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Short communication. An *in vitro* approach for ecotoxicity testing of toxic and hazardous wastes

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Abstract

In waste-characterization, chemicals with a low acute toxicity but with a high long-term hazard such as PBTs (persistent, bioaccumulable, and toxic chemicals) are difficult to identify, leading to an inaccurate assessment of their risk to the environment. This study assesses the sensitivity of *in vitro* assays with rainbow trout cell line RTG-2 as a tool for the ecotoxicological testing of hazardous wastes using a battery of bioassays, including a neutral red (NR) assay for predicting cytotoxicity, a β-galactosidase (βgal) assay for predicting cellular defence, and two sub-lethal damage assays: ethoxy resorufin-O-deethylase (EROD) and glutathione S-transferase (GST) to check for induction of cytochrome and for alteration of antioxidant defences respectively. Six samples of complex waste, covering a wide range of liquid (A to C) and solid (1 to 3) matrices were tested. The EC50-values (NR) ranged from 0.09 mL/100 mL (B) to 2.01 mL/100 mL (2). A hormesis effect (βgal) at low concentrations was observed for the A (0.03-0.18 mL/100 mL), 1 (0.03-0.29 mL/100 mL) and 2 (0.03-0.29 mL/100 mL) samples. A dose-dependent stimulation of enzymatic activity was observed for the B [(EROD (0.58-4.68 mL/100 mL), βgal (0.58-4.68) and GST (0.14-1.17 mL/100 mL)] and C [(EROD (0.29-4.68 mL/100 mL) and βgal (2.34-4.68)] samples. No effects were observed at the highest concentration tested (74 mL/100 mL) for sample 3. The *in vitro* approach can be considered to be an efficient, rapid and cost effective screening system to provide basic information on toxic and hazardous waste for further analysis and risk evaluation.

Additional key words: bioassays, industrial wastes, risk evaluation, RTG-2.

Resumen

Comunicación corta. Aproximación *in vitro* en el análisis de ecotoxicidad de residuos tóxicos y peligrosos

La caracterización de residuos se complica cuando contienen PBTs (sustancias tóxicas persistentes y bioacumulables) y sustancias con baja toxicidad aguda y mucha peligrosidad crónica, impidiendo la evaluación del riesgo ambiental. Este trabajo utiliza la línea celular de trucha RTG-2 para estudiar la sensibilidad de los ensayos *in vitro*, como herramienta en la evaluación ecotoxicológica de residuos peligrosos. La batería de bioensayos incluye la medida de citotoxicidad mediante el test de rojo neutro (RN), de protección celular mediante la actividad β-galactosidasa (βgal) y la medida de la alteración del citocromo y de las defensas antioxidantes mediante las actividades etoxi resorufin-O-dietilasa (EROD) y glutation S-transferasa (GST) respectivamente. Se ensayaron tres residuos líquidos (A a C) y tres sólidos (1 a 3) representando un amplio rango de muestras. Los valores de EC50 (RN) variaron entre 0,09 mL/100 mL (B) y 2,01 mL/100 mL (2). Se detectaron fenómenos de hormesis (βgal) a bajas concentraciones en las muestras A (0,03-0,18 mL/100 mL), 1 (0,03-0,29 mL/100 mL) y 2 (0,03-0,29 mL/100 mL). Se detectó aumento de las actividades enzimáticas en las muestras B [(EROD (0,58-4,68 mL/100 mL), βgal (0,58-4,68 mL/100 mL) y GST (0,14-1,17 mL/100 mL)] y C [(EROD (0,29-4,68 mL/100 mL) y βgal (2,34-4,68 mL/100 mL)], mientras que con la muestra 3 no se observaron efectos a la concentración más alta (74 mL/100 mL). La propuesta *in vitro* puede ser un método eficaz, rápido y barato para proporcionar información básica que permita orientar análisis posteriores y evaluar el riesgo de los residuos tóxicos y peligrosos.

Palabras clave adicionales: bioensayo, residuos industriales, riesgo ambiental, RTG-2.

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Pollution of water by toxic and hazardous wastes containing chemicals and biologically active substances is an issue of current importance. Chemicals, due to their persistence, toxicity and bioaccumulation potential, can produce effects in the very long term and through trophic chains. Biologically active substances can produce low acute toxicity but a high long term hazard. The EEC Hazardous Waste Directive (OJ, 1991) defines 14 H-criteria for the characterization of hazardous wastes. Among these is H-14 «Ecotoxic» meaning that the sample could be a risk for one or more compartments of the environment.

At present the ecotoxicological hazards posed by H14 substances are evaluated by the response of appropriate organisms representing different trophic levels exposed under controlled laboratory conditions. However, the generation of new data using the Organisation for Economic Co-operation and Development (OECD) methods is costly, very time consuming and requires considerable animal use. The European Chemicals Policy suggests a change in the testing strategy (IHCP, 2004), recognising the need to develop «Intelligent Testing Strategies» allowing the survey of as many of the ecotoxicological properties of waste samples as possible. This is essential for the design of suitable elimination/management procedures.

The Madrid Region produces 300,000 Mg yr^{-1} of toxic and hazardous wastes that require a characterization phase, mostly during treatment and disposal, allowing the identification of priority pollutants. The initial ecotoxicological evaluation of wastes is an area of expanding possibilities for the use of *in vitro* bioassays (Kirso *et al*., 2007). A further advantage of cell biochemical measurement over fish tests is the array of endpoints that can be easily and rapidly measured with an *in vitro* test, for detecting specific and/or general damage at the cellular and sub-cellular level, thus leading to much more specific toxicity characterisation of the sample than a lethality test.

In this study, the feasibility of the application of *in vitro* screening procedures and rapid assessment techniques in the hazard determination for toxic industrial wastes was examined, using a battery of cellular biochemical responses, to identify samples of interest and provide basic information for further analysis.

Table 1. EC50 values (mL/100 mL) and its 95% confidence intervals (CI) for the neutral red (NR) test of the six samples assayed

Samples	EC50	CI	
A Halogenated solvent (dry cleaner's and distillation process)	0.81	0.58	1.31
B No halogenated solvent	0.17	0.07	0.81
C Organic flammable water (iron and steel, chemical industries)	0.67	0.41	1.44
1 Sepiolite RS-866	3.30	2.07	7.79
2 Cosmetic RS-867	3.26	2.43	4.92
3 Silica RS-868	—	—	—

Three industrial whole liquid waste samples and three different industrial solid waste materials (Table 1) were assayed. Soluble fractions of the solid samples were prepared in reconstituted water (BOE, 1989). The liquid wastes and aqueous extracts from the solid wastes were subsequently used to reconstitute 10X EMEM media. This procedure allowed exposure of RTG-2 cells to concentrations of around 75% of the original aqueous sample. Untreated controls (water reconstituted 10X EMEM media) were run in parallel.

Cell viability was quantified using the neutral red (Borenfreund and Puerner, 1985) assay. In addition, total cell protein was measured (Knox *et al*., 1986) using the Kenacid blue protein (KBP) assay. The presence of co-planar organic toxic chemicals was indicated by the induction of cytochrome CYP1A, which was measured as 7-ethoxyresorufin-O-deethylase (EROD) activity (Babin and Tarazona, 2005). Cellular defence was predicted by a β-galactosidase (βgal) assay (Babin and Tarazona, 2005) and the alteration of antioxidant defences by a glutathione S-transferase (GST) activity assay (García-Alfonso *et al*., 1998). The EROD and βgal parameters were estimated from the areas under the curve, using OECD method 201 (OECD, 1993). A TECAN-Genius spectrofluorometer was used to quantify fluorescence (kinetic way) and absorbance endpoints.

The cytotoxicity assessment of the wastes revealed that the mixtures investigated were very toxic, as indicated by the low concentrations at which five of the six test solutions had a 50% effect (EC50; Table 1).

Abbreviations used: EMEM (minimal Eagle medium with Earle's BSS), EROD (ethoxy resorufin-O-deethylase), GST (glutathione S-transferase), KBP (Kenacid blue protein), LOEC (lowest observed effect concentration), NOEC (no observed effect concentration), NR (neutral red), PBT (persistent, bioaccumulable, and toxic chemical), RTG-2 (rainbow trout gonadal cell), βgal (β-galactosidase).

Although it is assumed that the endpoints used for measuring the cytotoxic potential of complex mixtures, NR and KBP, are equivalent (Barile, 1994), the shape of the NR and KBP curves shows that the use of NR levels as a cytotoxicity index was a more sensitive parameter of cytotoxicity than the protein estimation (data not shown). This is consistent with previous work where EC50 values in the NR assay were equally or more sensitive than the KBP assay (Castaño *et al*., 1995).

Due to the wide dose range tested (74-0.03 mL/100 mL), samples A (0.03-18 mL/100 mL), 1 (0.03-0.29 mL/100 mL) and 2 (0.03-0.29 mL/100 mL) revealed hormesis characteristics (Figs. 1 and 2) where increased fluorescence was observed at low, non-cytotoxic concentrations in the βgal assay. The dose stimulatory response for these three samples was no more than twofold greater than the control response, while the width of stimulatory response was less than 100-fold in dose range immediately contiguous with the toxicological no observed effect concentration (NOEC). This accords with the study of Calabrese and Blain (2005). Hormesis represents a possible beneficial overcompensation in response to disruption in homeostasis to adverse stimulus. Hormesis effects have been observed from a broadly diversified spectrum of chemical classes (Anderson, 2005; Calabrese and Blain, 2005).

For enzymatic activity either stimulation or inhibition are considered significant signals of cellular stress, and both effects were observed in this study. βgal activity was induced by the B and C samples. The lowest observed effect concentration (LOEC) for stimulation was at 0.58 mL/100 mL for B and 2.34 mL/

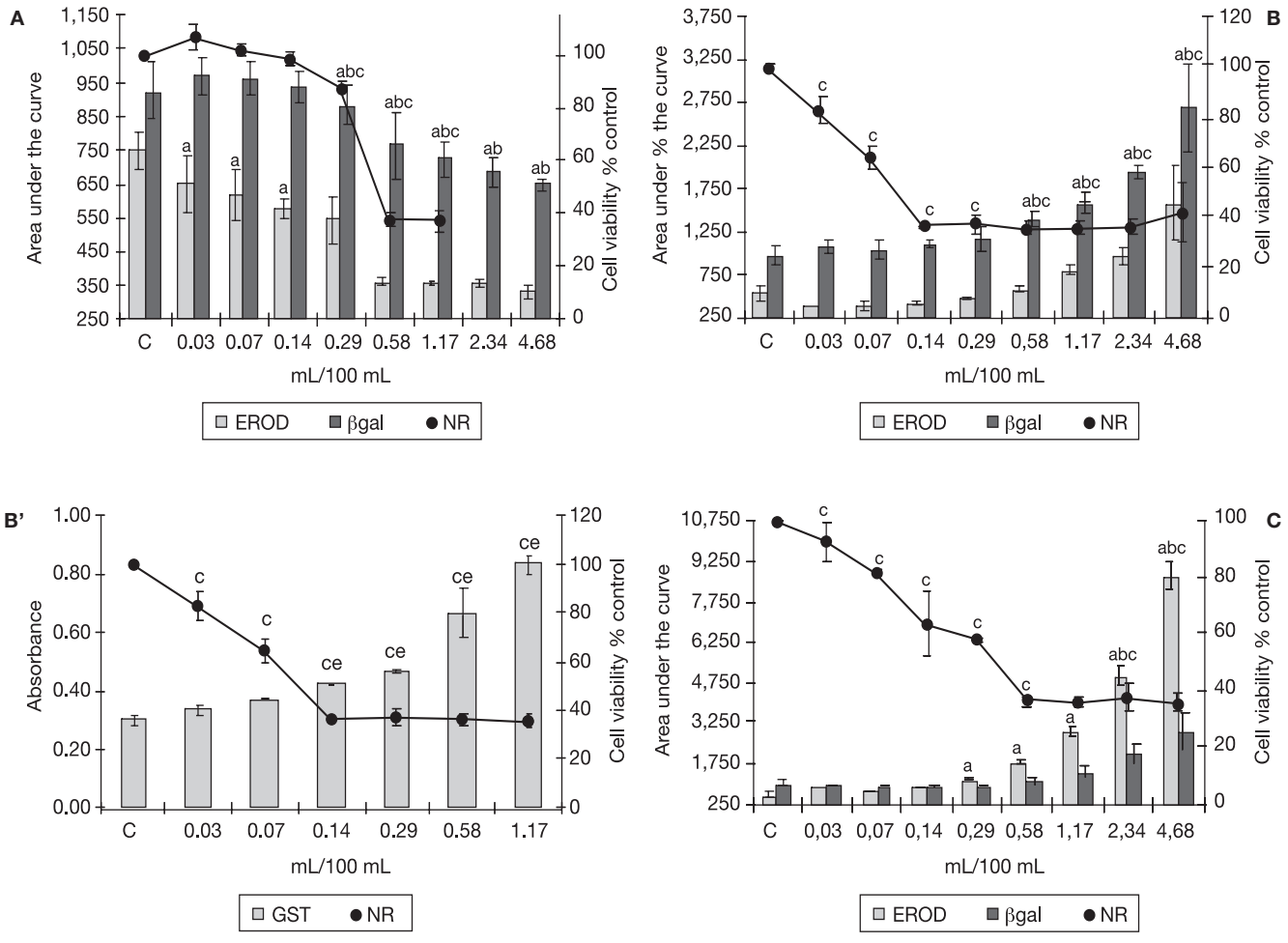

Figure 1. Enzymatic activity and cell viability of trout gonadal RTG-2 cells, exposed to increased A, B and C liquid sample concentrations. The data are expressed as mean ± SD of three independent experiments (three replicates per assay). a (EROD), b (βgal), c (NR) and e (GST) values are statistically different (one-way ANOVA) from the control (C, EMEM medium) ($p < 0.05$).

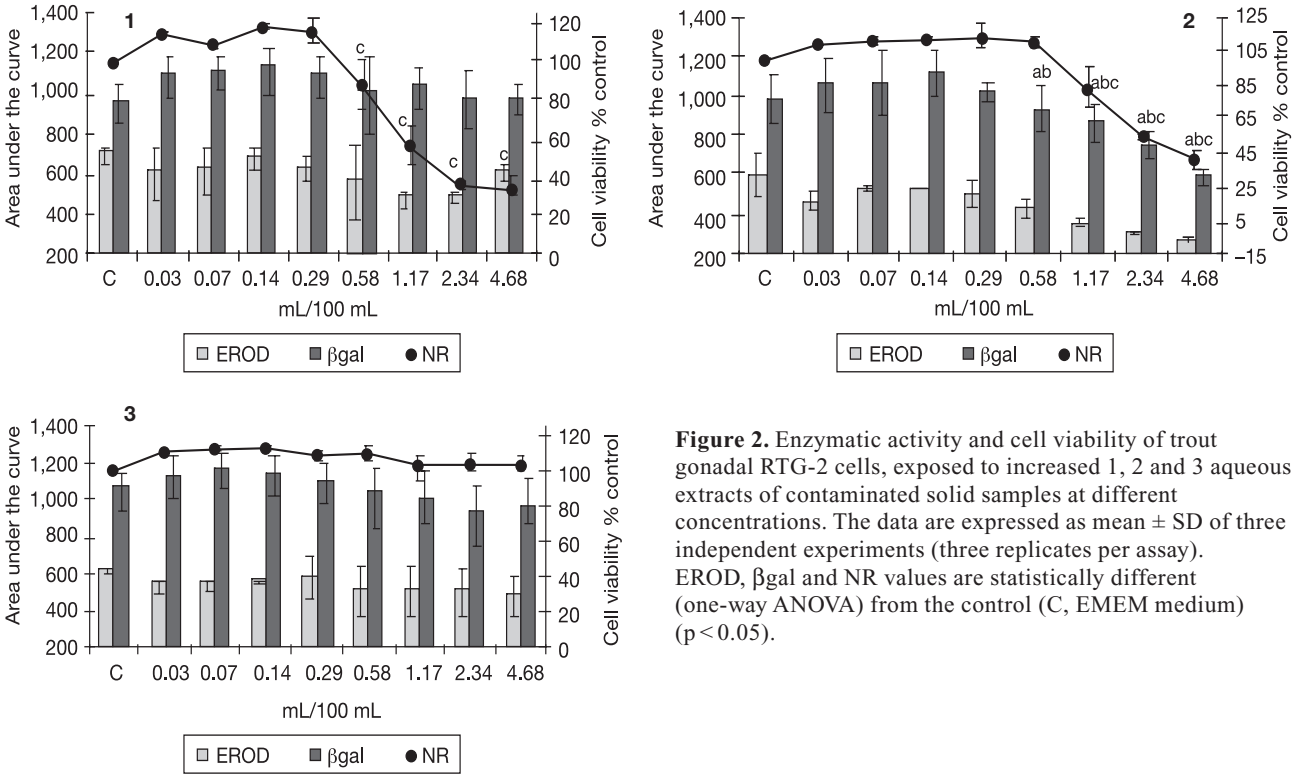

Figure 2. Enzymatic activity and cell viability of trout gonadal RTG-2 cells, exposed to increased 1, 2 and 3 aqueous extracts of contaminated solid samples at different concentrations. The data are expressed as mean ± SD of three independent experiments (three replicates per assay). EROD, βgal and NR values are statistically different (one-way ANOVA) from the control (C, EMEM medium) ($p < 0.05$).

100 mL for C (Fig. 1), the increase of βgal activity has not been described and will need further study and evaluation.

The EROD induction, mediated through the aryl hydrocarbon receptor (AhR), is a typical response for co-planar molecules. In the present test battery EROD measurements are not used for addressing CYP1A activity, but rather as a cost/effective and sensitive metabolic endpoint, where both stimulation and inhibition offer useful information. EROD activity was stimulated by the B and C samples. The LOEC for stimulation was 0.58 mL/100 mL for B and 0.29 mL/100 mL for C (Fig. 1). Samples A and 2 induced dose-related inhibition responses of EROD and βgal activity (Figs. 1 and 2). Inhibition was observed at the lowest tested concentration of 0.03 mL/100 mL for sample A. The inhibition of EROD by sample A, at concentrations lower than those inducing reductions in NR (0.03-0.14 mL/100 mL) could be explained by the chemical inhibitors present in the sample. The EROD inhibitors giving decreased P450 levels, and their xenobiotic-metabolizing activity was described by Soltis and Colby (1998) and Willett *et al*. (2001). EROD and βgal inhibition responses were also observed at concentrations producing dramatic effects on cell viability (NR) by samples A (0.29 mL/100 mL) and 2 (1.17 mL/100 mL) (Figs. 1 and 2). For extracts of sample 2, the βgal assay was more sensitive than the NR assay, and effects were observed at concentrations of 0.58 and 1.17 mL/100 mL, respectively (Fig. 2).

GST has physiological role in initiating detoxification of potential alkylating agents, thereby neutralizing their electrophilic sites and rendering the products more water-soluble. Cytosolic GST activity was only increased by sample B (Fig. 1). The LOEC for stimulation was at 0.07 mL/100 mL. Several studies have shown that PAHs, BNF and PCBs mixtures increase hepatic GST activity *in vitro* between 1.5 and 2-fold (Celander *et al*., 1993; Sandbacka and Isomaa, 2000; Billiard *et al*., 2004).

The liquid waste samples were more toxic than the solid waste samples when evaluating all endpoints. This was probably because the ecotoxic potential of the solid samples is only caused by the fraction mobilised by water.

The use of *in vitro* bioassays allowed the simultaneous handling of several endpoints and a large number of replicates at a very competitive cost. This study shows that batteries of *in vitro* tests can be applied to a full range of liquid and solid wastes. When results from

individual endpoints are obtained in a test battery, there is a high probability of characterizing the chemicals with specific and general mechanisms of action, in terms of their net effect rather than a dose's effect on a single factor. Toxicity of waste samples and extracts is usually related to a combination of chemicals acting through similar and/or different mechanisms depending on waste composition. A battery covering generic, metabolic and specific endpoints as well as a wide dose range can be particularly relevant for detecting hazards associated with the presence of biologically active chemicals. The stimulation of EROD or βgal at low exposure doses followed by inhibition of the same activities at higher exposure levels is an example. In conclusion, its versatility, speed and simplicity lead to recommend assays based on batteries of tests to provide basic (eco)toxicological information for mixtures of contaminants in toxic, hazardous wastes.

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