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Toxicological Evaluation of Waste-Water Samples to Appropriately Sensitized Cultured Fathead Minnow Cells Compared with the Microtox Assay

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Much toxicological research has been performed during the last years on the replacement of animals by cultured cells. The prediction of the human toxicity is one of the final goals in this case. The development of appropriate ecotoxicity assays is another important task. Indeed, the continuously increasing production and use of chemicals result in heavily contaminated fresh-, coastal- and seawater in most of the industrialized regions of the world. Different cultured fish cell lines have thus far been used for measuring the cytotoxicity of several groups of chemicals (Babich and Borenfreund 1991; Segner 1998).

We have previously shown that a good correlation exists between in vitro cytotoxicity data to cultured fathead minnow (FHM) fish cells and fish lethality data for a series of 50 chemicals belonging to very different chemical classes (Brandao et al. 1992). A comparable correlation was observed for the in vitro toxicity of 45 pesticides to goldfish GF-scale (GFS) cells (Saito et al. 1991). A good correlation between the cytotoxicity of inorganic metal compounds to FHM cells and the in vivo toxicity to *Daphnia magna* was also observed (Dierickx and Bredael-Rozen 1996), but the FHM assay was less sensitive than fish and *Daphnia* assays. In order to allow the use of the FHM assay for environmental water samples, its sensitivity was highly increased by adding sodium dodecyl sulfate (SDS) and buthionine sulfoximine (BSO), a glutathione synthesis inhibitor (Griffith 1982), to the assay system (Dierickx 1998). However, when so sensitised FHM cells were used to measure the toxicity of surface-water samples, it appeared that too many false-positives were obtained, at least when compared to the Microtox assay (Dierickx et al. 2000), and ascribed to the addition of SDS. We, therefore, searched for assay conditions without SDS resulting in an intermediate toxic effect between the original (Brandao et al. 1992) and the highly sensitive assay (Dierickx 1998). This adapted assay system was then applied for measuring the toxicity of waste-water samples, from which the toxicity was also measured by the Microtox method.

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MATERIALS AND METHODS

FHM cells are an established fish cell line (American Type Culture Collection no. CCL42) derived from tissue posterior to the anus from fathead minnow (*Pimephales promelas*). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% non-essential amino acids, 100 units/mL penicillin, and 0.1 mg/mL streptomycin (complete medium) and incubated in a humidified atmosphere of 5% CO_2 at 34°C.

The cytotoxicity was measured by the neutral red uptake inhibition assay of Borenfreund and Shopsis (1985), slightly adapted as described (Brandao et al 1992). In this study the cells were seeded into 64 wells of a titer plate (Nunc, Roskilde, Denmark) at $9x10^4$ cells in 0.2 mL complete medium per well, with or without 400 μM BSO (Sigma). It was shown in preliminary experiments that BSO in the medium reduced the glutathione content in the cells without affecting the growth of the cells. After incubation for 24hr the cultures were treated with 0.2 mL aliquots of different concentrations of the freshly prepared test compounds in complete medium (8 wells/concentration), also with or without 400 μM BSO. The cells were treated for 24hr and further analyzed as decribed (Borenfreund and Shopsis 1985; Brandao et al 1992). The relative toxicity of the six model compounds is established by the determination of the NI_{50}, the concentration of test compound required to reduce neutral red uptake by 50%. Standard deviations of less than 5% were obtained in completely independent assays.

The waste-water samples were taken in Southern Belgium, between February and December 2000. The samples were sterilised by filtration through a Millex-GS filter (0.22 μM; Millipore, Bedford, MA, USA).

Table 1. Waste-water sample identification.

Sampling code	Kind of plant/industry
P	Seawage treatment plant
Q	Seawage treatment plant
R	Seawage treatment plant
S	Paper industry
T	Wood panel production
U	Textile industry
V	Chemical industry
W	Metal industry
X	Metal industry
Y	Metal industry
Z	Metal industry

The toxicity of the water samples to FHM cells was measured in the above described conditions. In practice, the test was adopted to permit the use of large amounts of water. Doubly concentrated complete

culture medium was prepared, with or without BSO. This doubly concentrated medium was diluted with an equal volume of ultrapure Milli-Q purified water for the treatment of the control cells, and with an equal volume of the waste-water samples (the 50% waste-water samples). One to two diluted (with normal complete medium, with or without BSO) samples were also tested (the 25% waste-water samples). Neutral red uptake was measured. For the waste-water samples the results were expressed as the neutral red uptake in % of the control cells. FHM cells were considered to be intoxicated when the neutral red uptake was reduced by 15% or more compared with that of control cultures.

The Microtox assay is based on inhibition of luminiscence of the bacterium *Vibrio fischeri* (Van der Wielen and Halleux 2000), and was performed as described (Dierickx et al. 2000). The results were quantified by the TU_{20} and TU_{50}, corresponding to respectively $100/EC_{20}$ and $100/EC_{50}$, EC_{20} and EC_{50} being the exposure concentrations causing 20 and 50% inhibition of luminiscence.

RESULTS AND DISCUSSION

Six chemicals (Table 2) were used to find assay conditions resulting in an intermediate toxicity between the original assay (Brandao et al. 1992), where $6x10^4$ cells/well were treated for 2hr, and the highly sensitive assay (Dierickx 1998), where $15x10^3$ cells/well were treated for 24hr in the presence of 200 μM SDS and 400 μM BSO. Assays with varying cell numbers, serum concentrations, and treatment periods were performed in several combinations. The addition of SDS or other surfactants was not longer considered to be of practical value. The results presented in Table 2 show that, on the mean, intermediate toxicity was obtained

Table 2. Comparative cytotoxicity of six chemical compounds in different assay conditions. The results are given as the EC_{50} values (mM) for neutral red uptake (NI_{50}) or total protein content (PI_{50}).

Chemical	NI_{50}(2hr)[a]	PI_{50}(24hr)[b]	NI_{50}(24hr)	
			- BSO	+ BSO
Cupric sulfate	3.2	0.081	0.62	0.17
Ethylenediamine	52	1.21	8.9	11
Sodium nitrite	191	9.29	80	65
n-Propanol	300	11.9	278	271
Citric acid	14	1.46	8.3	10
Oxalic acid	17	2.98	0.9	0.6

[a]Results from Brandao et al. (1992).
[b]Results from Dierickx (1998).

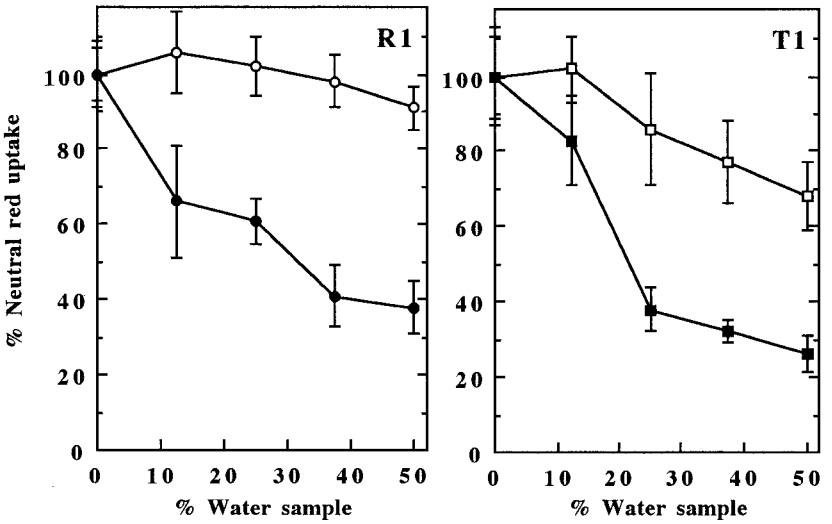

Figure 1. Influence of the dilution of water samples R1 and T1 on the neutral red uptake in FHM cells. Open symbols : without BSO, closed symbols : with 400 μM BSO.

when $9x10^4$ cells/well were treated for 24hr (2 last columns), and that the toxicity was in several cases higher in BSO-treated cells compared to normal cells. Oxalic acid, however, was more toxic than in the previous highly sensitive assay. The $9x10^4$ cells/well /24hr treatment conditions were used to measure the toxicity of waste-water samples. These were taken over an 11 month period in 2000. The 11 sampling points covered different kinds of industrial activity (Table 1). Most of the toxic samples showed a dose-dependent toxicity for a serial dilution of the water samples. This is illustrated for samples R1 and T1 in Fig. 1.

The toxicity results of both the FHM and the Microtox assay for all 51 waste-water samples are given in Table 3. Since only few samples were toxic to FHM cells in the abscence of BSO, the 25% water results are not given for these conditions. The presence of nutrients can, at least partly, explain the increased neutral red uptake which was observed for several samples. In the absence of BSO only 7 water samples reduced the neutral red uptake by 15% or more, from which two (P3 and T4) were not toxic in the presence of BSO. Since the FHM assay without BSO is not sensitive enough, we further only considered the cytotoxicity results in the presence of BSO: 16 samples were toxic using 25% water and 23 samples using 50% water. The additional 7 toxic samples using 50% water reflect the higher sensitivity of the assay in these conditions. A dose-response comparable to that in Fig. 1 was obtained for most toxic samples, but some exceptions were observed (samples Q5, T3, U1, W4, and X5). In the Microtox assay 36 samples were toxic based on the

Table 3. Toxicity results for the whole series of water samples. The cytotoxicity results in FHM cells are expressed as the neutral red uptake in % of the control cells.

Sample		% Neutral red uptake in FHM			Microtox®	
		- BSO	+ BSO			
N°	Date	50% water	25% water	50% water	TU_{20}[a]	TU_{50}[a]
P1	02/21	108 ± 6	94 ± 11	110 ± 26	-	-
P2	04/11	111 ± 6	113 ± 8	107 ± 14	-	-
P3	06/27	85 ±14[b]	128 ± 21	155 ± 32	-	-
P4	11/04	97 ± 5	92 ± 10	92 ± 13	-	-
Q1	02/28	112 ± 6	124 ± 25	128 ± 38	11	-
Q2	06/06	119 ± 6	106 ± 20	80 ± 11[b]	220	69
Q3	08/22	86 ± 14	81 ± 22[b]	51 ± 22[b]	370	140
Q4	10/03	99 ± 6	97 ± 13	99 ± 7	-	-
Q5	11/28	99 ± 3	47 ± 6[b]	49 ± 6[b]	-	-
R1	03/20	121 ± 11	87 ± 21	25 ± 8[b]	17	4.1
R2	05/23	114 ± 12	146 ± 25	114 ± 18	5.5	-
R3	09/05	128 ± 21	110 ± 30	87 ± 24	31	5
R4	10/03	111 ± 10	107 ± 3	103 ± 14	6.1	-
R5	12/05	103 ± 11	95 ± 8	87 ± 4	5.9	-
S1	02/07	105 ± 5	110 ± 11	113 ± 18	4.6	-
S2	06/13	109 ± 10	96 ± 13	114 ± 17	3.4	-
S3	05/12	115 ± 9	103 ± 13	85 ± 5[b]	2.4	-
S4	11/14	92 ± 15	94 ± 9	90 ± 9	5.9	-
T1	02/07	75 ± 16[b]	52 ± 10[b]	27 ± 4[b]	50	8.7
T2	06/13	107 ± 22	115 ± 14	85 ± 9[b]	75	15
T3	09/19	110 ± 8	63 ± 16[b]	63 ± 11[b]	87	14
T4	12/12	78 ± 6[b]	92 ± 5	90 ± 7	290	65
U1	02/15	94 ± 17	24 ± 5[b]	26 ± 9[b]	5.7	3.1
U2	08/29	110 ± 8	102 ± 13	110 ± 18	2.9	-
U3	10/24	97 ± 3	78 ± 14[b]	65 ± 16[b]	2.6	-
U4	12/12	95 ± 13	90 ± 5	92 ± 6	17	7.7
V1	03/20	103 ± 4	101 ± 15	84 ± 14[b]	-	-
V2	05/23	106 ± 19	82 ± 12[b]	62 ± 10[b]	-	-
V3	09/05	114 ± 24	119 ± 23	115 ± 25	-	-

Table 3. *Continued*

Sample		% Neutral red uptake in FHM			Microtox®	
		- BSO	+ BSO			
N°	Date	50% water	25% water	50% water	TU_{20}[a]	TU_{50}[a]
V4	11/07	98 ± 9	85 ± 11[b]	62 ± 12[b]	-	-
V5	12/19	107 ± 5	95 ± 4	91 ± 5	-	-
W1	02/14	110 ± 5	99 ± 22	104 ± 10	-	-
W2	04/04	114 ± 6	96 ± 18	91 ± 15	13	-
W3	06/20	133 ± 27	120 ± 24	88 ± 14	-	-
W4	10/24	96 ± 9	83 ± 11[b]	84 ± 7[b]	68	13
W5	12/19	95 ± 9	112 ± 20	90 ± 16	16	-
X1	02/14	107 ± 5	125 ± 19	98 ± 12	-	-
X2	04/04	110 ± 7	87 ± 12	99 ± 16	7.2	-
X3	06/20	97 ± 15	130 ± 27	130 ± 33	18	3.3
X4	10/03	100 ± 8	94 ± 15	80 ± 10[b]	22	2.6
X5	12/05	105 ± 12	80 ± 3[b]	87 ± 13	-	-
Y1	02/21	106 ± 6	96 ± 19	88 ± 19	3.2	-
Y2	04/11	106 ± 21	74 ± 16[b]	64 ± 12[b]	12	2.5
Y3	06/27	91 ± 12	149 ± 25	147 ± 41	3.8	-
Y4	05/12	102 ± 16	94 ± 12	50 ± 27[b]	6.3	-
Y5	11/14	96 ± 5	86 ± 14	82 ± 5[b]	2.3	-
Z1	02/28	71 ± 9[b]	32 ± 15[b]	22 ± 7[b]	590	120
Z2	06/06	79 ± 19[b]	37 ± 17[b]	18 ± 5[b]	2000	320
Z3	08/22	82 ± 17[b]	35 ± 19[b]	21 ± 6[b]	450	130
Z4	09/19	90 ± 10	80 ± 10[b]	66 ± 8[b]	164	60
Z5	11/28	85 ± 5[b]	32 ± 9[b]	29 ± 6[b]	200	47

[a]The TU_{20} and TU_{50} represent the number of times the water sample has to be diluted to obtain the EC_{20} and EC_{50}, respectively; - = lower than the quantitation limit of 2.2.
[b]Toxic.

TU_{20} and 19 based on the TU_{50}. Thus, the number of 23 toxic samples in the FHM assay is situated in between the two Microtox calculations.

When the toxicity of the 51 water samples is compared in both the FHM assay and in the Microtox (Table 4) a high number of samples was toxic

or not toxic in both assays. On the basis of the water samples which are only toxic in one of both assays, the Microtox assay based on the TU_{20} was more sensitive than the FHM assay, and the latter was somewhat more sensitive than the Microtox assay when based on the TU_{50}. It should be stressed, however, that stronger criteria for toxicity were chosen in the Microtox assay than in the FHM assay.

Table 4. Comparison of the toxicity of the 51 waste water samples in the FHM assay (in the presence of BSO) and in the Microtox assay. The numbers of water samples are given.

	Toxic in FHM assay & Microtox	Only toxic in Microtox	Only toxic in FHM assay	Not toxic in FHM assay or Microtox
Based on TU_{20}	19	17	4	11
Based on TU_{50}	15	4	8	24

Altogether our results show that the FHM assay in the presence of BSO allows to detect the toxicity of environmental water samples with a sensitivity which is qualitatively comparable to that of the Microtox method. The advantages of this assay are that no concentration or fractionation of the water sample is necessary and that any in vitro cytotoxicity laboratory can perform this kind of assay, in contrast to the special equipment and experience needed to perform *Daphnia* and fish tests. Further improvement of this assay can be expected by introducing the measurement of more specific additional biochemical parameters as lipid peroxidation, cytochrome- and xenobiotic-dependent enzyme activities (Brack et al. 2000; Strmac and Braunbeck 2000).

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