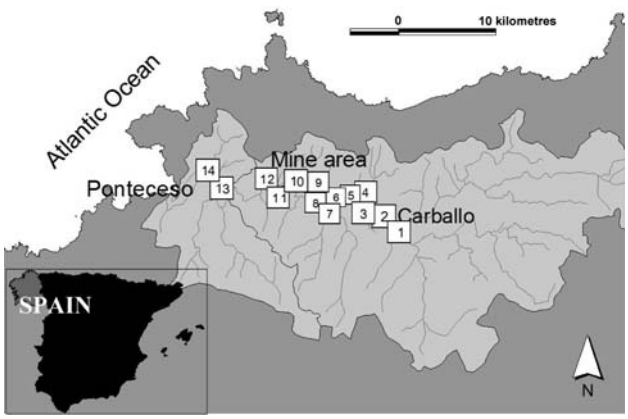

Fig. 1 Anllóns basin localization and sampling points. There are also indicated the main sources of pollution: Carballo and Ponteceso (urban pollution) and the mine area (arsenic pollution)

whose extraction activities favored the accumulation of As in sediments. Sample 14, collected in Ponteceso (7,000 inhabitants) is affected by urban pollution. On the other hand, non-point sources of pollution affect to a lesser extent the basin, mainly due to the contributions of P coming from agricultural activities.

The climate is wet (1,200 mm on average), with a mean temperature of 9°C in winter, and 20°C in summer. The river runs over schists in the steepest, upper area, turning into a smooth profile in the middle area of the river, characterized by basic rocks (gabbros and amphibolites). Finally, the lower stretch of the river runs over granite of two micas, followed by biotitic gneiss at the mouth. The land use of the area is a mixed forest of *Eucaliptus globulus, Eucaliptus alba and Pinus pinaster* (60% of the total cover), cultivated lands (18%), pastures (12%), bushes (9%) and urban uses (1%). The sampling collection was developed in 2004 (from June to September), and 14 samples were taken in the depositional areas, between the locality of Carballo and the river mouth, covering a distance of approximately 30 km. Each deposition site was sampled at 4–6 points, so that composite samples were taken. The samples were collected from the top 5 cm with a small plastic shovel and taken to the laboratory in a hermetic plastic container (prewashed and rinsed with deionised water MilliQ). Once in the laboratory pore water was removed by 20 min. of centrifugation at 2,000 rpm and the solid samples were freeze-dried and sieved by 2 mm.

To investigate the toxicity of sediment samples, the standard Toxicity Characteristic Leaching Procedure (TCLP), according to EPA Method 1311 (USEPA 1992), was used in river bed sediments. The toxicity test is carried out using *Vibrio fischeri.* The first step is based on an extraction of the soluble elements by using a 1:16 ratio of sediment:water. Later, the pH of the dissolution is measured and adjusted to 4.5 ± 0.1 by using acetic acid. After 24 h of extraction, the volume is adjusted following the next equation:

$$V = 20V - 16W - A$$

where V is the volume of deionized water, W is the weight of the residue used for the extraction and A is the volume of acetic acid used during the extraction. After the extraction step, samples are centrifuged at 2,000 rpm during 15 min and the liquid is filtered by 0.45 μm. The analyses were run in a Microtox® system. Bacteria were exposed to concentrations of 45%, 22.5%, 11.25% and 5.625% (v/v) elutriate diluted with Microtox® test medium (Azur Environmental Ltd.). The inhibition of luminescence was measured after 5 and 15 min and results were expressed as the percentage of inhibition of luminescence after 15 min ($EC_{50\text{-}15}$). To check the process a quality

control was used by employing 25 mg L^{-1} of 3–5 dichlorophenol ($EC_{50\text{-}15}$ expected = 3.36 ± 10%; $EC_{50\text{-}15}$ obtained = 3.57). All the analyses done were within the quality requirements. At the same time, chemical composition of the elutriate was determined by ICP-OES. The elements determined were Fe, Zn, Al, Pb and As. Hg, Cd and Cu were not detected in the area under study, so they were not determined in the elutriate extracts. The weak acetic acid elutriate (TCLP elutriate) was developed to assess leachability of solid waster under landfill conditions, but can be used as surrogate to estimate bioavailability. In addition, the acid extractable fraction of the same elements was determined also by ICP-OES by using HCl 1N. Many authors considered that this extractant solubilizes the available fraction (Snape et al. 2004; Moalla et al. 2006).

The phytotoxicity of the sediment samples was determined by using a modified version of the Zucconi test (Zucconi et al. 1985). Ten seeds of three species, namely annual rye-grass (*Lolium multiflorum* L.), garden cress (*Lepidium sativum* L.) and water cress (*Nasturtium officinalis* L.), with three replicates, were used for the test. Although several species have been traditionally used for evaluating phytotoxicity, there are no standardized seed species in use worldwide (Warman 1999). It is recommended (OECD 1984) that at least one monocotyledon and one dicotyledon species should be used in these tests. In this study, garden cress was chosen as it is one of the mostly employed seeds in phytotoxicity studies. Water cress was chosen as it is an aquatic plant which grows in the Anllóns River, typical of wet or swamped areas, characteristic of river banks. Annual rye-grass was also included as a representation of the monocotyledon species. After 5 days of incubation in the dark, the seed germination percentage and root length of seedlings immersed in the sediments extracts as well as in deionized water were determined. The values obtained for the deionized water were used as the control. The GI was calculated as follows: GI = G (La)/Lc, where G is the number of germinated seeds expressed as percentage of control values, La is the average value of root length in the sediment extracts and Lc is the average value of root length in the control. The statistical analyses were performed by using the SPSS software package (v11.0 for Windows). To evaluate the relationship between variables the Pearson product-moment correlation coefficients were calculated.

Results and Discussion

The results obtained for the inhibition test showed an average value of 21% of $EC_{50\text{-}15}$ and the highest luminescence decay was at sampling points 2, 3, 6, 10 and 11 (42.5, 62.0, 30.2, 60.5 and 50.2%, respectively), whereas the minimum values were observed at points 4 (4%), 5, 8, 9 and 12 (<1.0%). The values obtained with the *Vibrio fischeri* inhibition test were considered toxic over the 50%. According to these results, sample 3 can be considered toxic, and its toxicity cannot be attributed to a particular element analyzed at this point, but to a synergic effect of all the elements. On the other side, samples 10 and 11 can also be considered toxic, and their toxicity can be attributed to the high As concentrations of the sediments.

In relation to the phytotoxicity test, *Lepidium sativum* showed the higher GI with a mean value of 143% [127%–173%], followed by *Lolium multiflorum* with a mean value of 123 [95%–162%] and, finally *Nasturtium officinalis* with a mean value of 95 [73%–116%] (Table 1). A phytotoxicity effect is observed at sampling point 1 for the water cress. According to Zucconi et al. (1985), and Emino and Warman (2004), GI values under 50% suggest a high phytotoxicity; GI values between 50% and 80% suggest moderate phytotoxicity, whereas GI values above 80% suggest no phytotoxicity. When GI exceeds the 100%, the material can be considered as phytonutrient or phytostimulant. This is the case of the samples under study, which showed a clear phytostimulant effect that can be attributed to the increased P and N concentrations in the bed sediments of the sampled sites. On the other hand, no significant correlations were found between the inhibition of luminescence and the phytotoxicity assay, so these analyses do not show a similar sensitivity to toxicants in sediments. Differences between the inhibition of luminescence and phytotoxicity assays were also found in studies of sewage sludges (Fuentes et al. 2006).

In order to check the toxicity of the heavy metals (Fe, Al, Zn, Pb and the metalloid As) of the samples, two extracts were evaluated: the TCLP extract and the HCl-extractable fraction (Table 2). The concentrations obtained by the TCLP were lower than the HCl-extractable fraction.

To evaluate the correlations between the variables analyzed, a Pearson product-moment correlation coefficient was applied. Results are shown in Tables 3a–b. The significance of each correlation coefficient is also displayed in the correlation table.

Regarding the inhibition of luminescence assay, in the TCLP extract, linear correlations cannot be established between the heavy metal concentration and the $EC_{50\text{-}15}$. In the HCl-extractable fraction arsenic showed a linear relationship with the $EC_{50\text{-}15}$, suggesting an influence in the *Vibrio fischeri* survival. The inhibition of luminescence can be affected by one metal in particular or by a mixture of several components. In addition, there can be other toxicants in the samples, as organic compounds, not determined in this study, affecting the inhibition of luminescence. This reveals the usefulness of this kind of

Table 1 Distribution of inhibition of luminescence (EC_{50-15}) and GI values for rye-grass, garden cress and water cress along the watercourse. The values of a reference station upstream are also provided. In the table are also indicated the mean, minimum and maximum values, as well as the variation coefficients (in parenthesis)

Sample	EC_{50-15} (%)	Rye-grass (GI)	Garden cress (GI)	Water cress (GI)
Reference	<1.0	108 (20)	133 (21)	116 (12)
1	8.2 (14)	124 (19)	127 (24)	73 (9)
2	42.5 (7)	123 (2)	168 (6)	100 (17)
3	62.0 (6)	108 (24)	123 (18)	94 (13)
4	4.0 (15)	95 (16)	145 (22)	84 (5)
5	<1.0	129 (16)	154 (3)	110 (2)
6	30.2 (12)	135 (6)	165 (18)	107 (9)
7	12.1 (12)	153 (15)	155 (15)	101 (14)
8	<1.0	116 (18)	173 (9)	101 (29)
9	<1.0	111 (3)	168 (17)	89 (21)
10	60.5 (6)	100 (1)	132 (11)	83 (9)
11	50.2 (8)	130 (4)	132 (16)	85 (29)
12	<1.0	136 (13)	147 (11)	89 (20)
13	8.5 (16)	162 (6)	136 (10)	82 (15)
14	11.8 (18)	125 (7)	129 (9)	99 (5)
Mean	21.0	125	147	93
Min	1.0	95	123	73
Max	62.0	162	173	110

Table 2 Chemical composition of the TCLP elutriate and HCl Elutriate along the watercourse. Element units are mg kg^{-1}. Fe and Al are given in g kg^{-1}

Sample	TCLP weak acetic acid elutriate					1N HCl elutriate				
	Fe	Al	Zn	Pb	As	Fe	Al	Zn	Pb	As
1	6.8	4.8	3.1	0.06	0.11	0.7	4.2	84	10.5	0.5
2	14.3	23.3	5.4	0.03	0.28	0.2	1.7	27	3.1	0.1
3	0.8	2.2	2.4	0.03	0.14	0.3	2.4	26	3.3	0.3
4	4.5	1.9	1.9	0.03	0.06	0.3	1.8	26	2.7	0.1
5	7.1	9.8	2.0	0.02	0.10	0.2	1.5	25	2.3	0.1
6	0.3	4.9	2.2	0.02	0.07	0.3	2.9	32	3.6	0.2
7	0.6	3.1	1.9	0.02	0.10	0.2	1.4	17	1.8	0.1
8	3.0	8.7	1.7	0.01	0.11	0.3	1.8	22	3.0	0.1
9	2.1	5.4	1.2	0.03	0.35	0.2	1.7	20	2.5	0.1
10	2.1	5.4	1.2	0.03	0.35	0.4	3.9	36	5.4	2.2
11	8.7	11.0	0.7	0.03	0.39	0.2	2.3	21	3.4	0.6
12	9.4	7.0	3.2	0.03	0.16	0.3	2.3	24	3.2	0.3
13	17.9	15.5	5.9	0.04	0.21	0.3	2.8	33	4.5	0.3
14	3.1	6.5	0.9	0.05	0.26	0.4	5.4	34	6.2	0.3
Mean	5.8	7.8	2.4	0.03	0.19	0.3	2.6	30	4.0	0.4
Min.	0.3	1.9	0.7	0.01	0.06	0.2	1.4	17	1.8	0.1
Max.	17.9	23.3	5.9	0.06	0.39	0.7	5.4	84	10.5	2.2
Detection limit (μg L^{-1})	4.6	28	1.8	42	53	4.6	28	1.8	42	53

analyses, to detect the effect of any contaminant present on the sediment, and to take account of possible synergic effects through the detection of their biological effects.

With respect to the phytotoxicity assays, GI for *Lepidium sativum* and *Nasturtium officinalis* showed a linear correlation ($r^2 = 0.57$). They are sensitive to the presence of Pb, showing negative correlations with this element in the TCLP extract ($r^2 = -0.66$ for *Lepidium sativum* and $r^2 = -0.65$ for *Nasturtium officinalis*) (Table 3). Among the three species assayed for the Zucconi test, water cress (NO) was the only plant which showed moderated phytotoxicity.

Since no phytotoxicity was observed, it is difficult to assess which solvent provides the most useful prediction of

Table 3 Correlation coefficients between the TCLP and the 1N HCl elutriates

(a)	TCLP weak acetic acid elutriate								
	Pb	As	Fe	Al	Zn	RG	LS	NO	EC_{50-15}
Pb^1	1								
As^1	0.14	1							
Fe^1	0.34	0.21	1						
Al^1	0.02	0.37	0.81**	1					
Zn^1	0.21	−0.15	0.78**	0.65*	1				
RG	0.08	−0.12	0.45	0.34	0.45	1			
LS	−0.66*	−0.16	−0.06	0.27	0.08	0.06	1		
NO	−0.65*	−0.30	−0.28	0.12	−0.14	0.17	0.57*	1	
EC_{50-15}	−0.08	0.44	−0.11	0.10	−0.06	−0.3	−0.4	−0.1	1
(b)	1N HCl elutriate								
	Pb	As	Fe	Al	Zn	RG	LS	NO	EC_{50-15}
Pb^2	1								
As^2	0.35	1							
Fe^2	0.98**	0.40	1						
Al^2	0.81**	0.45	0.80**	1					
Zn^2	0.95**	0.24	0.94**	0.62*	1				
RG	−0.05	−0.33	−0.13	−0.1	−0.04	1			
LS	−0.56	−0.43	−0.59	−0.6	−0.45	0.06	1		
NO	−0.57	−0.42	−0.60	−0.3	−0.54	0.17	0.57*	1	
EC_{50-15}	0.03	0.55**	0.04	0.16	−0.05	−0.3	−0.4	−0.1	1

[n = 14 except for EC_{50-15} (n = 10)]

* Significant correlation at 0.05 level (bilateral)

** Significant correlation at 0.01 level (bilateral)

trace metal phytoavailability. However, both solvents appear to extract different compartments of the metals in the sediments. In this sense, the diluted HCl seems to extract the metals associated with the Fe and Al oxides, as can be seen in the correlations obtained ($r^2_{Fe–Pb} = 0.98$; $r^2_{Al–Pb} = 0.81$; $r^2_{Fe–Zn} = 0.94$; $r^2_{Al–Zn} = 0.65$). The TCLP extracted also the Zn fraction correlated with Fe and Al, probably associated to oxides ($r^2_{Fe–Zn} = 0.78$; $r^2_{Al–Zn} = 0.65$), but extracted a more labile fraction of Pb, which is not associated with the Fe and Al oxides and which seemed to cause a phytotoxic effect on *Lepidium sativum* and *Nasturtium officinalis*. In this sense, the TCLP, a diluted acetic acid extract, seemed to extract a fraction more accessible to plants, and which represents more accurately the phytoavailability of metals to plants.

The Anllóns River drains a rural basin which is essentially non-polluted. There are in the basin three main sources of pollution: sites 1 (urban pollution), 10–11 (mining activities) and 14 (urban pollution). Samples 10 and 11 showed inhibition of luminescence (60.5% and 50.2%, respectively) probably due to the high As concentrations accumulated in the sediments through the mining activities. However, sites 1 and 14 did not show inhibition of luminescence, instead the high sources of pollution of these urban areas. On the other side, site 3 showed the highest inhibition of luminescence (62.0%) attributed to non-point sources of pollution. This was an unexpected result, as no sources of pollution had been detected at this point. This result evidences the importance of this kind of analysis, which can detect synergic effects of various contaminants, whose individual effect over the environment lacks of importance. With regards to the phytotoxicity results, the three species assayed (ryegrass, garden cress and water cress) showed lower sensitivity. Moreover, the bed sediments can be considered as phytostimulant. However, it is important to note that Pb showed, at sampling point 1, the highest concentrations in either the weak or strong acid digestion, and that there was a significant correlation between Pb and phytotoxicity.

Comparing both sets of data, it can be deduced that the inhibition of luminescence assay using *Vibrio fischeri* showed more sensitivity to the toxicants available in the sediments than phytotoxic assays.

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