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A New Biological Model System for the Determination of Industrial and Agricultural Effluents Polluted the Aquatic Environment *In vitro*

Yousif A. Elhassaneen
Department of Nutrition and Food Science, Faculty of Home Economics, Minufiya University, Shebin El- Kom, Egypt

Abstract: Industrial and agricultural effluents represent wide spread contaminants of the aquatic environment. In a trial to assess the toxic effects of these pollutants, primary liver cell's culture of Spot fish (*Leiostomus xanthurus*) was used as an alternative new biological model of the whole organisms. The obtained results indicated that some cytotoxic effects such as lysosomes (NR), mitochondria (MTT) dysfunction and cell wall integrity (CV) were observed on the liver cells at the lower concentrations of this toxic chemicals. For NR, MTT and CV assays, the absorbance measurements (as a percent of control) were ranged 36.2-115.8, 37.8-104.2 and 22.6-109.2; 41.7-126.3, 44.3-124.1 and 25.7-126.5 and 53.2-132.7, 57.7-137.3 and 32.6-133.2 for paper, petroleum industries and agricultural effluents, respectively. Consequently, CV is more sensitive to industrial and agricultural effluents when compared with other assays. On the other side, the influence of such effluents ort the biochemical assays such as growth assay (GA, as determined by protein analysis), protease activity (PA) and lactic dehydrogenase activity (LDH) of isolated liver cells were determined in a trial to make a comparison between biochemical and cytotoxicity assays. The present data demonstrated that all of cytotoxic assays i.e. NR, MIT and CV corresponded well to those with GA, PA and LDH assays. The results of this study can constitute a milestone toward application of the simple inexpensive laboratory biological-model system for screening of the acute toxicities of aquatic pollutants.

Key words: Effluent, pollution, aquatic environment, liver cells

Introduction

Many different industrial and agricultural effluents enter aquatic environments and pose a potential threat to the different organisms. One of the most problem associated wfth the deposition of chemical pollutants into fresh and marine environments is the development of appropriate techniques and methodologies for determination of adverse changes in the quality of these environments. Much of the previous studies usually using LC_{so} bioassay of adult organisms such as fish for assessing the acute toxicity of aquatic pollutants. In spite their usefulness of such *in vivo* bioassay testing to bridge the gap between the aquatic environments and laboratory problems, some major disadvantages such as higher costs, long period of time, difficult control of the environment, hardest characterization and homogeneity of sample, and the current trend to reduce the number of living animals for research made this work is not popular (Rodgers, 1984; Freshney, 1994). Consequently, there is a great need to develop an alternate biological models which can be used in hazard assessment and biological monitoring of these toxic chemicals.

The use of cultured fish cells named cell lines i.e., culture from the organ consist of numerous generations of the cells originally present in the primary culture, to evaluate the cytotoxicity of aquatic pollutants received some attention (Kocan *et al.,* 1981; Bols *et al.*, 1985; Elhassaneen *et al.*, 1996). This technique suffered from many defects i.e. genetic instability, dedifferentiation, biotransformation and so many factors that may be changed the properties and characteristics of these cell lines after long-term culture. Subsequently, several questions arises about the possibility of using this model to make a comparison between *in vitro* and *in vivo* studies. For these reasons, several attempts have been tried to prepare liver primary culture of isolated hepatocytes from fish because they often considered the main target for chemical toxins. One of the most problems limited the developing of this attention was how to keep these cultures alive and maintaining their functions for extended period of time (Kojima *et al.*, 1985; Klaunig *et al.*, 1985; Mommsen and Lazier, 1986; Baksi and Frazier, 1988).

Recently, a protocol was developed by our investigation (Elhassaneen, 1996) to isolate liver cells from fish (Spot, *Leiostomus xanthurus*). This model of cells possess obvious advantages over whole organism and cell line like attach to each other and to the culture substrate, survive for relatively extended periods of time (more than 11 days), proliferated forming confluent monolayer, genetic stability, diversity of origin, ability to screen large numbers of samples and low cost. In addition to, our previous investigations (Elhassaneen, 1996; Elhassaneen *et al.*, 1996; Hassan *et al.*, 1996) showed that, the isolated cultured liver cells were respond to numerous environmental mutagens and carcinogens *in vitro.* These previous studies suggests that this model of cells could be used as an experimental tool to study the possible toxic effects of some industrial i.e. paper and petroleum refinery operations and agricultural effluents polluted the aquatic environment.

Materials and Methods

Industrial and agricultural effluents: The test samples used in experiment were collected from Mostored area, Shobra El-Khema, Qaluobia, a sites where the paper industries and petroleum refiniiry operations are present. While, the agricultural effluent samples were obtained from Shebin El-Kom, Menofia Governorate, an area where the farming activity are much applied. On the other side, the control samples were collected from wells around of these places. The samples were collected in dark bottles, filtered and stored at -4°C until used.

Liver cells culture: %Fish liver cells were isolated according to the technique described by Elhassaneen (1996) as shown in Fig. 1. In brief, Spot fish (*Leiostomus xanthurus*) were anesthetized in trichina methane sulfonate and dissected under aseptic conditions. Livers were excised to petri dish containing 5 ml Hank's Balanced Salt Solution (HBSS). Other tissues unless liver were cut away and the HBSS was removed. The livers were minced with a sterilize scissors and resuspended in 0.25 percent trypsin-0.02 percent ethylene diaminotetra-acetic acid (EDTA) solution. The suspension was then transferred to a magnetic stir for 20 min and strained through four layers of sterile cheesecloth to remove large tissue fragments. The single cell suspension was placed in a sterile

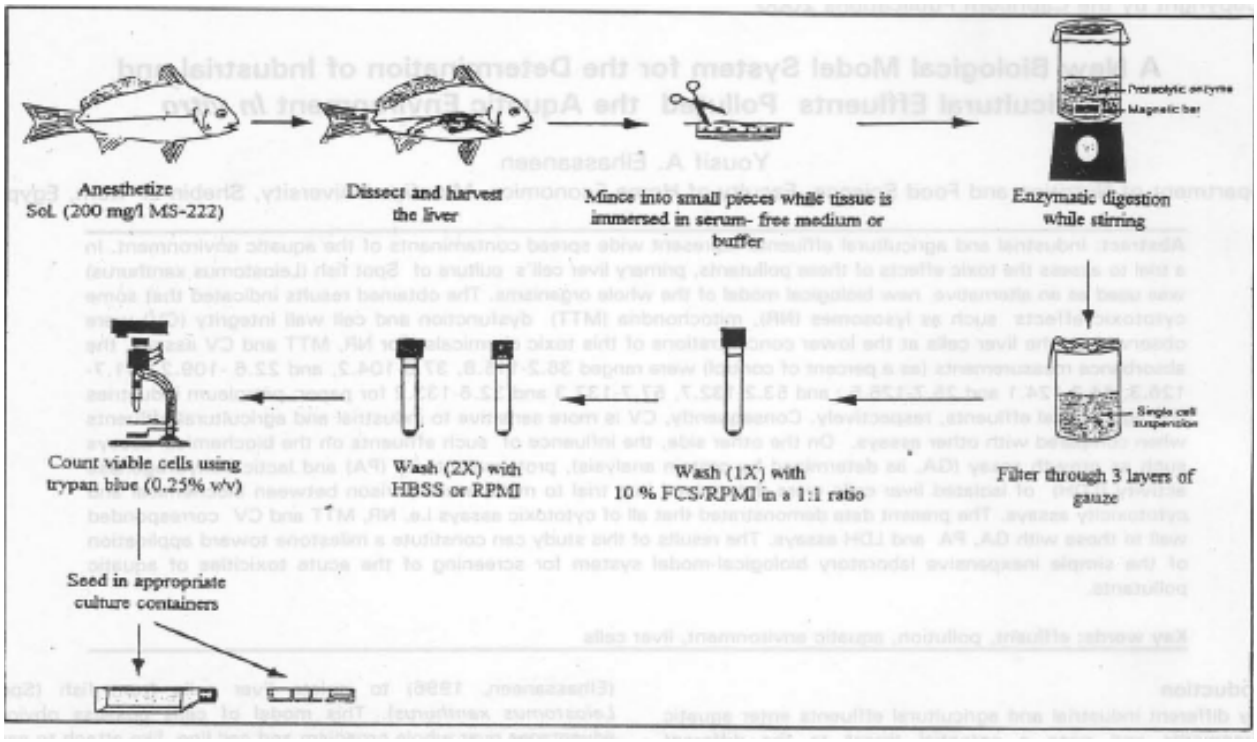

Fig. 1: Schematic diagram of a hepatocyte primary culture

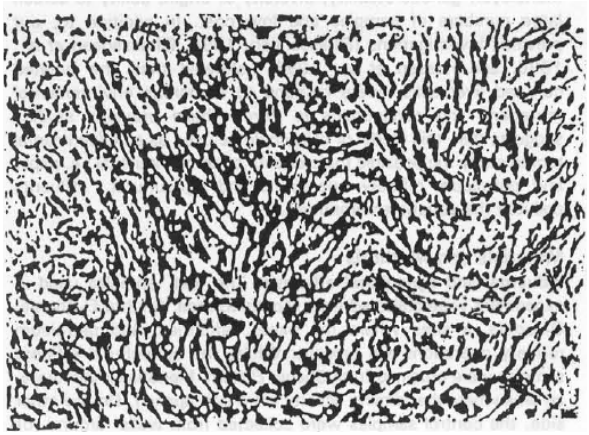

Fig. 2: Isolated liver cells, derived from Spot fish *(Leiostomus xanthurus),* were propagated at 27°C in RPMI-1640 medium supplemented with 10% fetal calf serum. A complete confluent for liver cells was achieved after 7 days of incubation (X 200)

15 ml centrifuge tube and washed at 1200 rpm for 5 min with a complete medium [10% Fetal Calf Serum (FCS)/RPMI 1640 medium adjusted to 330 mOs/kg and supplemented with 25 mM HEPES buffer, 2 mM L (+)glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin]. The supernatant was discarded and the cell pellet was washed two times with HBSS or 2% RPMI. Cell pellet were resuspened in a complete medium and counted by using of 0.4 tryoan blue exclusion stain.

Experimental design: Seven ten fold dilution of each effluent will be done in RPMI-1640 complete medium (make 1 ml for each dilution). Set up eight 96 well flat bottom tissue culture plates as follow: For media control, add 200 ml RPMI-1640 complete medium; for cell control, add 100 ml of cell suspension 1×10^5 cells) plus 100 ml RPMI-1640 complete medium; for samples, add 100 ml of cell suspension (1×10^5 cells) plus 100 nil of toxicant dilution to each well. After 7 days of incubation at 27°C (5% 00_2), plates will be removed from incubator into laminar flow hood and liver functions assays procedure will be applied.

Liver functions assays: Lysosomes, mitochondrial activity and cell membrane integrity in liver cells were determined by neutral red (NR), tetrazolium blue (MTT) and crystal violet (CV) assays according to the methods described by Borenfreund and Puerner (1984), Borenfreund *et al*. (1988) and Saotome *et al*., (1989), respectively. Liver cells protein which represent the growth assay (GA) of cells was measured by the methods of Babich *et al*., (1986). Protease activity (PA) assays which can be used as an immunological indicator for the liver cells was determined by the method of Rinderknecht *et al*., (1968) which using hide powder azhur (HPA) as an enzymatic substrate. Finally, Lactic dehydrogenase activity (LDH) was described by Gowenlock (1988).

Statistics: All experiments were performed at least three times, using six wells for each dilution of effluent. Data for the liver cells functions curves were presented as the arithmetic mean ± SD. Comparative cytotoxicity and biochemical assays of effluent i.e. the concentrations of effluent were needed to reduce absorbance of the NR, MTT and CV by 10% (NR_{90}, MTT_{90} and CV_{90} values) and by 50% (NR_{50}, MTT_{50}, and CV_{50} values) were computed by linear regression analysis of the data as percentage of control versus the logarithmic concentration of the toxicant.

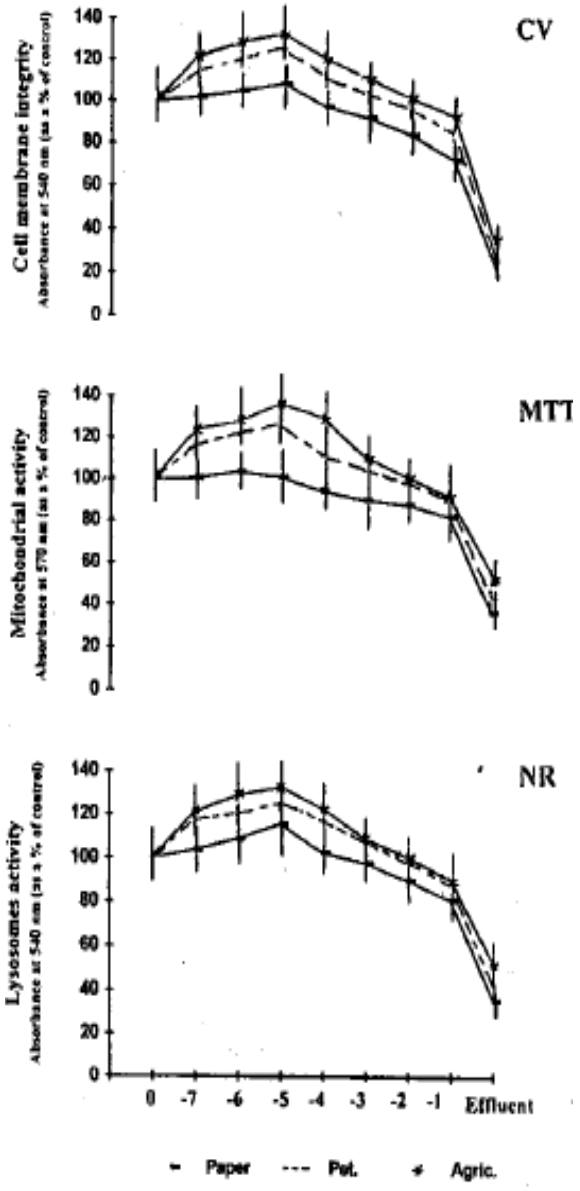

Fig. 3: Comparative cytotoxicity, as assayed by neutral red (NR), tetrazolium (MTT) and crystal violet (CV) techniques, of 7 days exposure of industrial and agricultural effluents to isolated fish liver cells. Lysosomes, mitochondria and cell wall membrane dysfunction, and hormesis are all evident. 100% represents the mean absorbance measurement of the three control cultures at the end of the exposure period, while data points represent mean absorbance measurement ± SD of three replicated for each exposure effluent dilution

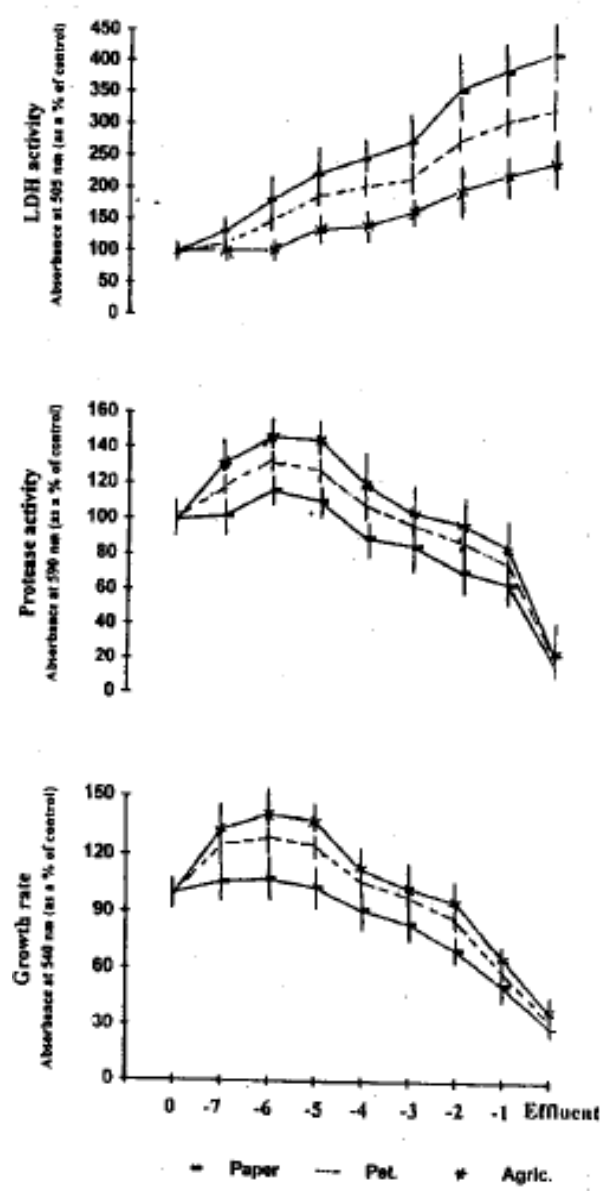

Fig. 4: Comparative biochemical effects, as assayed by growth rate (GA), protease activity (PA) and lactic dehydrogenase activity (LDH) techniques, of 7 days exposure of industrial and agricultural effluents to isolated fish liver cells. cell death, mitotic inhibition, hormesis and immunity deficiency are all evident. 100% represents the mean absorbance measurement of the three control cultures at the end of the exposure period, while data points represent mean absorbance measurement ± SD of three replicated for each exposure effluent dilution

Results

Primary spot fish liver cells were used as an experimental tool for studying the toxic and biochemical effects of some industrial and agricultural effluents. A complete confluent for liver cells was achieved after three days growth in RPMI-1640 complete medium (Fig. 2). For NR, MTT and. CV assays which determined the lysosornes activity, mitochondria! activity and cell membrane integrity of liver cells, the data were standardized by expressing absorbance aata in the presence of each effluent as a percentage of that in the control medium. Figure 3 represent typical NR, MTT and CV assays in spot liver cells exposed to paper and petroleum industry as well as agricultural effluents. The absorbance measurements of these assays (as % of control) were 38.2-115.8, 37.8-104.2 and 22.6-109.2; 41.7-126.3, 44.3-124.1 and 25.7-1 26.5 and 53.2-132.7, 57.7-137.3 and 32.6-133.2 for paper

Table 1: Comparative cytotoxicity of industrial and agricultural effluent as determined by different assays

Test effect	NR assay		MTT assay		CV assay	
	NR_{90}	NR_{50}	MTT_{90}	MTT_{50}	CV_{90}	CV_{50}
Paper industry effluent	0.001	0.64 ±	0.20 ±	0.730 ±	0.0037 ±	0.46 ±
	0.0002	0.14	0.004	0.11	0.00015	0.04
Petroleum industry effluent	0.98 ±	0.82 ±	0.082 ±	0.897 ±	0.064 ±	0.64 ±
	0.021	0.19	0.001	0.18	0.007	0.18
Agricultural effluent	0.188 ±	**	0.191 ±	**	0.281 ±	0.91 ±
	0.032		0.03		0.02	0.20

*Mean concentrations (% of effluent) required to reduce absorbance by 10% (NA_{90}, TT_{90} and CV_{90}) and by 50% (NR_{50}, MIT_{50} and CV_{50}), **Out the dimensions of the curve.

and petroleum industries and agricultural effluents. Consequently, it could be easily concluded that, NR assay is more sensitive to all industrial and agricultural effluents under this investigation than others assays. These data are in agreement with that obtained by Borenfreund *et al*. (1988) who studied the cytotoxic effects of some toxic chemicals and drugs used for cancer chemotherapy by using of fibroblast cell line. They found that the absorbance measurements for the NR assay, as noted earlier, about twice those for the MIT assay. The influence of industrial and agricultural effluent on the growth assay which performing by protein determination (GA), protease activity (PA) and lactic dehydrogenase activity (LDH) of liver cells were determined in a trial to make a comparison between biochemical and cytotoxicity assays (Fig. 4). The present data demonstrated that the dose-response curves with the NR, MTT and CV assays corresponded well to those with GA, PA and LDH assays.

On the other side, the concentrations (%) of each effluent causing initial values, the concentration of toxicant that caused 10 percent decrease in absorbance (NR_{90}, MTT_{90} and CV_{90}) and midpoint values, the concentration of toxicant that caused 50 percent decrease in absorbance (NR_{50}, MTT_{50} and CV_{50} as compared to control values, for each cytotoxicity test varied with the specific assay (Table 1). In NR, MTT and CV , the midpoint toxicities for all tested effluents occurred at ranged concentrations 1% of effluent), 0.64-0.82, 0.730-0.897 and 0.46-0.91, respectively. According to these data, the sequence of sensitivity for the different cytotoxicity assays in tested toxic substance were CV > NA > MTT.

Discussion

The utilizing of fish cell culture in *in vitro* cytotoxicity assays have been used to evaluate the acute toxicity of chemical and to better understand the biochemical basis of chemical toxicity. Because fish liver is frequently a target tissue of toxic chemicals, numerous investigators have been used isolated liver cells from fish for toxicological and biochemical studies (reviewed in Baksi and Frazier, 1990). But, one of the most problems limited the developing of this attention was how to keep these cultures alive and maintaining their functions for extended period of time. These set backs proposed the using of primary culture of liver cells in toxicological research directly after established as well as for a short time exposure i.e. 1-2 hr. Recently, a protocol was developed by our investigation (Elhassaneen, 1996) to isolate liver cells from fish (Spot, *Leiostomus xanthurus*). This model of cells has been successfully used as an experimental tool in studying the biotransformation of some xenobiotic compounds and cytotoxicity testing of some toxic chemicals (Elhassaneen, 1996; Elhassaneen *et al*., 1996; Hassan *et al*., 1996). In the present study we tried to ascertain the validity of this model of cells as a useful tool for studying the toxic and biochemical effects of some industrial and agricultural effluents.

From the above data it could be observed that, there are some variations between the sensitivity of different cytotoxicity assays (Fig. 3). These Variations may be resulted from the difference of the idea which each assay based on. For example, the NR assay is based on the uptake of neutral red a supravital dye and its accumulation in the, lysosomes of viable uninjured cells (Borenfreund and Puerner, 1984). While, the MTT assay is based on the reduction of soluble yellow tetrazolium salt to a blue insoluble tetrazolium-formazan product by mitochondria! succinic dehydrogenase (Mosmann, 1983). So, the lower sensitivity of the mitochondria in tested chemical may be due to the poor solubility of the MTT formazan product and/or lower amount of tetrazolium salt reduced by mitochondria. Also, MTT assay does not include a fixation step, so, cells tend to separate from the surface of the culture plate during the formazan solubility procedure (Denizot and Lang, 1986).

In general, by using three cytotoxic testing i.e. NA, MTT and CV we were able to demonstrate three different toxic responses as a consequence of exposure to these industrial effluents (Fig. 3). Briefly, The first type of response was the inhibition of cell division, which characterized by stabilization or slightly increase the initial count of cultured cells even with increasing the concentration of toxicant. Kocan *et al*. (1985) attributed this type of response to the cellular dysfunction or damage. The second type of response was the cytotoxicity or cell death, which could be characterized by the decreasing of the growth assay with the increasing of the concentration of tested toxicant substances (Fig. 4). Kocan *et al*. (1985) demonstrated that cytotoxicity can be resulted from cells dying and/or inhibition of cell proliferation. Hormesis represented the third response which means increasing occurs in cells number over the controls at low concentrations of the toxic substance but the toxic effect does not manifest itself until a higher critical dose level is reached (Laughlin *et al*., 1981). This data are in accordance with our previous investigations when isolated liver cells were exposed to some toxic chemical i.e. heavy metals or naphthalene (Elhassaneen, 1996).

For analysis the cytotoxicity data it was apparently necessary to determined the concentration of each different effluents causing initial cytotoxicity (NR_{90}, MTT and CV_{90}, values) and those causing midpoint cytotoxicity (NR_{50}, $MTT_{50}$and CV_{50} values). Such comparative data (Table 1) were necessary to distinguish between the responses of lysosomes (NR assay), mitochondria (MIT assay) and cell wall membrane (CV assay) to low levels of toxic agents. For example, the midpoint cytotoxicity values for paper industry effluents recorded lower levels for all assays followed by petroleum industry and agricultural effluents, respectively.

On the other side, numerous studies indicated that biochemical assays such as protease activity, LDH activity and protein analysis are believed to play an important role in toxicological studies of liver cells (Gowenlock, 1988; Neurath, 1989). The present data (Fig. 4) clearly demonstrated a high degree of similarity between the growth rate and the protease activity assays. In related to LDH activity assay, high increasing was noticed as the all tested

effluents applied. Gowenlock (1988) mentioned that increased activity of LOH are found in some forms of liver disease such as, ineffective hepatitis, tumors and necrosis. Also, very high levels of LDH were noticed in severe liver necrosis following exposure to tetrachloro-diphenyl-p-dioxin (TODD, usually represent a minute compound in paper industry effluent).

In conclusion, the response of the isolated fish liver cells in the six cytotoxicity and biochemical assays with other major advantages i.e. easy to apply and low cost, make this primary culture a good biological -model candidate for screening of the acute toxicities of aquatic pollutants.

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