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Ecotoxicological assessment of bromobenzene using a test battery with five model systems

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Abstract

Bromobenzene (BrB) is used as a solvent for crystallization and as an additive to motor oils and may be released into the environment through various waste streams. However, there is limited available information about the toxic hazard of BrB in the aquatic environment. Consequently, the ecotoxicological effects induced by BrB were investigated using five model systems with representants from four trophic levels. The battery included bioluminescence inhibition of the bacterium *Vibrio fischeri*, growth inhibition of the alga *Chlorella vulgaris* and immobilization of the cladoceran *Daphnia magna*. Total protein content, neutral red uptake and MTS metabolization were reduced, while lysosomal function, succinate dehydrogenase activity, G6PDH activity and leakage, metallothionein levels and EROD activity were stimulated in PLHC-1 and RTG-2 fish cell lines. The most sensitive bioindicator was the bioluminiscence of *V. fischeri*, with an EC_{50} of 0.04 mM BrB at 15 min and a non-observed adverse effect level of 0.02 mM BrB. There is a large difference in sensitivity to BrB among the model systems probably due to the metabolic capacity of the different species. PLHC-1 cells were more sensitive to BrB than RTG-2 cells. The most prominent morphological effects observed were hydropic degeneration, loss of cells and of the perinuclear pattern of distribution of lysosomes. Therefore, BrB should be classified as toxic to aquatic organisms.

Keywords: Bromobenzene; *In vitro*; Ecotoxicity; Cytotoxicity; Contamination; Alternatives

1. Introduction

Bromobenzene (BrB) is a colourless liquid with a characteristic aromatic odour. It is used as an industrial solvent, as an intermediate in organic synthesis and as an additive to motor oils. It can be present in atmospheric, terrestrial and aquatic environments, being biologically non-degradable with an estimated log K_{ow} of 2.99. As there is limited ecotoxicological information available about BrB, apart from the reported 0.23 mM LC_{50} at 96 h for fathead minnow (Marchini et al., 1993), it is convenient to investigate its potential aquatic effects.

BrB can be absorbed by ingestion, by inhalation and through intact skin. BrB is a classic hepatotoxic agent whose bioactivation is crucial to the development of liver injury (Wong et al., 2000). Liver toxicity is due to the transformation of BrB to reactive intermediates (2,3- and 3,4-bromobenzene epoxides) by the cytochrome P450 system (Lau and Zannoni, 1981). The binding of these reactive metabolites to proteins like glutathione S-transferase, liver

Abbreviations: BrB, bromobenzene; EC_{50}, mean effective concentration; EROD, ethoxyresorufin-*O*-deethylase; G6PDH, glucose-6-phosphate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; NOAEL, non-observed adverse effect level; SDH, succinate dehydrogenase.

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fatty acid binding protein or carbonic anhydrase is highly correlated with pathological effects (Koen et al., 2000). The reactive epoxides are metabolized in several steps, being probably detoxified by epoxide hydratase and glutathione transferase, and then excreted in urine, causing a decrease in glutathione levels leading to oxidative stress and lipid peroxidation (Heijne et al., 2004).

Model systems and bioassays are currently used in experimental ecotoxicology and environmental toxicology to provide information for risk assessment and registration of chemicals, as well as to investigate their effects and mechanisms of action (Repetto et al., 2003). Environmental toxicology is a multidisciplinary science that encompasses several diverse areas of study, such as biology, chemistry, ecology or microbiology. The tools of environmental toxicology include biological assays, which provide essential knowledge concerning the biological responses of individual organisms to pollutants (Yu, 2004).

The aim of this study was to evaluate the acute ecotoxicological hazard of BrB with a representative and cost-effective test battery comprising organisms representing four trophic levels of the aquatic ecosystem, formed by five ecological model systems with several endpoints measured at different exposure time periods. The battery included bioluminescence inhibition of the bacterium *Vibrio fischeri* (decomposer), growth inhibition of the alga *Chlorella vulgaris* (1st producer) and immobilization of the cladoceran *Daphnia magna* (1st consumer). Although daphnia and alga tests are accepted in most environmental legislations, including OECD Guidelines for the testing of chemicals, the vibrio test has only been adopted by several regulations for the characterization of hazardous wastes.

Cell morphology, total protein content, neutral red uptake, MTS metabolization, lysosomal function, succinate dehydrogenase activity (SDH) and glucose-6-phosphate dehydrogenase (G6PDH) leakage and activity were studied in two different fish cell lines: PLHC-1 derived from a hepatocellular carcinoma of the topminnow *Poeciliopsis lucida* and RTG-2 derived from rainbow trout normal gonad cells of *Oncorhynchus mykiss* (2nd consumers). In addition, metallothionein levels and Ethoxyresorufin-*O*-deethylase (EROD) activity were studied. Fish cell lines are useful tools for ecotoxicological evaluation of many chemicals. The use of *in vitro* methods in environmental testing, particularly those employing fish cell cultures, is an area of expanding possibilities in the ecotoxicological evaluation of mixtures, for controlling chemicals, emissions, effluents and hazardous wastes (Repetto et al., 2003; Castaño et al., 2003).

2. Materials and methods

2.1. Toxicant exposure

A range of different concentrations of exposure solutions of bromobenzene (Sigma®) was prepared before use directly in the different culture media, according to the appropriate assay, sonicated for 30 min and sterilized by filtration through a 0.22 μm Millipore® filter. After replacing the medium with the exposure solutions, the systems were incubated for the adequate exposure time period.

2.2. Model systems

2.2.1. Vibrio fischeri

Bioluminescence inhibition in the marine bacterium *V. fischeri* was evaluated according to Cordina et al. (1993) by using freeze-dried bacteria incubated at 15 °C from Microtox® test (Microbics Corp., Carlsbad, USA).

2.2.2. Chlorella vulgaris

Growth inhibition of the alga *C. vulgaris var viridis*, kindly provided by Dr. Muñoz-Reoyo (CISA, Spain) was evaluated in 96-well culture plates seeded with 200 μl/well of a 1,000,000 cells/ml algae culture in exponential

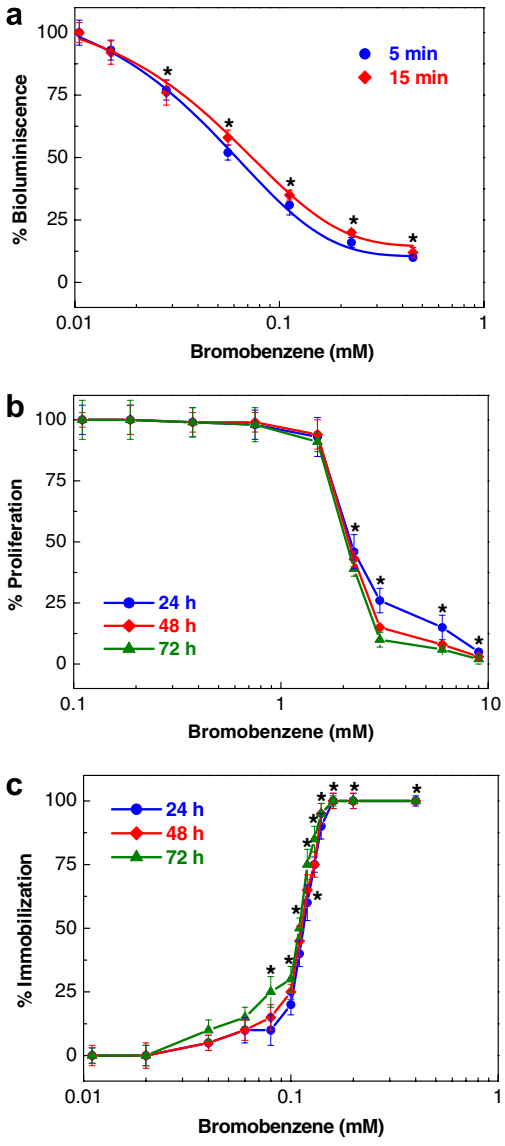

Fig. 1. Effects of exposure to different concentrations of bromobenzene studied as (a) bioluminescence inhibition of the bacterium *Vibrio fischeri* at 5 (●) and 15 min (◆); (b) proliferation of the alga *Chlorella vulgaris* and (c) immobilization of the cladoceran *Daphnia magna* at 24 (●), 48 (◆) and 72 h (▲). Data expressed in % of unexposed controls. * Indicates significant difference from control value ($p < 0.05$).

growth phase in Bold's Basal Medium, using constant agitation and a temperature of 22 °C, under a water-saturated sterile atmosphere containing 5% CO_2 and a cold light source of 8000 lux. Absorbance at 450 nm was read on a Multiscan RC plate reader (Labsystem, Helsinki, Finland). As a quality criteria, the control cultures had to grow at least tenfold in 48 h (Ramos et al., 1996).

2.2.3. Daphnia magna

D. magna, kindly provided by Dr. Muñoz-Reoyo (CISA, Spain), was maintained at 20 °C and fed with *C. vulgaris*. Acute toxicity immobilization tests were performed in standard reference water according to OECD Guideline 202 (2004) in replicate groups of 10 neonates per 25 ml, in 70 ml polystyrene flasks (Costar, Cambridge, MA, USA).

2.2.4. PLHC-1 cells

The hepatoma PLHC-1 cell line was derived from a hepatocellular carcinoma induced with 7,12-dimethylbenz(*a*)anthracene in an adult female *Poeciliopsis lucida*, a topminnow from the Sonoran desert (ATCC®# CRL-2406). The cells retain some of the characteristic morphology of primary liver hepatocytes, are epithelial, present and average population doubling time of 39.4 h, express aryl hydrocarbon receptors and basal and inducible P450IA activity (Ryan and Hightower, 1994; Fent, 2001). PLHC-1 cells were grown at 30 °C in a humidified incubator containing 5% CO_2 and propagated in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% foetal bovine serum (Gibco™), L-glutamine (BioWhittaker), sodium pyruvate (BioWhitaker) and non-essential amino acids (BioWhittaker). PLHC-1 cells in exponential growth phase were plated applying 0.2 ml of 450,000 cells/ml in each well of 96-well tissue culture plates (Costar). After 24 h at 30 °C, the cultures received 0.2 ml medium containing the test chemical and were incubated for a further 24, 48 or 72 h. For the morphological study, PLHC-1 cells were seeded in Lab-Tek® tissue culture 8 well chamber slides applying 0.5 ml of 500,000 cells/ml (Nunc, Inc., Naperville, IL) previously coated with Matrigel™ (BD Biosciences). They were then exposed to BrB for 24, 48 and 72 h, fixed in 70% methanol and stained with Mayer's hematoxylin and eosin or subjected to *in situ* hybridization (TUNEL) to detect induction of apoptosis (Enzo, Diagnostics, Farmingdale, US).

2.2.5. RTG-2 cells

The RTG-2 salmonid fish cell line, derived from normal gonad cells of a rainbow trout (Oncorhynchus mykiss) was kindly provided by Dr. Castaño (ISCIII, Spain). The cells present long spindle-like and fibroblast-like morphology, an average population doubling time of 72 h and basal and inducible P450IA activity (Araujo et al., 2000). The cell line was grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% foetal bovine serum (Biochrom), L-glutamine (BioWhittaker), and non-essential amino acids (BioWhittaker). RTG-2 cells in exponential growth phase were plated in 0.2 ml of 40,000 cells/ml in each well of 96-well tissue-culture plates (Costar). After 48 h at 20 °C, the culture medium was replaced with 0.2 ml test medium and then incubated for a further 24 or 48 h (Castaño et al., 2003). For the morphological study, RTG-2 cells were seeded in Lab-Tek® tissue culture chamber slides at applying 0.5 ml of 50,000 cells/ml per well (Nunc, Inc., Naperville, IL). They were then exposed to BrB for 24 and 48 h, fixed in 70% methanol and stained with Mayer's hematoxylin and eosin or subjected to *in situ* hybridization

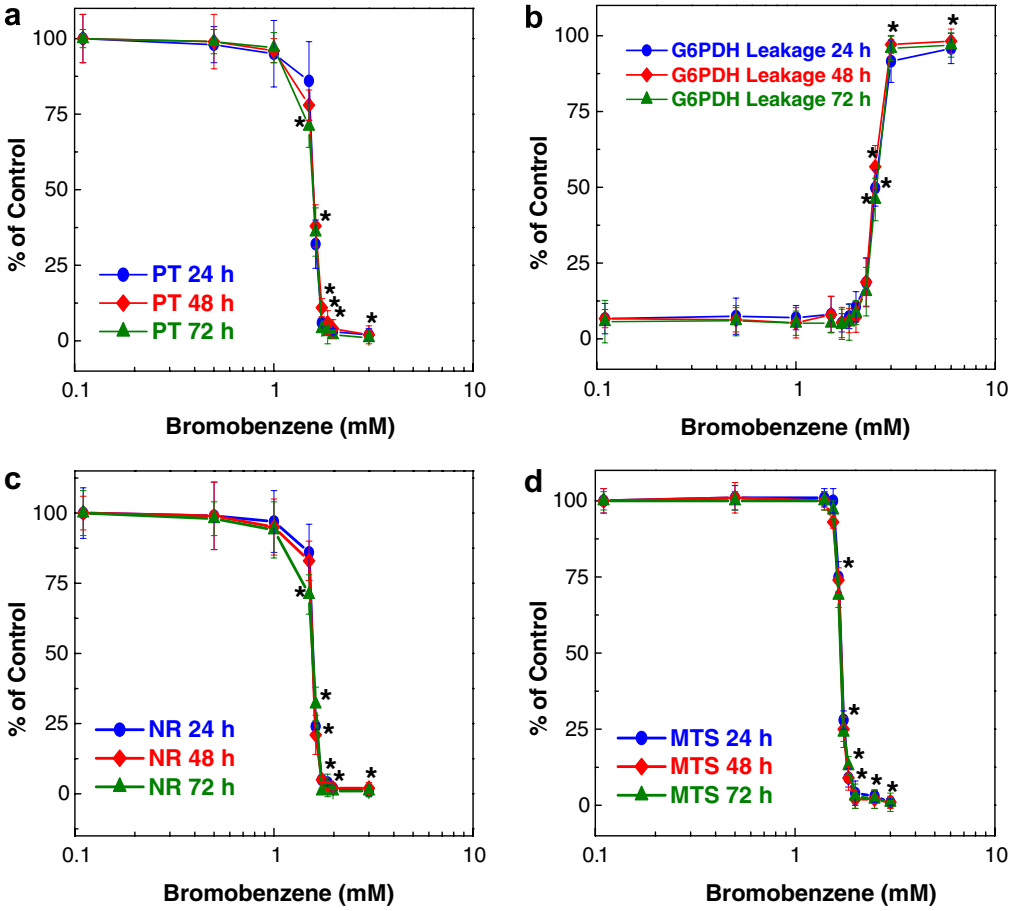

Fig. 2. Effects of bromobenzene on PLHC-1 fish cell cultures (a) total protein content, (b) G6PDH leakage, (c) neutral red uptake and (d) MTS metabolization after exposure to different concentrations for 24 (●), 48 (◆) and 72 h (▲). Data expressed in % of unexposed controls. * Indicates significant difference from control value ($p < 0.05$).

(TUNEL) to detect induction of apoptosis (Enzo, Diagnostics, Farmingdale, US).

2.2.6. Fish cell bioindicators

Total cellular protein content was quantified *in situ,* using Coomassie brilliant blue G-250 (Repetto and Sanz, 1993) in the same 96-well tissue-culture plates in which exposure originally took place (Jos et al., 2003; Repetto et al., 2001). Absorbance at 620 nm was read on a Multiscan RC plate reader (Labsystem, Helsinki, Finland). The lysosomal uptake of the supravital dye neutral red was evaluated according to Babich and Borenfreund (1987) and lysosomal function was calculated expressing the uptake in relative form to the protein content of the culture (Repetto and Sanz, 1993). After 3 h of incubation with neutral red in complete medium and a brief fixation with formaldehyde–$CaCl_2$, the dye was extracted with acidified ethanol for 20 min and quantified at 540 nm. After a brief wash with deionized water, protein content was quantified as above. The MTS tetrazolium reduction assay was performed according to a procedure based on Baltrop et al. (1991). The MTS tetrazolium compound is bioreduced by cells into a coloured formazan product that is soluble in tissue culture medium. G6PDH activity, in cells and in culture medium, was determined as described by Garcia-Alfonso et al. (1998). Metallothionein induction in cells was determined using the Cadmium/Haemoglobin affinity assay (Eaton and Cherian, 1991). EROD activity, a catalytic measurement of cytochrome P4501A induction, was determined by a direct fluorometric method described by Hahn et al. (1996).

2.3. Calculations and statistical analysis

All experiments were performed at least three times and at least in duplicate per concentration. Values for enzyme activities, lysosomal function and metallothionein levels were corrected for cell culture total protein content to avoid misinterpretation due to the influence of the chemical tested on cell proliferation and cell detachment. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. Mean effective concentration (EC_{50}) values were determined by probit analysis.

3. Results

The acute ecotoxicological effects of BrB were investigated using five model systems of four trophic levels. The battery included representants of one decomposer (*V. fischeri*), one 1st producer (*C. vulgaris*), one 1st consumer (*D. magna*) and two 2nd consumers (the hepatoma fish cell line PLHC-1 and the fibroblastic fish cell line RTG-2).

3.1. Effects on Vibrio fischeri, Chlorella vulgaris and Daphnia magna

The most sensitive model system was the inhibition of bioluminescence of the bacterium *V. fischeri*, with an EC_{50} value of 0.04 mM at 15 min (Fig. 1a). This finding is similar to the results obtained by Kaiser and Palabrica (1991). On the contrary, the least sensitive model system to BrB was the inhibition of the proliferation of the fresh-water algae *C. vulgaris* in 96-well microtiter plates, with an EC_{50} value of 2.35 mM at 24 h (Fig. 1b). Nevertheless, the immobilization of the cladoceran *D. magna* was

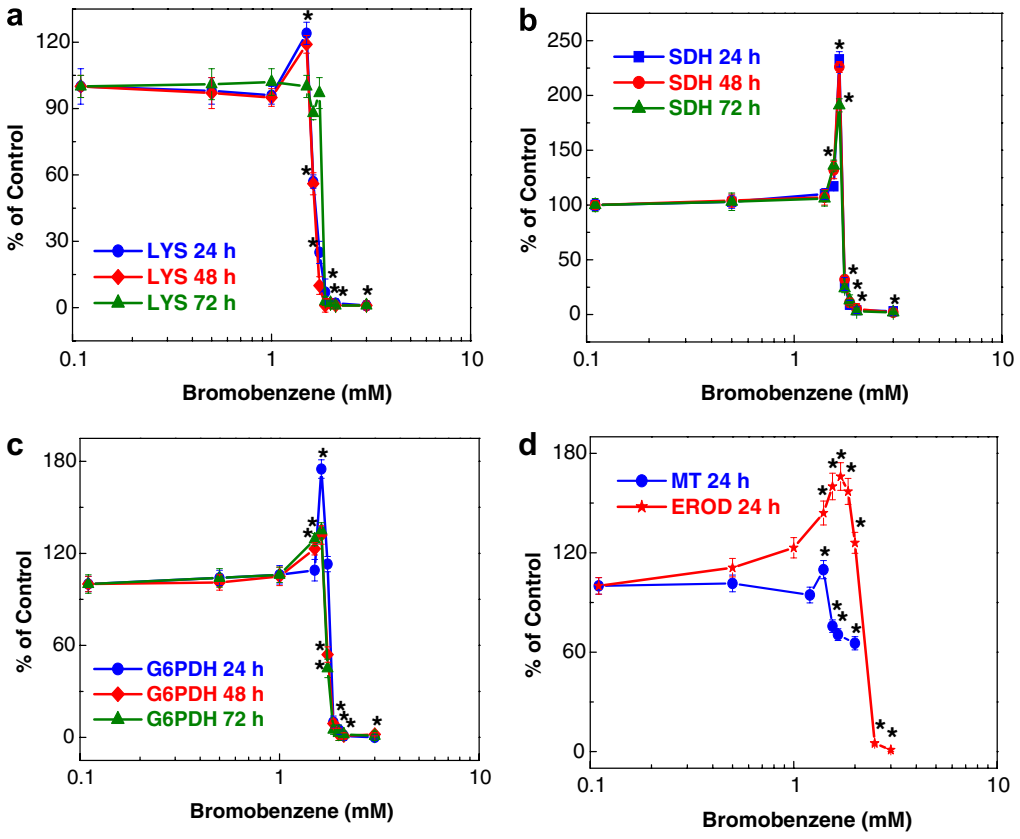

Fig. 3. Effects of bromobenzene on PLHC-1 fish cell cultures (a) lysosomal function, (b) succinate dehydrogenase activity and (c) G6PDH activity for 24 (●), 48 (◆) and 72 h (▲); (d) metallothionein (●) and EROD (∗) levels after 24 h exposure. Data expressed in % of unexposed controls. ∗ Indicates significant difference from control value ($p < 0.05$).

also a very sensitive model system to BrB, showing EC_{50} values of 0.11, 0.10 and 0.09 mM after 24, 48 and 72 h of exposure, respectively (Fig. 1c).

3.2. Effects on the hepatoma fish cell line PLHC-1

The effects of different concentrations of BrB were also investigated using the hepatoma fish cell line PLHC-1 at morphological, basal cytotoxicity (total protein content, G6PDH leakage, neutral red uptake and MTS metabolization) and biochemical levels (lysosomal function, SDH activity, G6PDH activity, metallothionein content and EROD activity). The cells present metabolic activity, a requirement to study the metabolism and the toxicity of environmental chemicals. The results obtained for the different endpoints evaluated on PLHC-1 cells are shown in Figs. 2 and 3. An intermediate sensitivity in comparison with the other models was found in PLHC-1 cells after 24 h of BrB exposure. The EC_{50} values obtained ranged from 1.58 mM for neutral red uptake to 2.58 mM for G6PDH leakage, with a progressive concentration-dependent increase in the leakage from 1.5 mM. The content of total protein and the uptake of neutral red were the most sensitive bioindicators in PLHC-1 cells, being drastically reduced at 24, 48 and 72 h. The lysosomal function and the metabolic markers SDH and G6PDH activities were also altered by BrB, being significantly stimulated from 1 up to 1.7 mM. A slight increase was observed after 24 h of exposure in metallothionein levels. EROD activity showed a 70% induction from 1.55 to 1.85 mM BrB.

Morphological changes, induced by BrB, were investigated in the hepatoma fish cell line PLHC-1 (Fig. 4). As described by Ryan and Hightower (1994), the control cultures retain some of the characteristic morphology of hepatocytes. PLHC-1 cells present polygonal form, sinuous borders, with secretion vesicles around the cellular surface and are disposed in a uniform monolayer. They have abundant deposits of glycogen, tight junctions near the apical surface and basolateral interdigitations. PLHC-1 cell cultures exposed to concentrations higher than 1 mM BrB for up to 72 h presented evident alterations, including reduction of cell number. The most prominent effect observed in cells exposed to 1.5 mM BrB was hydropic degeneration of the cytoplasm (cellular swelling) and the decrease in secretion vesicles. With 1.7 mM BrB, loss of cells, general damage, apoptotic bodies and reduction of the lysosomal function were observed.

3.3. Effects on the fibroblastic fish cell line RTG-2

The fifth model system selected was the salmonid cell line RTG-2. The results obtained for the different endpoints evaluated are shown in Figs. 5 and 6. BrB also presented a dose-dependent toxicity in RTG-2 cell cultures.

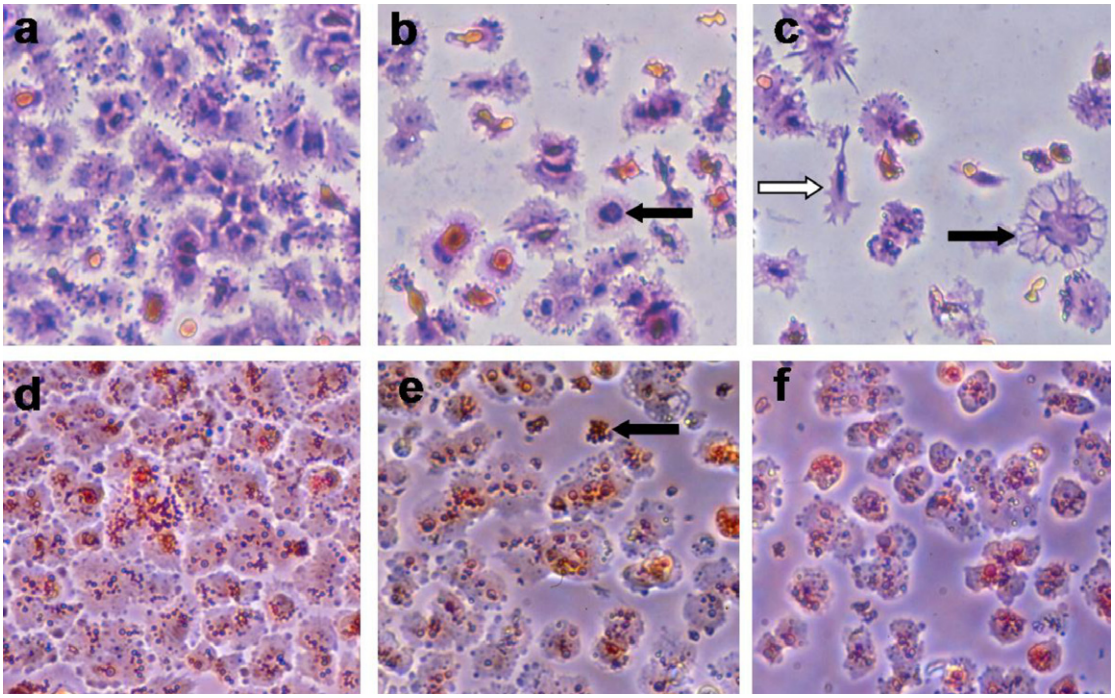

Fig. 4. Morphology of PLHC-1 cell cultures (×1600) stained with Mayer's hematoxylin and eosin (a–c) or neutral red (d–f): (a) Control culture of PLHC-1 cells presents polygonal form, sinuous borders, with secretion vesicles around the cellular surface. (b) Culture treated with 1 mM bromobenzene for 24 h showing loss of cells, cytoplasmic vesicles and hydropic degeneration of the cytoplasm (cellular swelling) (→). (c) Multiples changes were observed in the cultures treated with 1.5 mM bromobenzene for 24 h, including decrease of the number of cells, hydropic degeneration of the cytoplasm (→) and presence of pyknotic nuclei and condensed cytoplasm (⇒). (d) Control culture of PLHC1 cells treated with neutral red. (e) Culture of cells exposed for 24 h to 1 mM bromobenzene showing a general decrease of the uptake of neutral red, though some cells present evident accumulations (→). (f) Culture exposed for 24 h to 1.5 mM bromobenzene with more evident damage, reduction in cell number, rounded cells and reduced uptake.

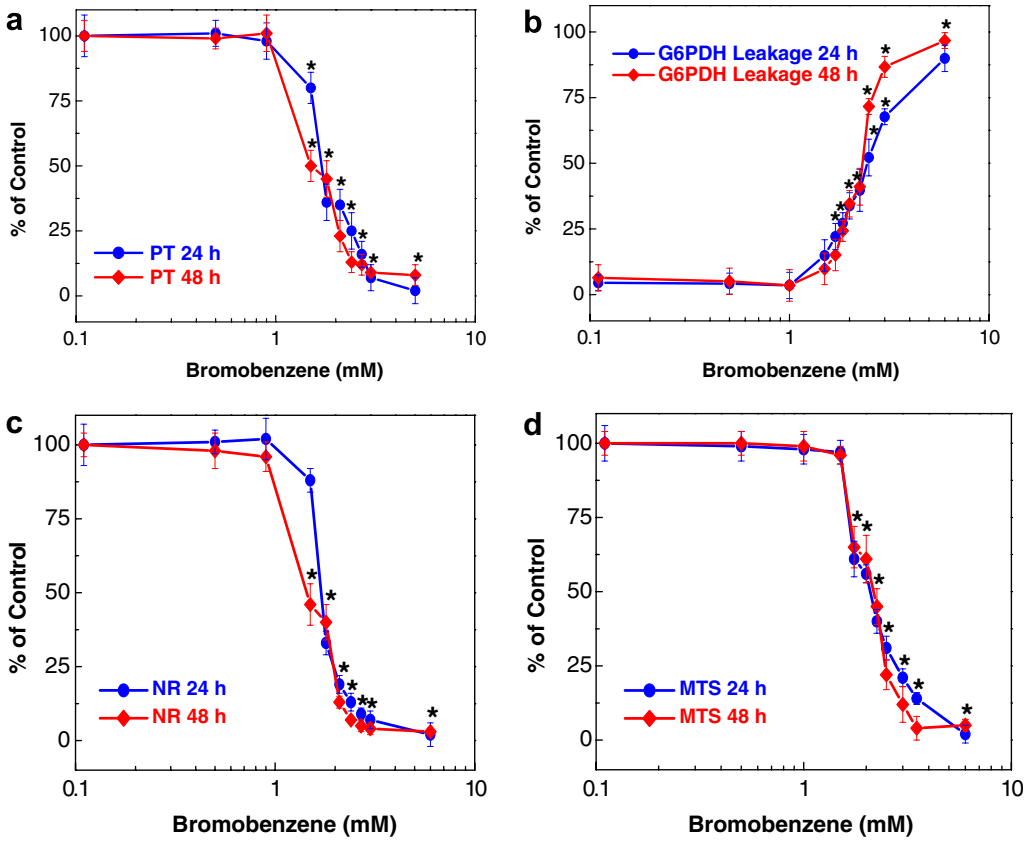

Fig. 5. Effects of bromobenzene in rainbow trout RTG-2 cells in (a) total protein content, (b) G6PDH leakage, (c) neutral red uptake and (d) MTS metabolization after exposure for 24 (●) and 48 h (◆). Data are expressed in % of each respective control treatment. ∗ Indicates significant difference from control value ($p < 0.05$).

However, this cell line was less sensitive than PLHC-1. With EC_{50} values between 1.77 and 1.85 mM, the uptake of neutral red was the most sensitive bioindicator, followed closely by the content of total protein. The lysosomal function and the activity of G6PDH were clearly stimulated from 1 mM at 24 h of exposure, showing a 50% rise at 1.5 mM. Nevertheless, no significant increase was detected in SDH activity. A progressive concentration-dependent increase in G6PDH leakage, a marker of cell death, was observed from 1 mM. A slight increase was detected in EROD activity in RTG-2 cells after 24 h of exposure, with a maximum level of induction with 1.85 mM.

Morphological changes induced by BrB were also investigated in RTG-2 cells (Fig. 7). The control cultures showed fusiform cells, arranged in plaques and disposed in parallel. They have well defined borders, eosinophilic cytoplasm and central nuclei. The morphological alterations were evident from 1.7 mM, the main changes observed being the induction of hydropic degeneration of the cytoplasm, cellular pleomorphism and reduction in cell number. A general loss of lysosomes and of their perinuclear pattern of distribution and a clear visualization of nucleolar structures was detected, possibly due to chromatine condensation around these organelles.

Table 1 includes the EC_{50} values for the different systems and biomarkers studied in the proposed ecotoxicological test battery. Considering all the data obtained, the sensitivity of the model systems decreased as follows: *V. fischeri* > *D. magna* > PLHC-1 cells > RTG-2 cells > *C. vulgaris*. Very small variations in the EC_{50} values for the different exposure periods were obtained in every studied model, demonstrating a lack of dependence of the ecotoxicological effects of BrB with regard to the exposure time. The estimated non-observed adverse effect levels (NOAEL) were 0.02, 0.06, 1.0, 1.1 and 1.6 mM BrB for *V. fischeri*, *D. magna*, PLHC-1 cells, RTG-2 cells and *C. vulgaris*, respectively.

4. Discussion

The use of BrB as a solvent for crystallization on a large scale and as an additive to motor oils may result in its release to the environment through various waste streams. In fact, it has been detected in industrial waste water (Bozzi et al., 2005). Although BrB presents an octanol/water partition coefficient <3 (2.99), it is a chemical of low degradability with bioaccumulation risk, due to bioaccumulation factors of 48 and 190 for fish and algae, respectively (Halfon and Reggiani, 1986).

There is limited available information about the toxic effects of this chemical in the aquatic environment. Consequently, the ecotoxicological effects induced by BrB were investigated using a suitable test battery of five model

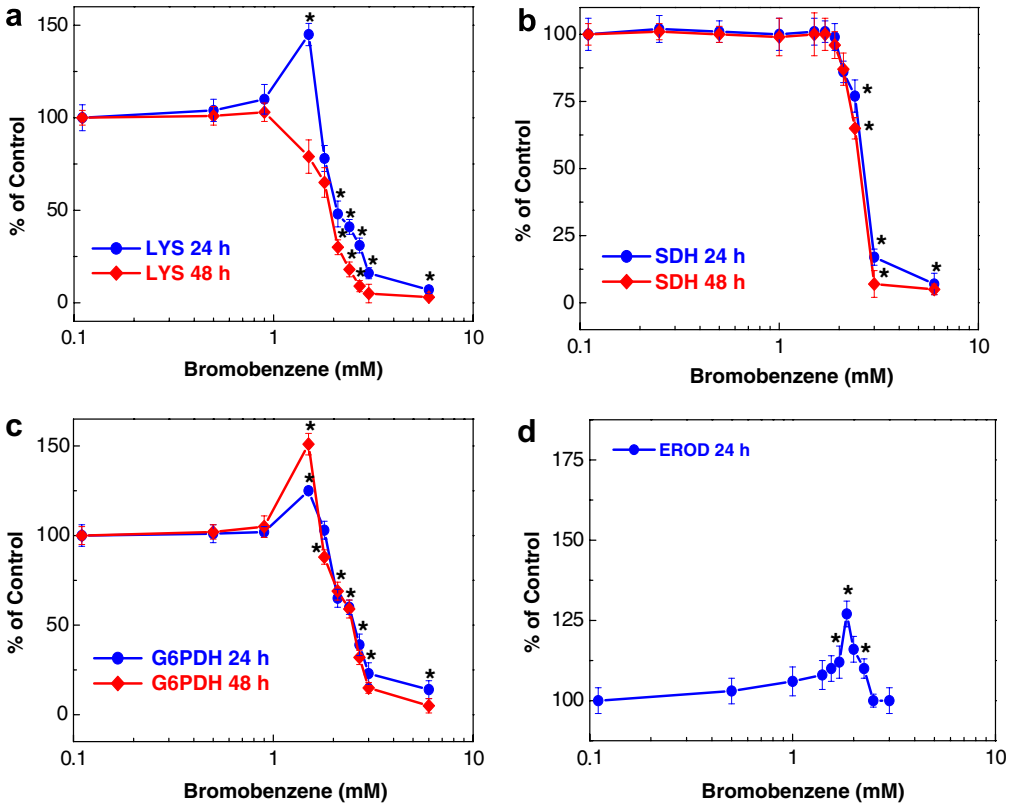

Fig. 6. *In vitro* effects of bromobenzene on RTG-2 cell culture (a) lysosomal function, (b) succinate dehydrogenase activity, (c) G6PDH activity for 24 (●) and 48 h (◆) and (d) EROD activity after 24 h (●) exposure. Data are expressed in % of each respective control treatment. * Indicates significant difference from control value ($p < 0.05$).

systems including two fish cell lines, and a total of thirteen endpoints.

The inhibition of bioluminescence of *V. fischeri* was the most sensitive model system, closely followed by the immobilization of *D. magna*. The strong toxicity of BrB to *V. fischeri* might be due to its interaction with mitochondrial oxidative phosphorylation. In fact, the high sensitivity of this bacterium to a variety of chemicals and environmental samples has been previously reported (Cordina et al., 1993; Jos et al., 2005). Nevertheless, the proliferation of the alga *C. vulgaris* presented the lowest sensitivity. This large difference of sensitivity to BrB between model systems may be due to the characteristic metabolic activities of the different species.

Taking into account the EC_{50} values obtained for the different biomarkers in the fish cell lines after BrB exposure, an intermediate sensitivity was observed in PLHC-1 cells, the content of total protein and the uptake of neutral red being the most susceptible endpoints. These bioindicators were also the most sensitive in the fibroblastic-like fish cell line RTG-2. Both cell lines presented a dose-dependent toxicity, as was previously reported in human hepatoma HepG2 cells (Duthie et al., 1994).

The extent of variation of each cytotoxic and biochemical biomarker, studied in both fish cell lines, exposed to 1.6 mM BrB at 24 h was compared (Fig. 8). The global results showed more changes in the bioindicators corresponding to PLHC-1 cells. Cell proliferation and neutral red uptake were inhibited though no significant cell death occurred, as indicated by the low leakage of G6PDH. In contrast, SDH and G6PDH were particularly stimulated.

The increase of SDH activity should be a response to the inhibition of mitochondrial respiratory function stated by Wong et al. (2000). The observed stimulation of G6PDH activity in PLHC-1 cells should indicate the induction of antioxidative defences. However, Schoonen et al. (2005) described the reduction of glutathione levels from 1 mM and the increase in reactive oxygen species from 3.2 mM BrB in several cell lines, as was previously described by Grewal et al. (1996) in mouse hepatocytes with 3 mM BrB.

The induction of lysosomal function in PLHC-1 cells can be related to the accelerated membrane phospholipid degradation described by Lamb et al. (1984) as the main cause of the alteration of the functional and structural integrity of hepatocytes. The increase of metallothionein levels is in agreement with the induction in the liver of rats treated with BrB reported by Wong and Klaassen (1981) and confirmed at the trascriptome level by Heijne et al. (2004) as the most expressed genes.

Comparing the results obtained in both fish cell lines, we concluded that PLHC-1 cells were more sensitive to BrB than RTG-2 cells. Among the possible explanations, the activation of the compound and the different metabolic profile of both fish cell lines should be considered. BrB is

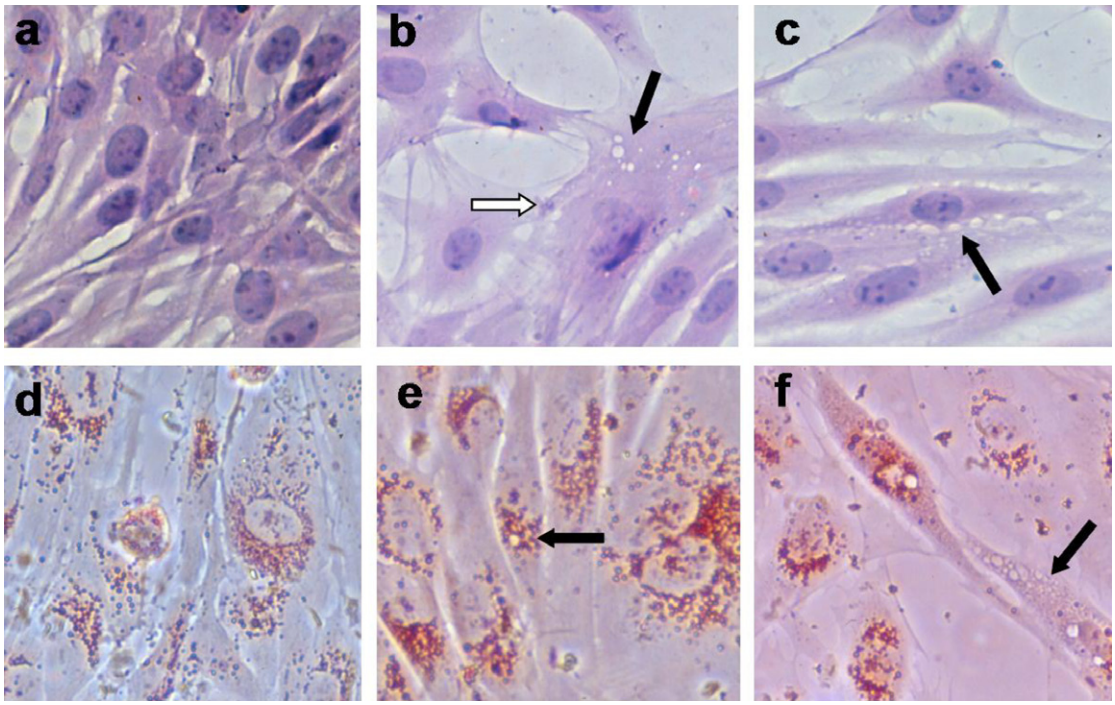

Fig. 7. Morphology of RTG-2 cell cultures (×1600) stained with Mayer's hematoxylin and eosin (a–c) or neutral red (d–f): (a) control culture of RTG-2 cells showing fusiform cells, with eosinophilic cytoplasm and central nucleus, arranged in plaques in parallel. (b) Cell culture exposed to 1.5 mM bromobenzene for 24 h showing loss of cells, induction of cellular pleomorphism, very evident hydropic degeneration of the cytoplasm (cellular swelling) (→) and one cell in apoptosis (⇒). (c) Culture of cells exposed for 24 h to 2 mM bromobenzene, with generation of vacuoles in the cytoplasm (→). (d) Control culture treated with neutral red. (e) The exposure to 1.5 mM bromobenzene for 24 h induced the process of death, showing a general loss of the perinuclear pattern of distribution of lysosomes and piknosis in a cell with irreversible damage (→). (f) After exposure to 2 mM bromobenzene for 24 h only a few cells remained alive presenting hydropic degeneration (→).

Table 1
Toxic effects of bromobenzene on the selected models and biomarkers of the proposed ecotoxicological battery

Model system	Origin	Indicator	24 h	48 h	72 h
Vibrio fischeri	Bacteria (decomposer)	Bioluminescence	0.04[a]	0.04[b]	–
Chlorella vulgaris	Unicel. Algae (1st producer)	Growth	2.35	2.20	2.18
Daphnia magna	Cladoceran (1st consumer)	Immobilization	0.11	0.10	0.09
PLHC-1 cell line	Topminnow (2nd consumer)	Protein content	1.59	1.58	1.57
		G6PDH leakage	2.43	2.52	2.58
		Neutral red uptake	1.58	1.58	1.57
		MTS metabolization	1.70	1.69	1.69
		Lysosomal function	1.61	1.62	1.79
		SDH activity	1.59	1.56	1.55
		G6PDH activity	1.54	1.73	2.58
		Metallothionein	[c]	–	–
		EROD activity	1.54	–	–
RTG-2 cell line	Rainbow trout (2nd consumer)	Protein content	1.85	1.70	–
		G6PDH leakage	2.45	2.32	–
		Neutral red uptake	1.77	1.58	–
		MTS metabolization	2.07	2.20	–
		Lysosomal function	2.04	1.93	–
		SDH activity	2.70	2.54	–
		G6PDH activity	2.50	2.54	–
		EROD activity	[c]	–	–

EC_{50} values (mM).
Ec_{50} (mM), concentration of test chemical that modified each biomarker by 50% (positive or negative) in comparison with appropriate untreated controls.
[a,b] Values referred to 5 and 15 min exposure times, respectively.
[c] Not modified.

believed to be relatively inert, requiring metabolic activation. It is metabolized by the cytochrome P450 to two toxic epoxide intermediates. Therefore, the systems that preferentially metabolizes BrB through the epoxide pathways

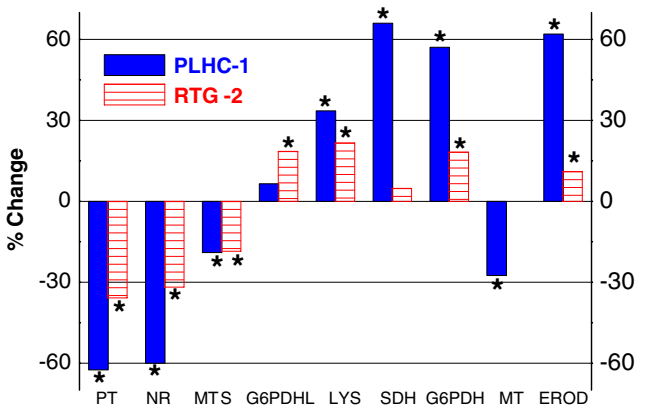

Fig. 8. Comparison of the extent of variation of each cytotoxicity and biochemical biomarker studied after 24 h exposure to 1.6 mM bromobenzene in PLHC-1 and RTG-2 cells. Toxicity indicators assessed in the *in vitro* test systems were: cell protein content (PT), G6PDH leakage (G6PDHL), neutral red uptake (NR), MTS metabolization (MTS), lysosomal function (LYS), succinate dehydrogenase activity (SDH), G6PDH activity (G6PDH), metallothionein levels (MT) and EROD activity (EROD). Data expressed in % of unexposed controls. * Indicates significant difference from control value ($p < 0.05$).

may be more susceptible to BrB-induced hepatotoxicity (Kerger et al., 1988).

We have found, from lower concentrations of BrB than those causing cytotoxic effects, a more marked stimulation of EROD activity in PLHC-1 than in RTG-2 cells. It can be correlated with the induction of cytochrome P450 isoforms reported by Heijne et al. (2004) to metabolize the chemical. However, the induction of mixed function oxidase facilitates the activation of the compound. Therefore, as PLHC-1 cells present 2.5 fold more basal EROD activity than RTG-2 cells, and EROD activity is in addition induced by BrB in a higher degree in PLHC-1 cells, they may generate a higher amount of active metabolites, producing more deleterious effects.

Several morphological alterations were observed after 24 h of exposure from 1.7 mM BrB in PLHC-1 and RTG-2 cells. The changes included hydropic degeneration of the cytoplasm and reduction of cell number and secretion vesicles. A general loss in lysosomal function and of the perinuclear pattern of distribution of lysosomes was observed. The presence of pyknotic nuclei and apoptosis in both type of cells was also present, as was confirmed by *in situ* hybridization (TUNEL). The reported oral administration of a single dose of BrB (5 mmol/kg) caused multiple cellular damage and necrosis in the Clara cells of the bronchiolar epithelium of C57B1/6 male mice (Forkert, 1985). In addition, the exposure of male Sprague–Dawley rats to BrB resulted in pronounced centrilobular necrosis, moderate pyknosis and minimal cytoplasmatic vacuolation (Chakrabarti, 1991).

A curious lack of time-dependent relationship was observed in the toxic effects of BrB in the different endpoints, the alterations being very similar for the different period times of exposure in each system studied. This peculiarity was not detected in more than 15 compounds previously evaluated with similar systems (Zurita et al., 2005; Jos et al., 2003; Repetto et al., 2001). In a study by Mennes et al. (1994) in rodents *in vivo* and in hepatocytes the effects were not influenced by the duration of the exposure period. In rats exposed to BrB (0.5, 2 and 5 mmol/kg), many genes were differentially expressed after 24 h, while hardly any change persisted after 48 h except with the highest dose (Heijne et al., 2004). Another investigation with male Fischer 344 rats showed that repeated treatment produced resistance to BrB hepatotoxicity (Kluwe et al., 1984). In addition, Chakrabarti (1991) proposed that an enhancement of BrB metabolism could partly explain a potential tolerance against acute hepatotoxicity.

Another characteristic effect was a sharp slope of the concentration-response curve for the different systems. It might be related to the *in vivo* threshold dose (1–2.5 mmol/kg) proposed by Chakrabarti and Brodeur (1984) due to the saturation of the metabolic pathways involving both the glutathione system and the formation of certain phenolic derivatives for its detoxification.

Considering the complexity of the results obtained, with different effects according to the model system studied and the exposure time period employed, where the sensitivity of each test system varied significantly with EC_{50} ranging from 0.04 to 2.58 mM, we concluded that a single bioassay will never provide adequate information for a suitable ecotoxicological evaluation. In the case of BrB, the variation in individual susceptibility may be related to species differences in the activity of de-activating pathways (Mennes et al., 1994). The most sensitive model system was the bacterium *V. fischeri*, with an EC_{50} of 0.04 mM BrB and a NOAEL of 0.02 mM BrB at 15 min, while the least sensitive was the green alga *C. vulgaris*, with an EC_{50} of 2.35 mM BrB and a NOAEL of 1.6 mM BrB at 24 h.

To conclude, following the EU guideline for classification, packaging and labelling of dangerous substances (Commission Directive 2001/59/EC), according to the obtained results, BrB should be classified as "R51/53 Toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment". The obtained result agrees with the present EU classification for this chemical, showing that bioassays batteries are valuable tools in the ecotoxicological assessment of chemicals.

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