

## Ecotoxicological evaluation of the additive butylated hydroxyanisole using a battery with six model systems and eighteen endpoints

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### Abstract

The occurrence and fate of additives in the aquatic environment is an emerging issue in environmental chemistry. This paper describes the ecotoxicological effects of the commonly used additive butylated hydroxyanisole (BHA) using a test battery, comprising of several different organisms and in vitro test systems, representing a proportion of the different trophic levels. The most sensitive system to BHA was the inhibition of bioluminescence in *Vibrio fischeri* bacteria, which resulted in an acute low observed adverse effect concentration (LOAEC) of 0.28  $\mu\text{M}$ . The next most sensitive system was the immobilization of the cladoceran *Daphnia magna* followed by: the inhibition of the growth of the unicellular alga *Chlorella vulgaris*; the endpoints evaluated in Vero (mammalian) cells (total protein content, LDH activity, neutral red uptake and MTT metabolization), mitotic index and root growth inhibition in the terrestrial plant *Allium cepa*, and finally, the endpoints used on the RTG-2 salmonid fish cell line (neutral red uptake, total protein content, MTS metabolization, lactate dehydrogenase leakage and activity, and glucose-6-phosphate dehydrogenase activity). Morphological alterations in RTG-2 cells were also assessed and these included loss of cells, induction of cellular pleomorphism, hydropic degeneration and induction of apoptosis at high concentrations. The results from this study also indicated that micronuclei were not induced in *A. cepa* exposed to BHA. The differences in sensitivity for the diverse systems that were used ( $\text{EC}_{50}$  ranged from 1.2 to  $>500 \mu\text{M}$ ) suggest the importance for a test battery approach in

*Abbreviations:* BHA, butylated hydroxyanisole;  $\text{EC}_{50}$ , mean effective concentration; G6PDH, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

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the evaluation of the ecological consequences of chemicals. According to the results, the levels of BHA reported in industrial wastewater would elicit adverse effects in the environment. This, coupled with its potential to bioaccumulate, makes BHA a pollutant of concern not only for acute exposures, but also for the long-term.

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## 1. Introduction

Butylated hydroxyanisole (BHA) is an antioxidant widely used to preserve and stabilize the freshness, nutritional value, flavour and colour of foods and animal feed products. It is also used in food packaging, cosmetics, pharmaceuticals, and rubber and petroleum products (JEFCA, 1996).

BHA (INS No. 320) is authorised in the European Union and USA, where it is listed as a common preservative and is considered generally recognized as safe (GRAS), but, in contrast, its use is not permitted in Japan. The main reason why the safety of this product is questioned is due to its controversial effects; for example, BHA may be referred to as an antioxidant, a pro-oxidant, an anticarcinogen, a carcinogen, and a tumour promoter (Iverson, 1999). BHA has shown anticarcinogenic activity in mice when is administered prior to the known or initiating carcinogen, but it could become a cancer promoter if is administered after the carcinogen (Iverson, 1999). Williams et al. (1990a) did not obtain evidence of genotoxicity for this compound. However, chronic feeding studies in rats resulted in a small increase in papillomas in the stomach (Williams et al., 1990b). Williams et al. (1999) concluded that BHA is a rodent carcinogen which is species-specific for all practical purposes, and not relevant to humans. The International Agency for Research on Cancer has evaluated BHA as 2B, a possible carcinogen to humans (IARC, 1987); it has been found sufficient evidence for carcinogenicity in experimental animals, but no data for humans. Apart from these effects, BHA has been reported to be an environmental endocrine disruptor (Jiménez, 1997).

Due to its widespread use, BHA may be released into the environment by various waste streams. This, along with its physical properties, makes it likely to be present in atmospheric, terrestrial and aquatic environments. In addition, BHA has low soil mobility, volatilizes slowly

from water and its bioconcentration factor (based on an estimated log  $K_{ow}$  of 3.5) is 269 (Meylan and Howard, 1995), presenting potential to bioaccumulate.

The occurrence of BHA in industrial wastewaters and surface waters has been reported (Davì and Gnudi, 1999), however, data concerning the ecotoxicological effects of additives in general and of BHA in particular are limited.

This paper investigates the environmental toxicity of BHA to different trophic levels, using a battery of bioassays that have been effective for other chemicals such as pesticides (Repetto et al., 2001) and pharmaceuticals (Jos et al., 2003).

Six ecotoxicological model systems with eighteen endpoints were used at different exposure time periods. The systems employed included bioluminescence inhibition in the marine bacterium *Vibrio fischeri* (decomposer); the inhibition of the growth in the alga *Chlorella vulgaris* (producer); micronuclei induction, mitotic index and growth inhibition in the plant *Allium cepa* (producer); and the immobilization of the cladoceran *Daphnia magna* (1st consumer). Total protein content, neutral red uptake, lactate dehydrogenase (LDH) activity and MTT metabolization were investigated in Vero African green monkey kidney cells (model of 2nd consumer). Neutral red uptake, total protein content, MTS metabolization, LDH leakage and activity, apoptosis induction and changes in morphology were studied in the RTG-2 cell line (model of 2nd consumer), derived from rainbow trout gonad (*Oncorhynchus mykiss*).

## 2. Materials and methods

### 2.1. Toxicant exposure

Stock solutions of BHA (Sigma) were prepared in ethanol. A range of different concentrations of exposure solutions were prepared in different media,

according to each experimental system, and sterilized by filtration through a 0.22 µm Millipore® filter. High quality deionised (Milli Q) water was used for preparation of the different media. The concentration of ethanol in all controls and exposure groups was 1%. After replacing the previous medium, the exposure solutions were added to the systems, and incubated for the adequate exposure period.

## 2.2. Model systems

### 2.2.1. *Chlorella vulgaris*

Growth inhibition of the alga *C. vulgaris var viridis* was evaluated in 96-well culture plates seeded with 200 µl/well of a 1,000,000 cells/ml algal culture in exponential growth phase, using constant agitation at 22 °C, under a water saturated sterile atmosphere containing 5% CO<sub>2</sub> 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 must grow at least 10 times in 48 h (Ramos et al., 1996).

### 2.2.2. *Allium cepa*

Bulbs of the onion *A. cepa* (15–30 g) were grown in the dark at 25 °C (González-Fernández et al., 1971). Root meristems of *A. cepa* were exposed for 48 h, fixed with ethanol/acetic acid and stained with acetic orcein (2%) according the method of Tjio and Levan (1950) and crushed in 50% acetic acid for the analysis of mitotic index and micronuclei induction (Jos et al., 2003). The assay was completed by measuring the length of the root bundles after 72 h of exposure. The control sets grew 8.8 times in 3 days.

### 2.2.3. *Vibrio fischeri*

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

### 2.2.4. *Daphnia magna*

*D. magna* clone A was maintained at 20 °C and fed with *C. vulgaris*. Acute toxicity immobilization tests with the cladoceran were performed in standard reference water according to OECD (1993) in replicate

groups of 10 neonates in 25 ml, contained in 70 ml polystyrene flasks (Costar, Cambridge, MA, USA).

### 2.2.5. Vero monkey cells

Vero monkey kidney cells were grown at 37 °C in 75 cm<sup>2</sup> plastic flasks (Costar, Cambridge, MA, USA) under a water-saturated sterile atmosphere containing 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin, 100 mg/l streptomycin and 2 mM glutamine (Biochrom, Berlin, Germany). Cells were seeded at a density of 14,000 cells/ml into 24-well culture plates and incubated for 24 h. After removing the cell culture medium and washing in phosphate buffered saline (PBS), the cell cultures were exposed to increasing doses of BHA.

To evaluate cell proliferation and/or detachment, the number of cells were quantified by measuring the total cellular protein content (TPC), using bovine serum albumin (Sigma) as standard, by the method of Bradford (1976). Neutral red uptake was evaluated according to Borenfreund and Puerner (1984) and intracellular LDH (EC1.1.1.27) activity as described by Vassault (1983). Cell viability was measured by the MTT reduction assay according to a procedure based on Carmichael et al. (1987) after 2 h incubation. Absorbancies were measured on a Spectrafluor microplate reader (Tecan, Austria).

### 2.2.6. RTG-2 cells

The RTG-2 salmonid fish cell line, derived from the gonad of rainbow trout (*O. mykiss*), was grown in Eagle's Medium supplemented with 10% foetal calf serum (Flow). RTG-2 cells in exponential growth phase were plated at a density of 8000 cells/well in 96-well tissue-culture plates (Costar). After 24 h at 20 °C, the culture medium was replaced with 0.2 ml test medium and then incubated for a further 24, 48 or 72 h (Castaño et al., 2000; Castaño et al., 2003).

Total protein content, a combined indicator of cell detachment and cell proliferation, was quantified in situ using Coomassie brilliant blue G-250 (Repetto and Sanz, 1993). Absorbance at 620 nm was read on a Multiscan RC plate reader (Labsystem, Helsinki, Finland). Neutral red uptake was evaluated according to Babich and Borenfreund (1987). The MTS tetrazolium reduction assay was performed according to a procedure based on Baltrop et al. (1991). The MTS tetrazolium

compound is bio-reduced by cells into a coloured formazan product that is soluble in tissue culture medium. LDH (EC 1.1.1.27) activity in cells and in culture medium was determined according to Duffy and Flint (1987). The production of NADH, during the conversion of lactate to pyruvate, and G6PDH activity was determined by the method described by García-Alfonso et al. (1998).

For the morphological study, RTG-2 cells were seeded in Lab-Tek<sup>®</sup> tissue culture chamber slides (Nunc, Inc., Naperville, IL) previously coated with Matrigel<sup>™</sup> (BD Biosciences). They were then exposed to BHA 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).

### 2.3. Calculations and statistical analysis

All experiments were performed at least three times and at least in duplicate per concentration. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. EC<sub>50</sub> values were determined by probit analysis.

## 3. Results and discussion

The effects of BHA were investigated using six ecotoxicological model systems with eighteen endpoints from a variety of organisms including vegetables, bacteria, a crustacean, and cell cultures from monkey and fish origin.

The inhibition of bioluminescence in *V. fischeri* was the most sensitive model system, with mean EC<sub>50</sub> values of 1.2  $\mu$ M for the three exposure periods (5, 15 and 60 min) (Fig. 1a). The strong toxicity of BHA to this bacteria might be due to two factors based around its interaction with mitochondrial oxidative phosphorylation. Firstly, BHA uncouples oxidative phosphorylation by increasing the permeability of the mitochondrial inner membrane to protons. Secondly it also inhibits respiration by a direct interaction with the electron transport chain (Fusi et al., 1991). These cellular respiration pathways are closely linked to those implicated in bioluminescence, so interference

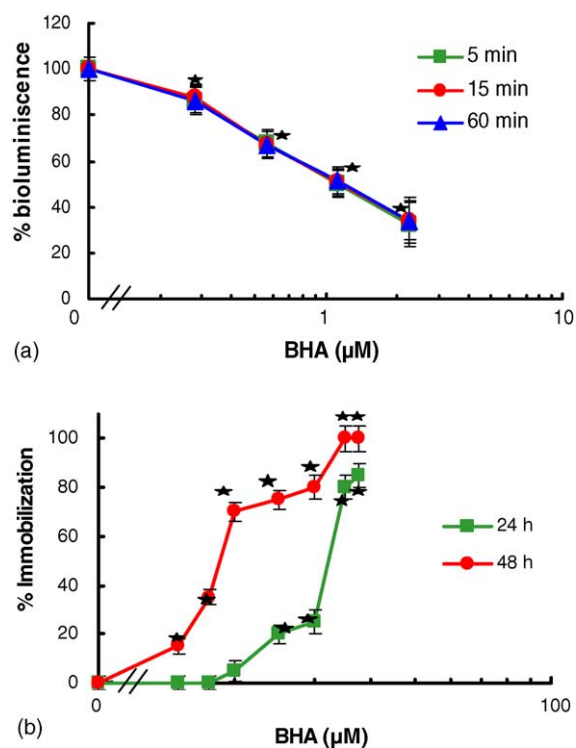


Fig. 1. (a) Bioluminescence inhibition of *Vibrio fischeri* bacteria after exposure to different concentrations of BHA for (a) 5 min (■), 15 min (●) or 60 min (▲); and (b) *Daphnia magna* immobilization after exposure to different concentrations of BHA for 24 h (■) and 48 h (●), respectively. Data expressed in (%) of each respective control treatment. \* Indicates significant difference from control value ( $p < 0.01$ ).

produced by a toxic compound, will also affect light production. Moreover, BHA has also been reported to elicit antimicrobial activity to *Aspergillus flavus* and *Bacillus* spp. (Efiuvwevwe and Efi, 1999), *Clostridium perfringens* (Klindworth et al., 1979) and *Vibrio vulnificus* (Sun and Oliver, 1994), which suggests that BHA would be effective as a food preservative.

The immobilization of *D. magna* was the second most sensitive test system with an EC<sub>50</sub> value of 31  $\mu$ M (24 h) and 20  $\mu$ M (48 h) (Fig. 1b). This was followed by the inhibition of the proliferation of the freshwater alga *C. vulgaris*, which resulted in an EC<sub>50</sub> value of 51  $\mu$ M (24 h) and 42  $\mu$ M (48 h) (Fig. 2a).

The growth of *A. cepa* was inhibited by 50% at concentrations of 194  $\mu$ M of BHA at 72 h of exposure and the mitotic index of meristematic cells at a concentration of 283  $\mu$ M at 48 h of exposure (Fig. 2b). The

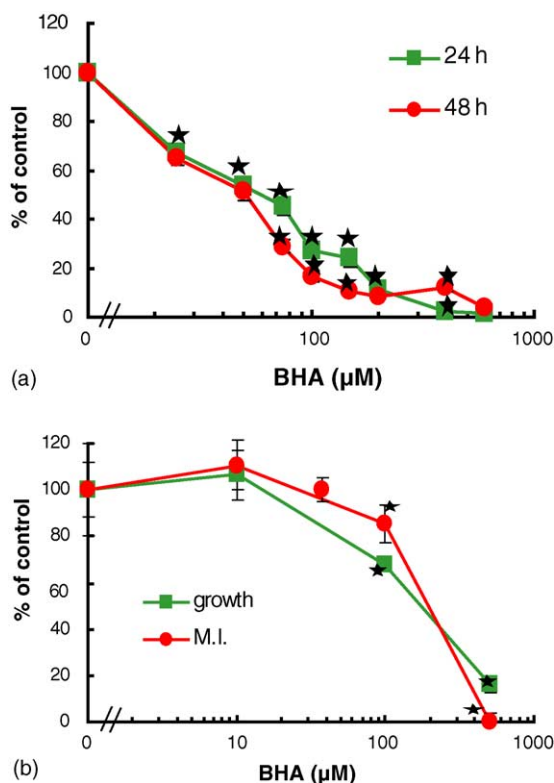


Fig. 2. (a) *Chlorella vulgaris* proliferation after exposure to different concentrations of BHA for 24 h (■) and 48 h (●); and (b) *Allium cepa* growth (■) and mitotic index (●) after exposure to different concentrations of BHA for 72 h and 48 h, respectively. Data are expressed in (%) of each respective control treatment. \* Indicates significant difference from control value ( $p < 0.01$ ).

observation of the general population of *A. cepa* root cells did not reveal any statistically significant difference in the frequencies of micronuclei, even when high concentrations were tested (500 µM). Micronuclei frequencies were calculated only in meristematic cells in order to compare all the cytogenetic parameters.

These findings are supported by the results obtained by Williams et al. (1990a,b) who did not find any evidence of genotoxicity using other kinds of tests (for example, the hepatocyte primary culture/DNA repair test, the Salmonella/microsome mutagenesis test, the adult rat liver epithelial cell/hypoxanthine-guanine phosphoribosyl transferase test or in the Chinese hamster ovary cell/sister chromatid exchange test). This indicates that BHA does not operate through a chemical DNA-reactive mechanism (Williams et al., 1999).

The results obtained for the different endpoints evaluated on the Vero monkey cells (Neutral Red Uptake, Total Protein Content, MTT metabolization and LDH activity) are shown in Fig. 3. An intermediate sensitivity in comparison with the other models was found, with EC<sub>50</sub> values between 87 µM for NRU and 27 µM for LDH activity after 48 h of BHA exposure.

The salmonid fish cell line, RTG-2, was the least sensitive system to BHA being necessary levels between 177 µM (EC<sub>50</sub> for Neutral Red Uptake at 72 h) and 574 µM (EC<sub>50</sub> for LDH activity at 24 h) to inhibit the measured endpoints by 50% (Fig. 4). At intermediate concentrations of BHA, some stimulations were observed for neutral red uptake, protein content and G6PDH and LDH activities, possibly due to the interaction with cell respiration.

Morphological changes, induced by BHA, were also investigated in RTG-2 cells (Fig. 5). The control cultures show fusiform cells, arranged in plaques and in parallel. They have well defined borders, eosinophilic cytoplasm and central nuclei. Morphological aberrations were evident after 24 h exposure to 200 µM of BHA, and were irreversible at 240 µM. The changes included loss of cells, induction of cellular pleomorphism, and hydropic degeneration of the cytoplasm (cellular swelling) at concentrations of BHA at 100 µM. The induction of apoptosis, confirmed by in situ hybridization (TUNEL), was evident after 48 h exposure at concentrations from 100 µM BHA. Yu et al. (2000) showed that BHA, at concentrations lower than 200 µM, did not exert a significant toxic effect in freshly isolated rat hepatocytes but, instead, stimulated the induction of phase II detoxifying enzymes. At apoptosis-inducing concentrations, BHA interacts with mitochondria and triggers mitochondrial permeability transition, resulting in the release of cytochrome c, which subsequently stimulates proteolytic activities of caspase-3, -8, and -9. Conversely, the protective action of low concentrations of BHA has been reported in the induction of apoptosis by cadmium and X-rays, but not by heat-shock (Galan et al., 2001).

Considering all the data obtained from the battery of tests (Table 1), the sensitivity of the test systems decreased as follows: *V. fischeri* > *D. magna* > *C. vulgaris* > Vero mammalian cell line > *A. cepa* ≈ RTG-2 fish cell line. Other chemicals have been found to produce a very different profile of effects on the diverse models considered (Repetto et al., 2001; Jos et al.,

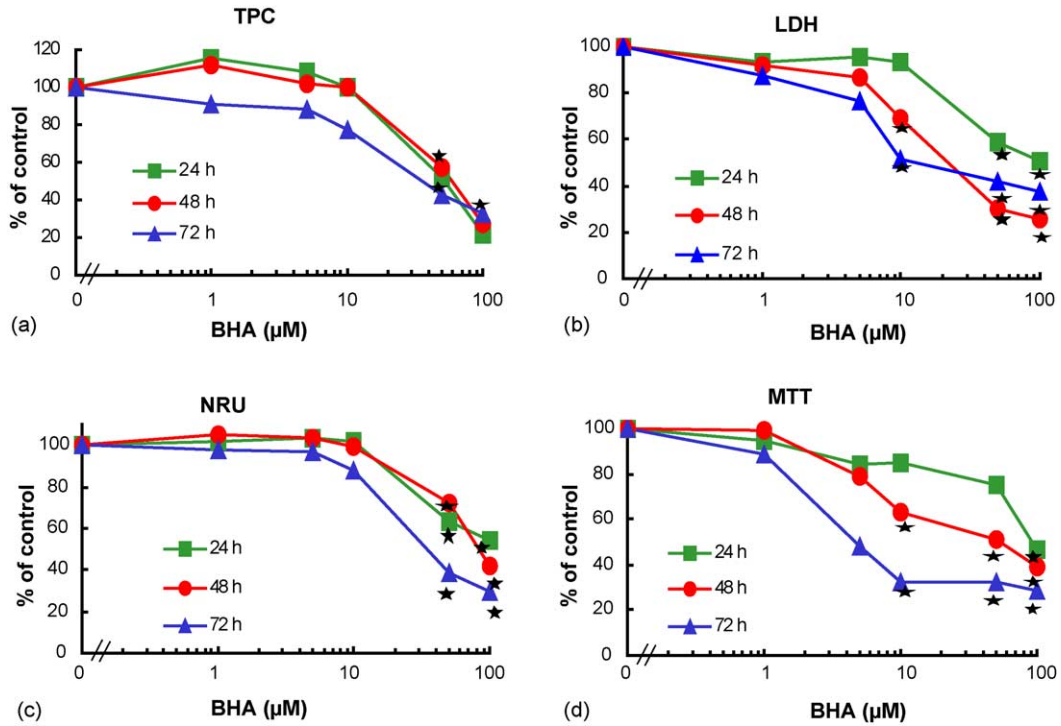


Fig. 3. Vero monkey cells (a) total protein content; (b) LDH activity; (c) neutral red uptake; and (d) MTT metabolization after exposure to different concentrations of BHA for 24 h (■), 48 h (●) or 72 h (▲). Data are expressed in (%) of each respective control treatment. \* Indicates significant difference from control value ( $p < 0.01$ ).

Table 1  
Toxic effects of butylated hydroxyanisole on the different models and bioindicators of the proposed ecotoxicological battery

| Model system              | Origin                       | Indicator             | 24 h              | 48 h              | 72 h              |
|---------------------------|------------------------------|-----------------------|-------------------|-------------------|-------------------|
| <i>Vibrio fischeri</i>    | Bacteria (Decomposer)        | Bioluminescence       | 1.15 <sup>a</sup> | 1.18 <sup>a</sup> | 1.19 <sup>a</sup> |
| <i>Chlorella vulgaris</i> | Unicel. Algae (Producer)     | Cell growth           | 51                | 42                | –                 |
| <i>Allium cepa</i>        | Terrestrial Plant (Producer) | Root growth           | –                 | –                 | 194               |
|                           |                              | Mitotic index         | –                 | 283               | –                 |
|                           |                              | Micronuclei induction | –                 | Not detected      | –                 |
| <i>Daphnia magna</i>      | Cladoceran (1st Consumer)    | Immobilization        | 31                | 20                | –                 |
| Vero cell line            | Monkey (2nd Consumer)        | Total protein content | 50                | 58                | 38                |
|                           |                              | Neutral red uptake    | 108               | 87                | 38                |
|                           |                              | MTT metabolization    | 95                | 50                | 4.5               |
|                           |                              | LDH activity          | 97                | 27                | 21                |
|                           |                              | LDH leakage           | 568               | –                 | –                 |
| RTG-2 cell line           | Rainbow Trout (2nd Consumer) | LDH activity          | 574               | –                 | –                 |
|                           |                              | Total protein content | 277               | 231               | 211               |
|                           |                              | Neutral red uptake    | 187               | 183               | 177               |
|                           |                              | MTS metabolization    | 218               | 199               | 181               |
|                           |                              | G6PDH activity        | 233               | 191               | 190               |

EC<sub>50</sub> values are measured in μM.

<sup>a</sup> Values refer to 5, 15 and 60 min exposure times respectively.

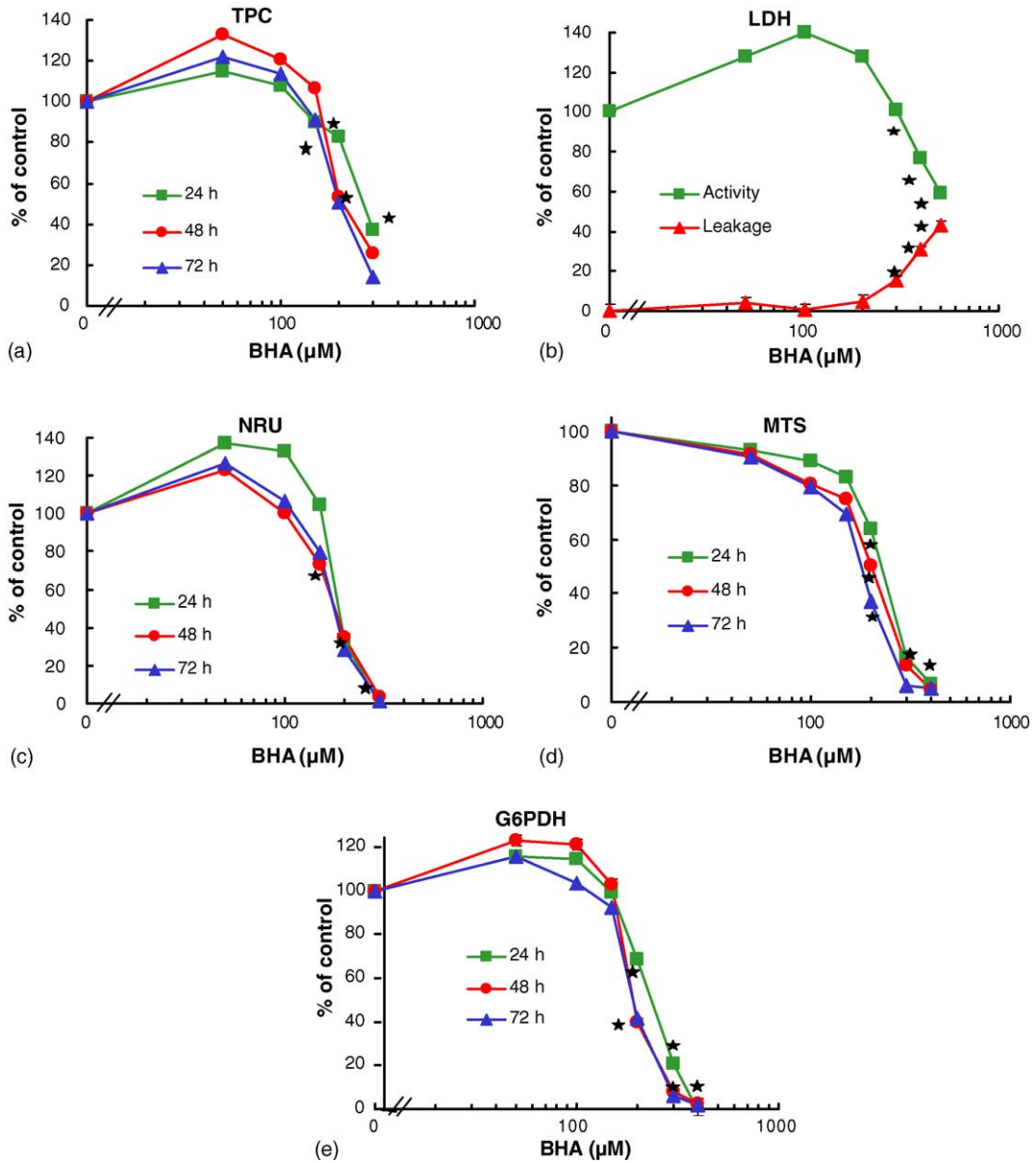


Fig. 4. Effects of BHA in rainbow trout RTG-2 cells in (a) total protein content; (b) LDH leakage and activity; (c) neutral red uptake; (d) MTS metabolization; and (e) G6PDH activity after exposure to different concentrations of BHA for 24 h (■), 48 h (●) or 72 h (▲). Data are expressed in (%) of each respective control treatment. \* Indicates significant difference from control value ( $p < 0.01$ ).

2003). The sensitivity of each of the tests exposed to BHA varied significantly with  $EC_{50}$  ranging from 1.2 to  $>500 \mu\text{M}$ . The complexity of the results, with varying effects according to the test system and exposure time period employed, suggests that a single bioassay

will never provide adequate information to protect the quality of the environment. Single species testing may over or underestimate the potential toxicity of a substance. Accordingly, recent research has focused on the development of representative, cost effective and

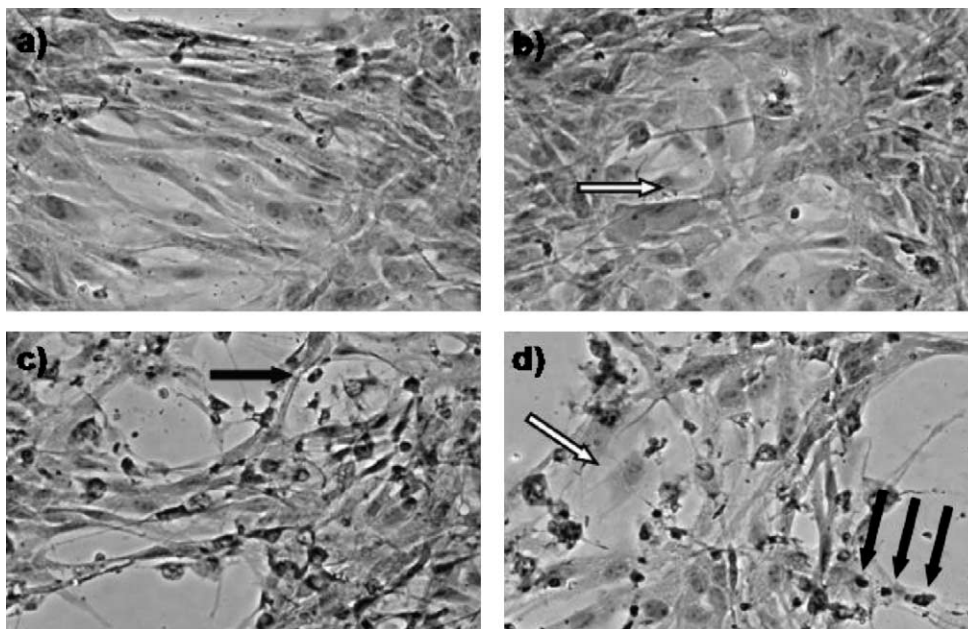


Fig. 5. Morphology of a (a) control culture of RTG-2 cells, showing fusiform cells, with eosinophilic cytoplasm and central nucleus, arranged in plaques in parallel. (b) Cell culture exposed to 100  $\mu\text{M}$  BHA for 24 h. Changes included loss of cells, induction of cellular pleomorphism, and very evident hydropic degeneration of the cytoplasm (cellular swelling) ( $\Rightarrow$ ). (c) The exposure to 270  $\mu\text{M}$  BHA for 24 h reduced the number of cells and caused the death of cells by necrosis and apoptosis ( $\rightarrow$ ). (d) After exposure to 200  $\mu\text{M}$  BHA for 48 h only a few cells remained alive with hydropic degeneration ( $\Rightarrow$ ) and the rest presents necrosis and apoptosis ( $\rightarrow$ ).

quantitative test batteries with model systems and indicators representative of a wide range of organisms. For the time being, a minimal ecotoxicological test battery should at least include bacteria, vegetables, invertebrates and mammalian and non-mammalian cells (Repetto et al., 2001).

In a 3 year study in the river Po (Italy), performed by Davì and Gnudi (1999), BHA and its isomer phenol, 1,1-dimethylethyl-4-methoxy, were the most abundant phenolic compounds detected maximally at concentrations of 45  $\mu\text{g}/\text{l}$  (0.25  $\mu\text{M}$ ). This concentration was very close to the acute low observed adverse effect concentration (LOAEC) obtained with the present battery, corresponding to 0.28  $\mu\text{M}$  for the extremely sensitive bioluminescence inhibition in *V. fischeri*.

In an industrial wastewater survey for organic pollutants in the US (Burse and Pellizzari, 1982), BHA was detected at 7  $\text{ng}/\mu\text{l}$  ( $\approx$ 39  $\mu\text{M}$ ) which, according to our results, would elicit important adverse acute effects in *V. fischeri*, *D. magna* and the Vero cells, since  $\text{EC}_{50}$  are clearly exceeded. This, coupled with its potential to

bioaccumulate, make BHA a pollutant of concern not only for acute exposures, but also for the long-term.

In another context, following the EU guidelines for classification, packaging and labelling of dangerous substances (Commission Directive 2001/59/EC), and in accordance with the results obtained in our battery, BHA would be classified as “N/dangerous for the environment” and “R51/53/toxic to aquatic organisms. It may cause long-term adverse effects in the aquatic environment”.

It looks clear that chemicals initially not considered as dangerous can also have a negative impact on the environment. The EU now has the task, through the new Chemicals Policy (EU, 2001), of establishing an effective environmental assessment strategy, where in vitro tests should play an important role.

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