



## A test battery for the ecotoxicological evaluation of pentachlorophenol

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### Abstract

Experimental bioassays are currently used in ecotoxicology and environmental toxicology to provide information for risk assessment evaluation of new chemicals and to investigate their effects and mechanisms of action; in addition, ecotoxicological models are used for the detection, control and monitoring of the presence of pollutants in the environment. As a single bioassay will never provide a full picture of the quality of the environment, a representative, cost-effective and quantitative test battery should be developed. The effects of pentachlorophenol were studied using a battery of ecotoxicological model systems, including immobilization of *Daphnia magna*, bioluminescence inhibition in the bacterium *Vibrio fischeri*, growth inhibition of the alga *Chlorella vulgaris*, and micronuclei induction in the plant *Allium cepa*. The inhibition of cell proliferation and MTT reduction were investigated in Vero cells. Neutral red uptake, cell growth, MTT reduction, lactate dehydrogenase leakage and activity were studied in the salmonid fish cell line RTG-2, derived from the gonad of rainbow trout. Pentachlorophenol was very toxic for all biota and cells. The system most sensitive to pentachlorophenol, was micronuclei induction in *A. cepa*, followed by *D. magna* immobilization, bioluminescence inhibition in *V. fischeri* bacteria at 60 min and cell proliferation inhibition of RTG-2 cells at 72 h. Inhibition of cell proliferation and MTT reduction on Vero monkey cells showed intermediate sensitivity. © 2001 Published by Elsevier Science Ltd. All rights reserved.

**Keywords:** Alternatives; Battery; Ecotoxicology; Pentachlorophenol; Cytotoxicity; Contamination

### 1. Introduction

Experimental model systems and bioassays are currently used in ecotoxicology and environmental toxicology both to provide information for risk assessment evaluation and to register new chemicals as well as to investigate their effects and mechanisms of action. In addition, ecotoxicological models are used for the detection, control and monitoring of the presence of pollutants in water, soil, and wastes, etc.

Most of the procedures used in regulatory and non-regulatory toxicology are carried out on mammals, but public pressure to minimize the use of vertebrates in ecotoxicity testing (Walker et al., 1998) and scientific interest in promoting the study of the effects of chemicals on both terrestrial organisms (including vegetables) and aquatic environments is growing. The use of invertebrate organisms, microorganisms, and plants—despite being far more abundant in nature than vertebrates—is still not well represented in ecotoxicology.

The most promising alternatives in environmental toxicology encompass the use of fewer organisms with limited sensitivity and/or which are not protected by legislation controlling animal experiments. These include bacteria, fungi, algae, plants and invertebrate animals; vertebrates used at early stages of development—fish, amphibians, reptiles, birds and mammals; also, the employment of in vitro methods using material from these organisms is of particular interest.

As a single bioassay will never provide a full picture of the quality of the environment, a representative,

*Abbreviations:* BSA, bovine serum albumin; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; EC<sub>50</sub>, mean effective concentration:concentration of test chemical that modified each biomarker by 50% in comparison with appropriate untreated controls; FCS, fetal calf serum; LC<sub>50</sub>, mean lethal concentration:concentration of test chemical that produced death or immobilization to 50% of exposed; LDH, lactate dehydrogenase; PBS, phosphate buffered saline.

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cost-effective and quantitative test battery should be developed (Isooma and Lilius, 1995; Bierkens et al., 1998). For the time being, a minimum ecotoxicological in vitro test battery should at least include bacteria, protozoans, algae, invertebrates, fish cell lines, or cells isolated from fish and other species.

The aim of this study was to design an ecotoxicological battery of several test systems and indicators able to detect different effects using a variety of endpoints. Such a battery of test systems and indicators would be representative of a wide range of organisms. The systems studied included immobilization of the cladoceran *D. magna*, bioluminescence inhibition in the marine bacterium *V. fischeri*, growth inhibition of the alga *Chlorella vulgaris*, and micronuclei induction in the plant *A. cepa*. The inhibition of cell proliferation and MTT reduction were investigated in Vero cells. Neutral red uptake, cell growth, MTT reduction, and lactate dehydrogenase leakage and activity were studied in the salmonid fish cell line RTG-2, derived from the gonad of rainbow trout (*Oncorhynchus mykiss*). Pentachlorophenol, a well-known pesticide, fungicide and non-selective contact herbicide mainly used as wood preservative, was applied to check the performance of the proposed battery as a whole. As pentachlorophenol is a widely investigated pesticide, and its effects on different organisms have been previously reported in separate studies, it was considered to be a good model compound, relevant from the environmental perspective. Recently, however, more than 30 countries have applied restrictions to the use of pentachlorophenol based on its toxicity, as well as the fact that it generates dioxins when exposed to the sun or on being incinerated.

## 2. Materials and methods

### 2.1. Toxicant exposure

Stock solutions of 10 mM pentachlorophenol (Merck, Darmstadt, Germany) were prepared in ethanol and maintained in darkness at room temperature. A range of different concentrations of exposure solutions were prepared before being used in different media according to each experimental system and sterilized by filtration through a 0.22 µm Millipore® filter. After replacing the previous medium, the exposure solutions were added to the systems, and incubated for the adequate exposure time period. Ethanol concentration in medium was 0.1%, including the control groups.

### 2.2. Model systems

*D. magna* clone A (a gift from Dr. Muñoz-Reoyo, CISA, Spain) were 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).

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).

Growth inhibition of the algae *C. vulgaris var viridis* was evaluated in 96-well culture plates with 2,000,000 cells/ml in constant agitation under a light source, and absorbancy at 450 nm was read on a Multiscan RC plate reader (Labsystem, Helsinki, Finland) (Ramos et al., 1996).

Vero monkey kidney cells were grown at 37°C in 75-cm<sup>2</sup> 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% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-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 pentachlorophenol. Cell viability was quantified by the MTT reduction assay according to a procedure based on Carmichael et al. (1987). MTT (5 mg/ml) was dissolved in PBS, sterilized by filtration through a 0.22 µm Millipore® filter and stored at 4°C. After the different treatments, cells were washed twice in PBS, then 1.5 ml of a solution of MTT (0.5 mg/ml in DMEM) were added to each well, they were then incubated for 2 h at 37°C to allow MTT metabolism. Absorbancies at 570 nm were measured in an UV/Vis spectrophotometer UV-1601 (Shimadzu, Tokyo, Japan). Cell proliferation was quantified according to total cellular protein, using bovine serum albumin (Sigma) as standard, by the method of Lowry et al. (1951). Absorbances at 660 nm were read in an UV/Vis spectrophotometer UV-1601 (Shimadzu).

RTG-2 salmonid fish cell line derived from the gonad of rainbow trout (*O. mykiss*) (a gift from Dr. Castaño, CISA, Spain) was grown in Eagle's medium supplemented with 10% FCS (Flow). RTG-2 cells were plated at a density of 15,000 cells/well in 96-well tissue-culture plates (Costar). After 72 h at 20°C, the culture medium was replaced with 0.2 ml medium containing the test chemical in solution and then incubated for 24 h (Castaño et al., 1995). As previously stated, cell proliferation was quantified in situ, according to total cellular protein, using Coomassie Brilliant Blue G-250 (Repetto and Sanz, 1993) in the same 96-well tissue culture plates in which exposure originally took place (Repetto et al., 1993). Absorbancy 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) and Repetto and Sanz (1993). LDH (EC 1.1.1.27) activity in cells and in culture medium was determined according to Duffy and Flint (1987) by following the production of NADH during the conversion of lactate to pyruvate.

Onion bulbs of *A. cepa* L (15–30 g in weight) were grown in darkness at a constant temperature of  $25 \pm 0.5^\circ\text{C}$ . The bases of the bulbs remained submerged in filtered tap water (renewed every 24 h) and aerated by continuous bubbling at a rate of 10–20 ml/min. (González-Fernández et al., 1971). The experiments were begun when meristem roots reached 15–20 mm. Three bulbs and four roots per bulb were used for each treatment. Root meristems of *A. cepa* were exposed for 24 h and then fixed in a mixture of ethanol:acetic acid (3:1, v/v) at  $4^\circ\text{C}$  overnight. Finally, they were stained with acetic orcein (2%) according to the method of Tjio and Levan (1950) and crushed in 50% acetic acid for the analysis of mitotic index and micronuclei. The mitotic index was calculated counting the number of cells in mitosis within a field in relation to the total number of cells. The micronuclei was calculated from the number of cells where a separate fragment of the nuclei was observed. A group of control bulbs were grown in filtered tap water and another group in filtered tap water with 10% ethanol. No differences in the rates of mitotic index and micronuclei were observed.

### 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 test.  $\text{EC}_{50}$  values were determined by probit analysis.

### 3. Results and discussion

The results of this study demonstrate that the crustacean *D. magna* is very sensitive to pentachlorophenol, showing a dose-dependent curve of immobilization with an  $\text{EC}_{50}$  of  $2.1 \mu\text{M}$  at 24 h and of  $2.0 \mu\text{M}$  at 48 h and  $1.5 \mu\text{M}$  at 72 h of exposure (Fig. 1). A similar range of effects was observed for bioluminescence inhibition using *V. fischeri* bacteria, with  $\text{EC}_{50}$  of 3.2, 2.3 and  $1.9 \mu\text{M}$  for 5, 15 and 60 min, respectively (Fig. 2). As pentachlorophenol is an uncoupler of oxidative phosphorylation, metabolic blockage produces a direct decrease in bioluminescence.

The proliferation of the freshwater algae *C. vulgaris* in 96-well microtiter plates was inhibited at intermediate concentrations, showing  $\text{EC}_{50}$  values from 29 to  $59 \mu\text{M}$  (Fig. 3). The effect of pentachlorophenol on algae is very much species specific, with 96-h  $\text{EC}_{50}$  values for the green algae *Scenedesmus quadricauda* of  $0.3 \mu\text{M}$  becoming higher than for other species (Hobbs et al., 1993). In the other vegetal system, a very important reduction in the mitotic index of Root meristems of *A. cepa* occurred from  $5 \mu\text{M}$ . In addition, the genotoxic effects produced in the terrestrial plant were very important, with increases of 180 times in the micronuclei rate (Table 1). Among the genotoxic studies with pentachlorophenol, Ehrlich (1990) reported that concentrations of up to  $75 \mu\text{M}$  of pentachlorophenol did not cause DNA single strand breaks and/or alkali labile sites in cultured Chinese hamster ovary (CHO) cells. On

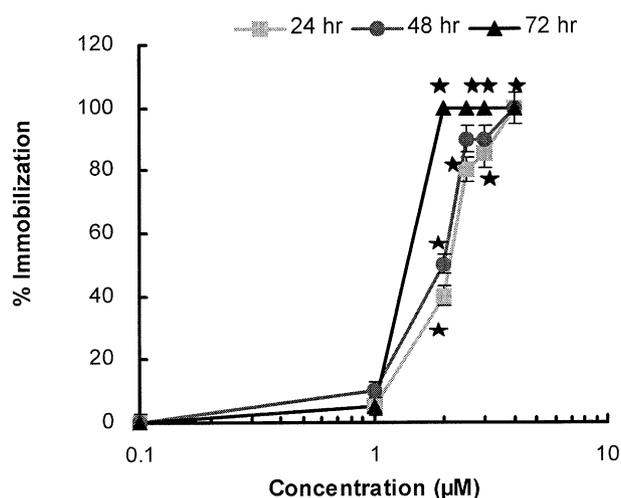


Fig. 1. *Daphnia magna* immobilization after exposure to different concentrations of pentachlorophenol for (a) 24 h (■), (b) 48 h (●) or (c) 72 h (▲). Data expressed relative to mean value in respective unexposed controls. \*Indicates significant difference from control value ( $P < 0.01$ ).

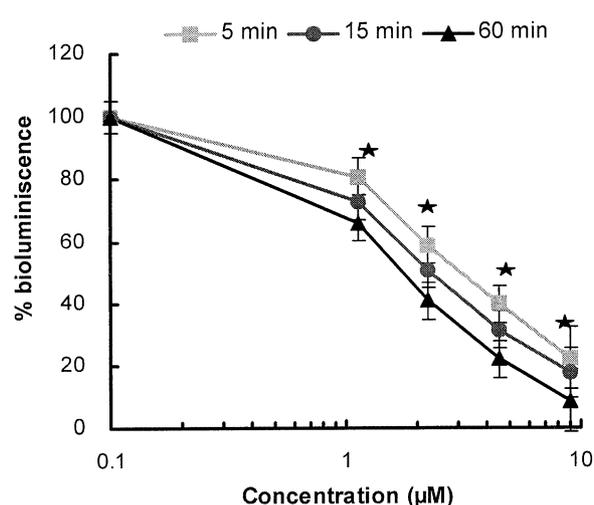


Fig. 2. Bioluminescence inhibition of *Vibrio fischeri* bacteria after exposure to different concentrations of pentachlorophenol for (a) 5 min (■), (b) 15 min (●) or 60 min (▲). Data expressed relative to mean value in respective unexposed controls. \*Indicates significant difference from control value ( $P < 0.01$ ).

the other hand, the metabolite of pentachlorophenol, tetrachlorohydroquinone, did cause DNA single strand breaks at lower concentrations. Ehrlich (1990) suggested that the reported formation of chromosomal aberrations in the peripheral lymphocytes of workers exposed to pentachlorophenol may in fact be caused by tetrachlorohydroquinone after the metabolization of pentachlorophenol. The possible metabolization in vitro is currently under investigation.

The metabolic effects of pentachlorophenol on Vero monkey cell MTT reduction and inhibition of cell proliferation were very different, according to the period of exposure, with  $EC_{50}$  values from 8.6 to 37.6  $\mu\text{M}$  for MTT reduction and from 6 to 34  $\mu\text{M}$  for cell proliferation (Fig. 4). The growth of CHO cells, another mammalian cell line, have been reported to be only slightly inhibited after 3 days in the presence of 37.5  $\mu\text{M}$ , indicating higher toxicity of the metabolite (Ehrlich, 1990).

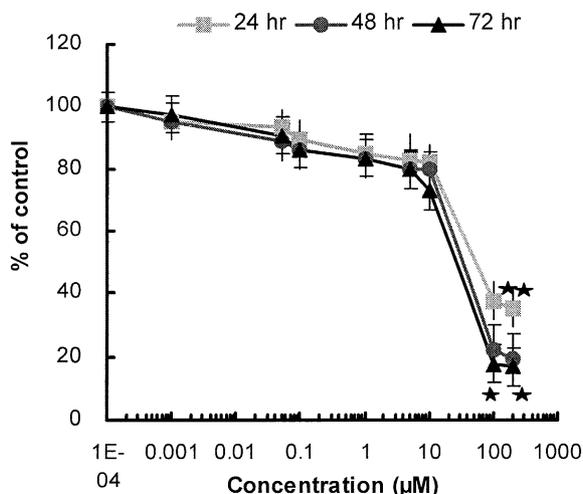


Fig. 3. *Chlorella vulgaris* proliferation inhibition after exposure to different concentrations of pentachlorophenol for (a) 24 h (■), (b) 48 h (●) or 72 h (▲). Data expressed relative to mean value in respective unexposed controls. \*Indicates significant difference from control value ( $P < 0.01$ ).

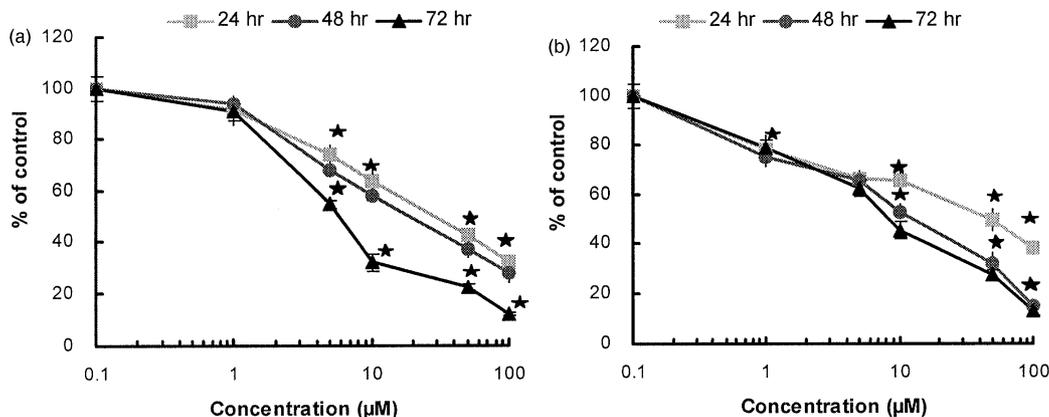


Fig. 4. Vero monkey cell (a) MTT reduction and (b) cell proliferation after exposure to different concentrations of pentachlorophenol for 24 h (■), 48 h (●) or 72 h (▲). Data expressed relative to mean value in respective unexposed controls. \* Indicates significant difference from control value ( $P < 0.01$ ).

The in vivo toxicity of pentachlorophenol varies greatly among different fish species. The reported  $LC_{50}$  at 96 h for bluegill sunfish is 0.2  $\mu\text{M}$  (EPA, 1980), 0.96 for *Pimephales promelas* (Genoni, 1997), 1.76 for fat-head minnow (Cleveland et al., 1982), and 0.35  $\mu\text{M}$  after 48 h of exposure for rainbow trout (McKim et al., 1987).

As pentachlorophenol was one of the compounds included in the Multicenter Evaluation of In Vitro Cytotoxicity Programme, results from 67 assays can be compared (Clemenson et al., 1998b). Apart from morphological effects on two human cell lines exposed for 168 h ( $EC_{50}$  0.2  $\mu\text{M}$ ), two vegetal systems, *A. cepa* 72-h growth and *Nicotiana sylvestris* pollen tubes 18-h growth, were the most sensitive assays, with  $EC_{50}$  0.25 and of 0.78  $\mu\text{M}$ , respectively. Slightly less sensitive were the Anostraca *Streptocephalus proboscideus* 24-h larval mortality ( $EC_{50}$  1.5  $\mu\text{M}$ ), the rotifer *Brachionus calyciflorus* 24-h larval mortality ( $EC_{50}$  1.6  $\mu\text{M}$ ), the 5-min bioluminescence inhibition in *V. fischeri* ( $EC_{50}$  2 or 5.12  $\mu\text{M}$ ), the Cladocera *D. magna* 24-h neonates immobilization ( $EC_{50}$  2.2 or 3.2  $\mu\text{M}$ ), and the Anostraca *Artemia salina* 24-h larval mortality ( $EC_{50}$  4.4  $\mu\text{M}$ ). After 24 h of exposure, an  $EC_{50}$  of 12  $\mu\text{M}$  was found for morphological alterations in the human cell lines Hep G2 and Chang liver cells, and higher for other indicators.

Table 1

Effects after 24 h of exposure of Root meristems of *Allium cepa* to different concentrations of pentachlorophenol, on mitotic index rate or micronuclei rate

[ $\mu\text{M}$ ]	M.I. (%)	Mn (%)
–	12.0 $\pm$ 1.6	0.1 $\pm$ 0.02
1	13.0 $\pm$ 0.3	0.1 $\pm$ 0.03
5	9.6 $\pm$ 0.8*	0.1 $\pm$ 0.3*
10	6.8 $\pm$ 0.8*	1.7 $\pm$ 0.1*

Data are expressed relative to mean value in respective unexposed controls.

\*Indicates significant difference from control value ( $P < 0.01$ ).

One important piece of information needed in risk assessment is the concentration range at which a chemical produces adverse effects on organisms living in the aquatic or terrestrial environment. Without this information we can neither make predictions nor establish safety factors. According to the basal cytotoxicity concept, a majority of chemicals cause toxicity by means of basal cytotoxicity, while a clear minority cause toxicity by interference with either organ-specific cell functions or extracellular bodily functions.

According to this reductionistic view, the toxicity of a compound can be broken down into a number of elements, each of which can be identified and quantified in appropriate model systems (Ekwall, 1994). It seems possible that a limited number of cell lines or isolated cells from invertebrates and vertebrates may be sufficient for basal cytotoxicity screening. In the case of aquatic organisms, extrapolation can be effected fairly easily, since chemical concentrations in water can be directly compared with concentrations in culture medium. For this reason, fish cell lines in particular are becoming more frequently employed as surrogates for whole-animal ecotoxicity testing with relevant (sub)-lethal endpoints (Castaño and Tarazona, 1995).

We have found that the RTG-2 rainbow trout cell line proliferation was inhibited after pentachlorophenol exposure for 72 h with an  $EC_{50}$  of 2  $\mu\text{M}$ , that neutral red uptake was less inhibited at 24 h with an  $EC_{50}$  of 90  $\mu\text{M}$ , and that LDH leakage was not increased up to 100  $\mu\text{M}$  ( $EC_{20}$  195  $\mu\text{M}$ ; Fig. 5), in agreement with the  $EC_{50}$  of 186  $\mu\text{M}$  reported  $^{86}\text{Rb}$  leakage after 3 h of exposure in rainbow trout hepatocytes (Clemmedson et al., 1996). Although MTT reduction was not modified, LDH activity was inhibited with an  $EC_{50}$  of 200  $\mu\text{M}$ . In fact, Castaño et al. (1995) reported a 48-h  $EC_{50}$  of 90  $\mu\text{M}$  for RTG-2 cell proliferation, which was reduced to 25  $\mu\text{M}$  when ATP content was used as an indicator. It was also reported (Clemmedson et al., 1998a) that the R1 rainbow

trout cell line presents an  $EC_{50}$  of 33  $\mu\text{M}$  for 144-h cell proliferation, increased to 36  $\mu\text{M}$  for 24 h, and to 38  $\mu\text{M}$  for neutral red uptake at 24 h. Other fish cell lines have been reported to be less sensitive to pentachlorophenol; the  $EC_{50}$  was 170  $\mu\text{M}$  on BF-2 Bluegill Sunfish cells for 24 h using neutral red uptake as a marker (Babich and Borenfreund, 1987); the  $EC_{50}$  for the hepatoma cell line PLHC-1 derived from topminnow exposed for 24 h was 200  $\mu\text{M}$  using neutral red uptake and 320  $\mu\text{M}$  using MTT reduction as endpoints (Fent and Hunn, 1996). Although sensitivity for pentachlorophenol seems to be low, probably due to either its specific mechanism of action or its biotransformation into tetrachloro-hydroquinone, different studies have demonstrated the ability of various fish cell lines to predict the toxicity of many chemicals on fish (Babich and Borenfreund, 1987; Castaño et al., 1995; Fent and Hunn, 1996) or cladoceran (Dierickx and Bredael-Rozen, 1996).

Toxic effects of pentachlorophenol on the different models and bioindicators included in the proposed ecotoxicological battery are summarized in Table 2. The system most sensitive to pentachlorophenol, was micronuclei induction of Root meristems in *A. cepa*, followed by the cladoceran *D. magna* immobilization, bioluminescence inhibition in *V. fischeri* bacteria at 60 min and cell proliferation inhibition of RTG-2 cells at 72 h. Inhibition of cell proliferation and MTT reduction on Vero monkey cells showed intermediate sensitivity. The less sensitive systems were inhibition of neutral red uptake and MTT reduction in the RTG-2 cells at 24 h and the inhibition of the growth of *C. vulgaris*. From the risk assessment point of view, in a range of 2  $\mu\text{M}$ , pentachlorophenol is very toxic for a variety of organisms, producing also genotoxic effects, requiring rigid control measures.

The complexity of the obtained results, with very different effects according to the test system and exposure period employed, shows that a single bioassay will never

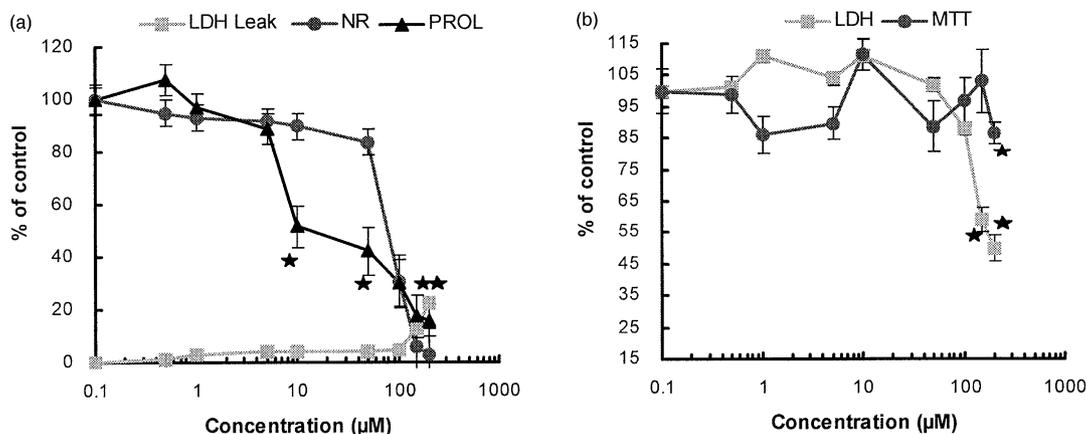


Fig. 5. RTG-2 Rainbow trout cells effects of pentachlorophenol on (a) LDH leakage (■), and neutral red uptake (●) after 24 h exposure, and cell proliferation inhibition at 72 h (▲) or (b) LDH activity (■) and MTT reduction (●) after 24 h exposure. Data expressed relative to mean value in respective unexposed controls. \* Indicates significant difference from control value ( $P < 0.01$ ).

Table 2  
Toxic effects of pentachlorophenol on the different models and bioindicators included in the proposed ecotoxicological battery

Model system	Origin	Indicator	Exposure period	EC <sub>50</sub> <sup>a</sup> (μM)
<i>Daphnia magna</i>	Cladoceran	Immobilization	24 h	2.1
<i>Daphnia magna</i>	Cladoceran	Immobilization	48 h	2.0
<i>Daphnia magna</i>	Cladoceran	Immobilization	72 h	1.5
<i>Vibrio fischeri</i>	Bacteria	Bioluminescence	5 min	3.2
<i>Vibrio fischeri</i>	Bacteria	Bioluminescence	15 min	2.3
<i>Vibrio fischeri</i>	Bacteria	Bioluminescence	60 min	1.9
<i>Chlorella vulgaris</i>	Unicel. algae	Growth	24 h	59
<i>Chlorella vulgaris</i>	Unicel. algae	Growth	48 h	38
<i>Chlorella vulgaris</i>	Unicel. algae	Growth	72 h	29
<i>Allium cepa</i>	Terrestrial plant	Mitotic index	24 h	> 10
<i>Allium cepa</i>	Terrestrial plant	Micronuclei rate	24 h	1.2
Vero cell line	Monkey	Cell proliferation	24 h	34
Vero cell line	Monkey	Cell proliferation	48 h	24
Vero cell line	Monkey	Cell proliferation	72 h	6
Vero cell line	Monkey	MTT reduction	24 h	37.6
Vero cell line	Monkey	MTT reduction	48 h	10.3
Vero cell line	Monkey	MTT reduction	72 h	8.6
RTG-2 cell line	Rainbow trout	LDH leakage	24 h	> 200
RTG-2 cell line	Rainbow trout	Neutral red uptake	24 h	90
RTG-2 cell line	Rainbow trout	Cell proliferation	72 h	2
RTG-2 cell line	Rainbow trout	LDH activity	24 h	200
RTG-2 cell line	Rainbow trout	MTT reduction	24 h	> 200

<sup>a</sup> Mean effective concentration: concentration of test chemical that modified each biomarker by 50% in comparison with appropriate untreated controls.

provide a full picture of the quality of the environment. A battery of test systems and indicators would be representative of a wide range of organisms. For the time being, a minimum ecotoxicological in vitro test battery should at least include bacteria, algae, invertebrates and fish cell lines.

Many opportunities to change the actual scene will be implemented in the near future. More efficient risk assessment procedures should be designed, integrating more flexible testing methods into tier testing schemes that employ only the necessary tools for each case. A careful selection of the most appropriate species, strains and development stages for testing can be combined with a reduction in the number of required assays and/or animals/assay. The development of mechanistic assays, based on relevant new endpoints for mammals, invertebrates (bacteria, fungi, algae, protozoans) and cell cultures, may be crucial to this process.

The regulatory acceptance of new ecotoxicological methods has been very slow, particularly for the evaluation of new chemicals. The *D. magna* immobilization assay and the algae growth are included in most environmental legislations, including OECD Guidelines for testing of Chemicals. However, the *V. fischeri* bacteria test has only been adopted by some regulations for the characterization of hazardous waste. Some alternative procedures (mainly in vitro) have been applied and integrated as useful tools in environmental contamination and toxicology and are now routinely used in areas such as pollutant monitoring and control by, both

scientists and the industry. Their continuing validation and acceptance as real alternatives to animal testing, and particularly to replace fish studies, should be promoted.

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