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# Acute Toxicity of Selected Metals and Phenols on RTG-2 and CHSE-214 Fish Cell Lines

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*In vitro* toxicity tests with fish cell lines appear as an alternative to single species bioassays (Babich and Borenfreund 1987a) and have been used successfully in different applications, both for single chemicals (Bols *et al.* 1985) and for environmental samples (Kokan *et al.* 1985; Halder and Ahne 1991; Vega *et al.* 1994), including Toxicity Identification Evaluation procedures (Tarazona *et al.* 1990).

Different fish cell lines, such as BF-2 (Babich *et al.* 1986a), RTG-2 (Tarazona *et al.* 1993), FHM (Rachlin and Perlmutter 1968) and R1 (Ahne 1985) have been employed in these toxicological studies.

The aim of the present study was to compare the sensitivity of two salmonid fish cell lines, RTG-2 and CHSE-214, in evaluating toxicity of seven compounds (three metals and four phenolic chemicals), by measuring three endpoints: cellular mass, cell viability and intracellular ATP content of the cells.

## MATERIALS AND METHODS

Chemicals tested were: cadmium nitrate, zinc nitrate, copper nitrate, 4-chlorophenol (4-CP), pentachlorophenol (PCP) (Merck, Germany) phenol (P) and 2,4-dinitrophenol (2,4-DNP) (Fluka, Switzerland). Metals were dissolved in the test medium while phenolic compounds were previously dissolved in methanol (4-CP and PCP) or ethanol (P and 2,4-DNP).

Toxicity was studied on two salmonid fish cell lines, RTG-2, derived from the gonad of rainbow trout (*Oncorhynchus mykiss*), and CHSE-214, an embrionary cell line derived from chinook salmon (*Oncorhynchus tshawytscha*). Both cell lines were cultured in Minimum Essential Medium (MEM), with Earle's salts and supplemented with 10% Foetal Calf Serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, 1.25 μg/mL fungizone and 2mM glutamine. Cells were seeded in 96-well culture microtiter plates, with an initial density of 15 x $10^3$ and

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25 x $10^3$ cells/well for RTG-2 and CHSE-214, respectivelly. Cells were incubated for 48 hr at 20°C in a sealed plastic bag in a 5% carbon dioxide-air atmosphere. After this acclimation period, in which cells raise exponential growth-phase, culture medium was removed and replaced by test medium, containing 1% instead 10% FCS, amended with different concentrations of chemical. In each experiment eight well per concentration and eight control wells were used. Experiments were run in triplicates. Final solvent concentrations were always below 1%, adequate solvent controls were included.

After 48 hr of exposure, cytotoxicity was determined measuring three endpoints: 1) protein content to evaluate the total cellular mass using the FRAME Kenacid Blue Protein assay (KBP) (Knox *et al.* 1986), 2) uptake of Neutral Red stain (NR) to evaluate the cell viability (Borenfreund and Puerner 1984), and 3) intracellular ATP content, by extracting ATP directly from cell monolayers (Castaño and Tarazona 1994).

The $EC_{50}$ and 95% confidence limits were calculated by computerized log-probit analysis according to Trevors (1987). Statistical treatment of the data was performed using the Statgraphics program. Significant differences between $EC_{50}$ values were amended by the $f_{1,2}$ method proposed by Litchfield and Wilcoxon (1949) according to APHA (1980). $p < 0.05$ was considered a significant level.

## RESULTS AND DISCUSSION

Table 1 shows the $EC_{50}$ values obtained for CHSE and RTG-2 cells. Concerning to the endpoint measured, for CHSE cells only phenol and PCP, two out of the seven tested chemicals, showed significant differences. On the other hand, RTG-2 showed differences for five out of the seven chemicals. Differences increased on phenols, which showed a 85% of the total.

Data show that endpoint responses varied with the chemical. In some cases, i.e. for 4-chlorophenol, all $EC_{50}$ values fall within the same order of magnitude, while for chemicals such as PCP, three orders of magnitude are required to cover the $EC_{50}$ range. This fact can be easily observed looking at the dose/response curves, which are represented in Figures 1A and 1B.

Significant differences between $EC_{50}$ values for CHSE and those observed for RTG-2 were found in 81% of the cases. In fact, all chemicals showed significant differences between both cell lines, at least, for one of the endpoints considered in this study.

A linear relationship between the $EC_{50}$ values obtained for both cell lines was observed. Fitting, 95% confidence limits and correlation coefficient appear in Figure 2. The slope, 1.33 with an standard error of 0.19 ($p < 0.001$), indicates that RTG-2 cells are slightly more sensitive than CHSE. Only 2,4-DNP have the three endpoints out of the confidence limits. In fact, CHSE cells were more

Table 1. $EC_{50}$ values obtained for CHSE and RTG-2 cell lines. Values are expressed in $\mu$g/mL. [a]significant differences with ATP; [b]significant differences with Neutral Red. ($p < 0.05$). *Significant differences with the $EC_{50}$ values for CHSE. Range values in parenthesis.

| | ATP | Neutral Red | Proteins |
|---|---|---|---|
| CHSE cell line | | | |
| cadmium | 6.5 (5.6-7.6) | 6.2 (5.3-7.3) | 6.6 (5.2-8.3) |
| zinc | 2.0 (1.7-2.3) | 1.9 (1.6-2.3) | 2.4 (2.0-2.9) |
| copper | 6.5 (5.9-6.8) | 6.3 (5.5-7.3) | 6.3 (5.8-7.8) |
| phenol | 1076.2 (974-1189) | [a]888.0 (770-1023) | [a,b]637.4 (597-666) |
| 2,4-dinitrophenol | 67.48 (46-100) | 71.7 (39.3-130) | 109.6 (74-162) |
| 4-chlorophenol | 166.0 (147-187) | 169.0 (149-191) | 181.0 (167-210) |
| pentachlorophenol | 81.5 (63-104) | 97.3 (73-129) | [a,b]148.3 (116-188) |
| RTG-2 cell line | | | |
| cadmium | *3.9 (2.6-5.9) | 6.2 (4.9-7.8) | [a]7.7 (6.1-9.8) |
| zinc | *13.7 (11.6-16.3) | *11.0 (6.9-17.8) | *15.1 (7.5-30.3) |
| copper | *11.9 (10-14.1) | *9.5 (7.7-11.7) | *12.7 (9.7-16.7) |
| phenol | *518.5 (482-556) | *461.7 (385-552) | [a,b]638.9 (573-721) |
| 2,4-dinitrophenol | *279.0 (249-313) | *,[a]165.0 (118-231) | *,[b]323.0 (261-401) |
| 4-chlorophenol | 173.7 (158-191) | *128.9 (11-147) | *,[a]144.1 (124-166) |
| pentachlorophenol | *6.7 (6.1-7.5) | *,[a]13.8 (11-16.6) | *,[a,b]24.3 (19.6-30) |

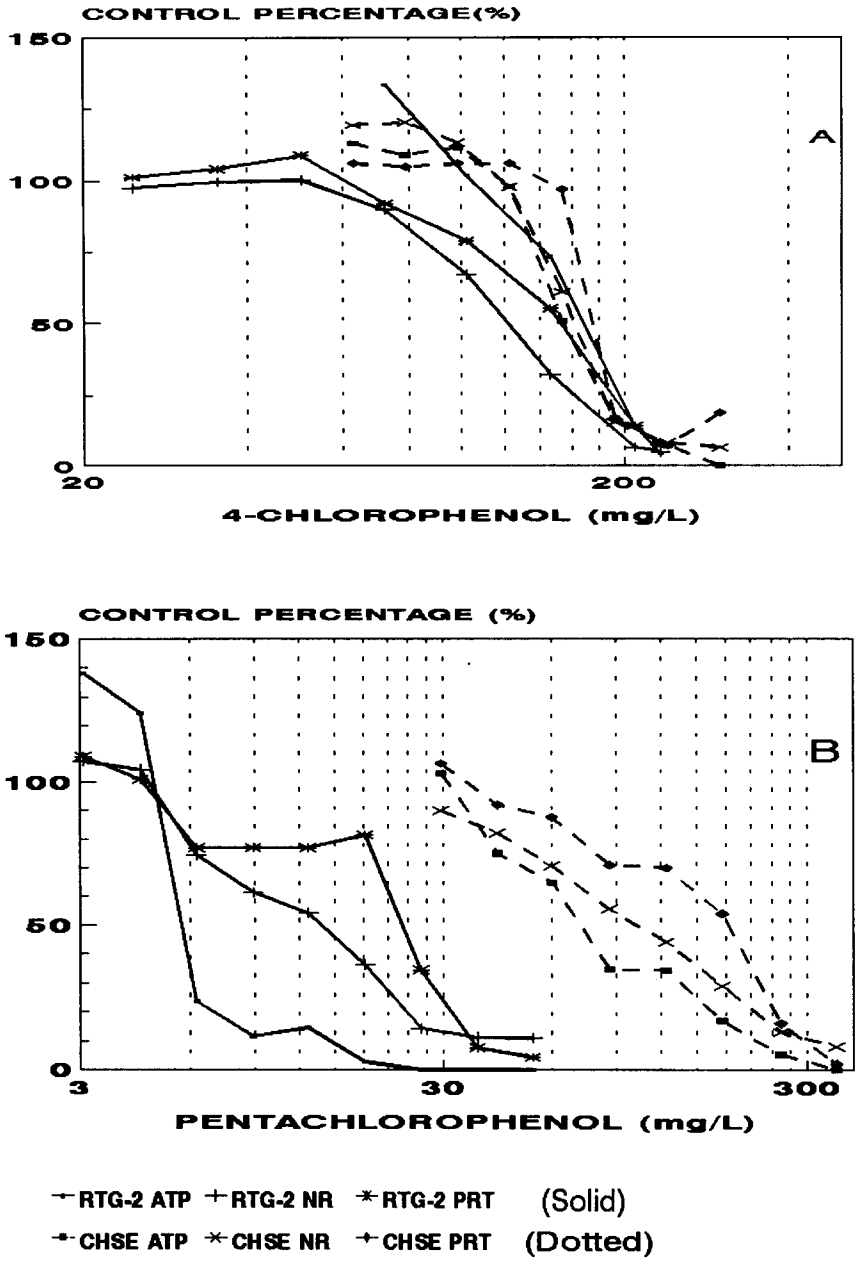



Figure 1. CHSE and RTG-2 dose-response curves for 4-chlorophenol (A) and pentachlorophenol (B). Response is represent as percentage of control cells.

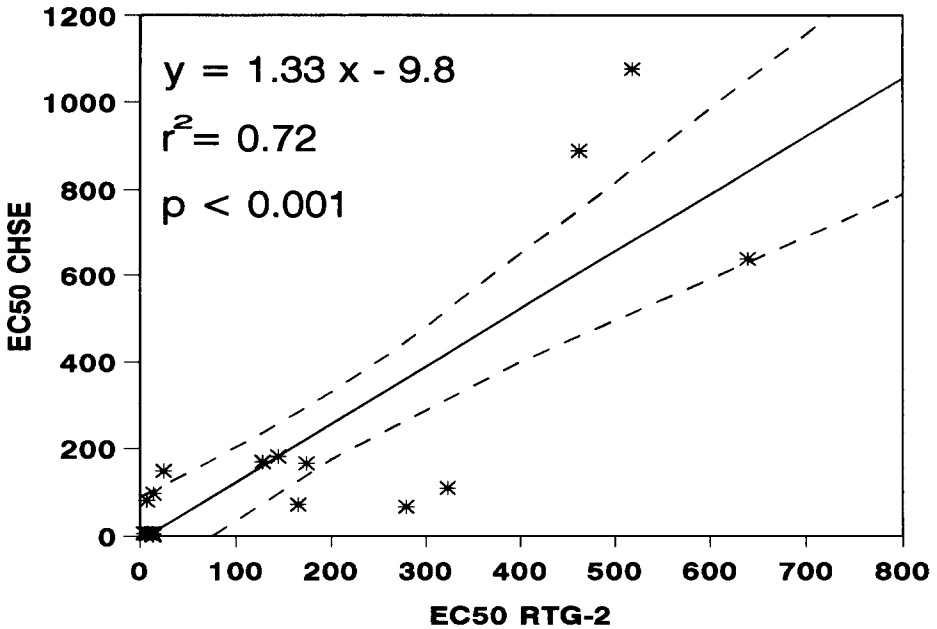


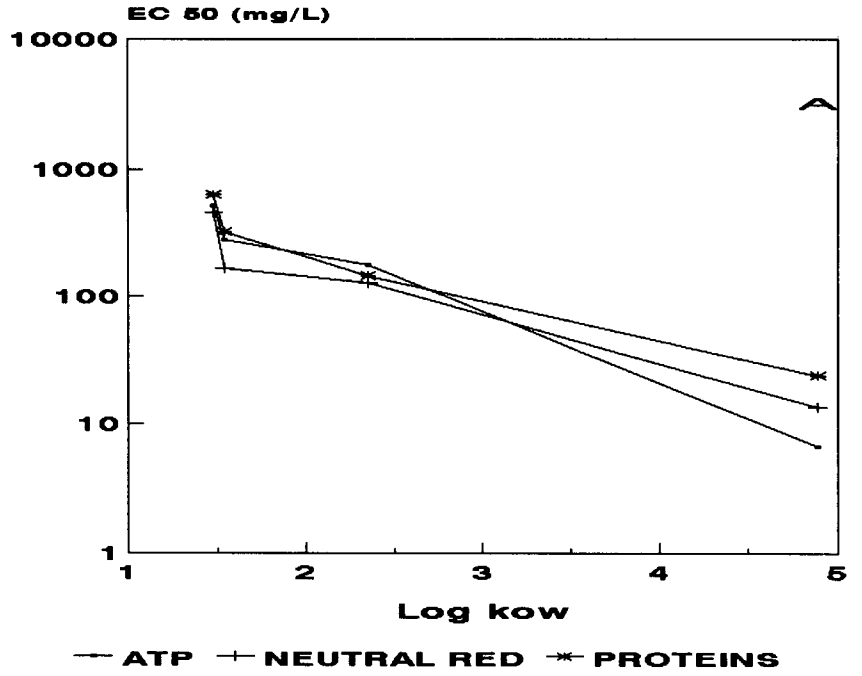

Figure 2. CHSE-214 vs RTG-2 $EC_{50}$s correlation ($\mu$g/mL). Dotted lines indicate confidence level for $p < 0.05$.

sensitive than RTG-2 for this particular chemical.

The lower sensitivity of CHSE cells should be due, at least in part, to the testing conditions. These conditions were optimized for RTG-2 cells in terms of both, reproductibility and sensitivity (Castaño, 1992). In fact, the sensitivity achieved with this experimental protocol is higher than that previously reported for RTG-2 (Bols *et al.* 1985). Babich *et al.* (1986b) compared RTG-2 and BF-2 cells using experimental conditions developed for BF-2, and also found a higher sensitivity for the fish line tested under optimal conditons.

Qualitative difference between both cell lines can be observed by plotting phenolic $EC_{50}$ values versus their log $K_{ow}$. Log-linear relationship for RTG-2 cells (Figure 3A) with correlation coefficients of 0.99; 0.96 and 0.97 ($p < 0.05$) for ATP, NR and proteins, respectively, were established. However the relationship was not observed for CHSE (figure 3B), which showed correlation coefficients lower than 0.5 for the three endpoints without statistical significance.

The relationship between toxicity and lipophilicity of phenols has been established for different aquatic organisms, including rainbow trout (Nendza and Klein, 1990), and are very common in QSAR indices. Thus a limited set of chemicals was used to check the usefulness of the fish cell lines. We have no reasons to



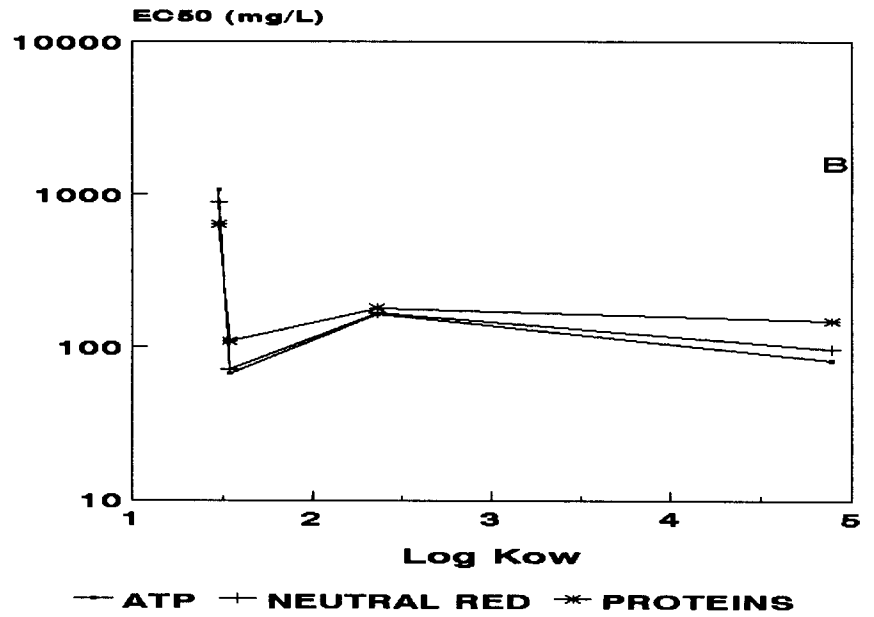




Figure 3. Log $K_{ow}$ versus cytotoxicity relationships for diferent phenols on RTG-2 cells (A) and on CHSE (B).

explain the difference observed between RTG-2 and CHSE other than the use of testing conditions specifically optimized for RTG-2 cells.

We have no reasons to explain the difference observed between RTG-2 and CHSE-214 other the use of testing conditions specifically optimized for RTG-2 cells. In this sense, different species often have different sensitivity (Cairns and Niederlenhner, 1987), and even different tissues (Bridges *et al.*, 1987).

Limits to the applicability of QSARs exist in cases where specific biological interactions can be expected (Babich and Borenfreund, 1987b). Figure 3 shows that the main problem related to the lack of relationship for CHSE regard the toxicity of 2,4-DNP. Problem with nitro-substituents are common in these kind of studies (Nendza and Klein, 1990); and it must be considered that 2,4-DNP was significantly more toxic to CHSE-214 than RTG-2.

*In vitro* cell cultures have demonstrated to be a suitable tool for assessing the toxicity of different chemicals to fish (Bols *et al.* 1985). Emphasis is to be layered on high sensitivity as well as on the proximity to higher organisms. A positive correlation of acute lethal potency in fish with *in vitro* cytotoxicity has been found by Fry *et al.* (1990). *In vitro* acute toxicity test can be performed much more easily and faster so it is an important achievement in the environmental assessment of toxicants. The difference observed between both cell lines demonstrate the need of a previous work of optimization before setting the testing conditions for toxicity tests with fish cell lines.

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