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Evaluation of Micronucleus Test's Sensitivity in Freshwater Fish Species

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Abstract: Nile tilapia (*Oreochromis niloticus*), Butterfish (*Poronotus triacanthus*) and Red-tailed tinfoil barb (*Puntius altus*), three fish species inhabiting in Southeast Asia freshwater ecosystems were evaluated for their use as pollution biomarkers using the micronucleus (MN) and nuclear abnormalities (NA) tests in erythrocytes. NA shapes were scored into blebbed nuclei (BL), lobed nuclei (LB), notched nuclei (NT) and binuclei (BN). Fish were exposed to lead (Pb), copper (Cu) and cadmium (Cd) for the period of 24, 48, 72 and 96 h. It was observed that, fish species showed significant sensitivity to the different heavy metals treatment. In general, the highest value of both MN and NA cells were significantly increased in the exposure to Pb followed by Cu and Cd. On the other hand, Nile tilapia was the most sensitive to the three heavy metals exposure. The frequencies of each NA shape were found in the all fish species and in the all treatments as following NT>LB>BN>BL. Results of MN and NA revealed the highest number after 48 h treatment in all cases and decreased within the longer time exposure. Our results demonstrated the suitability of Nile tilapia for genotoxicity of pollution biomarkers using the micronucleus and nuclear abnormalities test.

Key words: Micronucleus test, nuclear abnormalities testm, fish, heavy metal

INTRODUCTION

The important environmental pollutants are those that tend to accumulate in organisms, those that are persistent because of their chemical stability or poor biodegradability. Heavy metals possess all of these characteristics and are one of the major contributors to the pollution of Thailand's natural aquatic ecosystem. Lead (Pb) is quite prevalent in the environment from batteries and lead-based paint as well as in water from industrial dumps (WHO, 1995). The problem of lead contaminated surface water and sediment in Klity Village, Kanjanabuti province, Thailand was reported in April 1998 (Tonmanee, 2001). Copper (Cu) is classified as an essential element for most living organisms but, in high concentrations, it can be a toxic pollutant (WHO, 1998). Cu is used to control fungal diseases in vineyard plants in France, South Africa (Schlotfeldt, 1992) and orange orchard in Thailand. High concentrations of Cu were detected in some aquatic ecosystems collecting vineyard runoff water (GERBE, 1996). High concentrations as high as 0.6 mg L^{-1} Cu were detected in orange orchard runoff in Prae province, Thailand (Duangduen *et al*., 1999). Cadmium (Cd) is an extremely toxic element of continuing concern because its environmental levels have risen steadily (WHO, 1992; Goering *et al*., 1995). They are being used in a wide variety to industrial processes in Thailand, for example, the use of Cd as a coloring agent, a stabilizer and in alloy mixtures.

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Fish can be useful models for analyzing the genotoxic potential of the aquatic environment as they are constantly in direct contact with water (Al-Sabti and Metcalfe, 1995). It has been suggested that a variety of biomarkers and bioassays in the laboratory and field studies be used in determining the effects of genotoxic pollution. These include the presence of DNA adducts, chromosomal aberrations, DNA strand breaks and measurement of micronuclei frequencies. Among the currently available test systems, the micronucleus assay is the most widely applied method due to its simplicity, reliability, sensitivity and proven suitability for fish species. Although originally developed for its application in mouse, it was subsequently modified by Hoofman and de Raat for the application in the laboratory to fish (Hoofman and Raat, 1982). The micronucleus test detects both clastogenic and aneugenic effects and therefore can detect the genotoxicity of a wide range compounds (Heddle *et al*., 1991). Several species of freshwater fish have been reported to be good targets for biomonitoring of rivers and lakes using the micronucleus test as a genotoxicity indicator: rainbow trout, *Oncorhynchus mykiss* (De Flora *et al*., 1993), brown trout, *Salmo trutta* (Sanchez-Galan *et al*., 1998). None of these species is appropriate to biomonitor Southeast Asia freshwater ecosystems because they are not native to them. Otherwise, in most cases the method of exposure employed has been intraperitoneal injection of the product assayed (Jiraungkorskul *et al*., 2007). Some experiments with exposure to pollutants by immersion have been described for different species: for example, Nile tilapia, *Oreochromis niloticus*, to glyphosate (Jiraungkorskul *et al*., 2002, 2003), Barb, *Puntius altus*, to cadmium (Jiraungkorskul *et al*., 2006a) and Butterfish, *Poronotus triacanthus*, to copper (Jiraungkorskul *et al*., 2006b).

Nile tilapia, *O. niloticus*, Butterfish, *P. triacanthus* and Red-tailed tinfoil barb, *P. altus*, are the commercialized freshwater fish that are not only widely known around the world, but they are also the popular fish in Thailand. They can provide the good models to study responses and possible adaptations of local fish populations to aquatic pollutants. In the present study, we evaluated the sensitivity of three fresh water species native to Southeast Asia (*O. niloticus*, *P. triacanthus* and *P. altus*) to three heavy metals, lead, copper and cadmium using the micronucleus test and the new parameter in erythrocytes as the incidence of abnormal shape nuclei.

MATERIALS AND METHODS

Animals

Nile tilapia (*O. niloticus*), Butterfish (*P. triacanthus*) and Red-tailed tinfoil barb (*P. altus*), 14.50±4.03, 14.80±3.96 and 14.77±2.15 g in body weight, respectively and 9.20±0.71, 9.26±0.74 and 9.02±0.61 cm in total length, respectively; were purchased from a commercial hatchery in Chonburi province, Thailand. Tap water was filtered with activated charcoal (Aquapur, thysen, FRG) to eliminate chemical contamination. The physicochemical characteristics of water were measured daily, according to the experimental procedures described in Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Conductivity was measured with Hanna instruments Model 3 DiST WP (Hanna Instruments Inc., USA). The pH was measured with a Cyberscan 510 (Eutech Instruments Inc., USA) and the temperature was measured with a glass mercury thermometer. A 16: 8 h light-dark cycle was maintained throughout.

Acclimatization to laboratory conditions for 7 days was done using dechlorinated tap water that had the following physicochemical characteristics: temperature = 27.0±1.5°C, pH = 6.5-6.8, total hardness = 65-75 mg L^{-1} (as $CaCO_3$), alkalinity = 75-80 mg L^{-1} and conductivity = 185-210 μmhos cm^{-1}. Chlorine residual and ammonia were below detection limits. Fish were fed twice a day with 37%-protein commercial fish food (Charoen Pokphand Group, Bangkok, Thailand). The quantity of food was 2% of the initial body weight per day.

Chemicals

The heavy metals were the products of Sigma, Germany as following: $Pb(NO_3)_2$ (CAS No.10099-74-8), $CuSO_4.5H_2O$ (CAS No.7758-99-8) and $CdCl_2.H_2O$ (CAS No.10108-64-2). Chemicals were directly diluted in water to obtain the desired exposure concentrations.

Lethal Toxicity Test

The lethal toxicity tests of heavy metals to fish were performed following the standard methods for the static non-renewal technique (APHA, 2005). After acclimatization period, ten fish were randomly transferred from the stock tank to the experimental aquaria. Fish were not fed 48 h before starting and 96 h during the experiment. Preliminary screening was carried out to determine the appropriate concentration range for heavy metals. The tests consisted of six groups exposed to these following heavy metal concentrations: 0, 80, 160, 240 and 320 mg L^{-1}. Each concentration was done in three replicates. The criteria for death were no gill movement and no reaction to gentle prodding. Fish mortality in each aquarium was recorded at the intervals of 24, 18, 72 and 96 h. Dead fish were immediately removed. Mortality and abnormal behavioral responses were recorded every 12 h, during 96 h. The results of the median lethality concentration (LC_{50}) at 24, 48, 72 and 96 h were computed using the probit analysis computer program (Finney, 1971).

Experimental Design

The dose chosen was 25% of the 96 h LC50 value from the acute toxicity test. Each fish species (n = 80) was randomly assigned to four equally sized groups as following: (1) distilled water; (2) $Pb(NO_3)_2$; (3) $CuSO_4.5H_2O$ and (4) $CdCl_2.H_2O$. The fish were kept separately in the glass flow-through aquaria (50×50×120 cm) with continuous aeration were filled with 200 L of dechlorinated tap water whose physicochemical characteristics were the same as those described previously.

Micronuclei (Mn) and Nuclear Abnormality (Na) Tests: Giemsa Staining

At different times (24, 48, 72 and 96 h), 5 fish of each group were anesthetized with 0.2 g L^{-1} MS-222 (tricaine methan sulphonate, Sigma, Germany, CAS No.886-86-2), weighed and measured. Peripheral blood samples were obtained by caudal vein puncture using a heparinized syringe. Blood was smeared immediately on clean grease free microscope slides, air dried for 12 h and then fixed in absolute ethanol for 20 min. Each slide was stained with 5% Giemsa solution for 30 min. Three slides were prepared for each group. From each slide 1000 cells were scored under 1000× magnification using a Nikon E600 light microscope and photographed using a Nikon DXM 1200 digital camera (Tokyo, Japan).

Micronuclei and Nuclear Abnormality Cells Scoring

Only the cells clearly isolated from the surrounding cells were scored. The criteria for the identification of MN were earlier described: (a) MN must be smaller than one-third of the main nuclei, (b) MN must be clearly separated from the main nuclei, (c) MN must be on the same plane of focus and have the same color. Cells having two nuclei with approximately equal sizes were considered as binucleates (Fenech *et al*., 2003). Nuclear abnormality shapes were scored into one of the following categories: blebbed nuclei (BL), lobed nuclei (LB), notched nuclei (NT)and binuclei (BN) (Carrasco *et al*., 1990). The result was expressed as the mean value (%) of the sum for all the individual abnormality observed.

Statistical Analysis

All data were expressed as means±SD. A two-way analysis of variance was used to determine the significance of micronucleus test. The least-significant difference (LSD) was used for determination of significant differences at $p<0.05$.

RESULTS

The results of the 24, 48, 72 and 96 h LC_{50} values for heavy metals in each fish species were calculated by the probit method and presented in Table 1.

Normal erythrocyte approximately diameter 7 μm was contained mainly elliptical nuclei (Fig. 1A). The small non-refractive circular or ovoid particles lying in the cytoplasm and resembling a nucleus with respect to staining properties were considered as micronuclei. The size of the micronuclei varied to some extent (between 1/25th and 1/5th that of nuclear size) but the number was always one. The position of the micronuclei in the cytoplasm also varied, some located very near to the nucleus or some located vary far even at the periphery of the cell (Fig. 1B-D). Some of the nuclei clearly deviated from their normal shape and were either blebbed (BL) (Fig. 1E-F), lobed (LB) (Fig. 1G-H), notched (NT) (Fig. 1I-J) and binucleated cells (BN) (Fig. 1K-L). All abnormalities of nuclei were scored. In briefly, cells with two nuclei were considered as binucleates. The two nuclei should be approximately equal size, staining pattern and staining intensity, within the same cytoplasmic boundary. Blebbed nuclei present a relatively small evagination of the nuclear membrane, which contains euchromatin. Evaginations larger than the blebbed nuclei which could have several lobes are classified as lobed nuclei. Nuclei with depth into a nucleus were recorded as notched nuclei.

It is evident that MN was not significantly induced in the control group at 24, 48, 72 and 96 h. Otherwise; the differences were statistically significant in treated group when compared with those of the control group at all the time intervals. These data revealed that MN significant greater number in fish treated with Pb followed by Cu and Cd. Although MN averages were always higher in Cu than Cd treatment, the apparent difference was not statistically significant in any time intervals. On the other hand; Nile tilapia was the most sensitive to the three heavy metals exposure. MN averages were also higher for butterfish than red-tailed tinfoil barb, but the apparent difference was not statistically significant in any case, except at 48 h in Pb treatment (Table 2).

Similarly to MN, the frequencies of NA were also significant greater number in fish treated with Pb followed by Cu and Cd. Otherwise, Nile tilapia was still the most sensitive to the three heavy metals exposure. The frequencies of NA in erythrocytes were analyzed separately. It was observed that the frequencies of each abnormality shapes in all treatments were found as followed: NT>LB>BN >BL. Results of MN and NA revealed the gradually increased with the time up to 48 h in all cases and decreased to some extent at 72 and 96 h (Table 3).

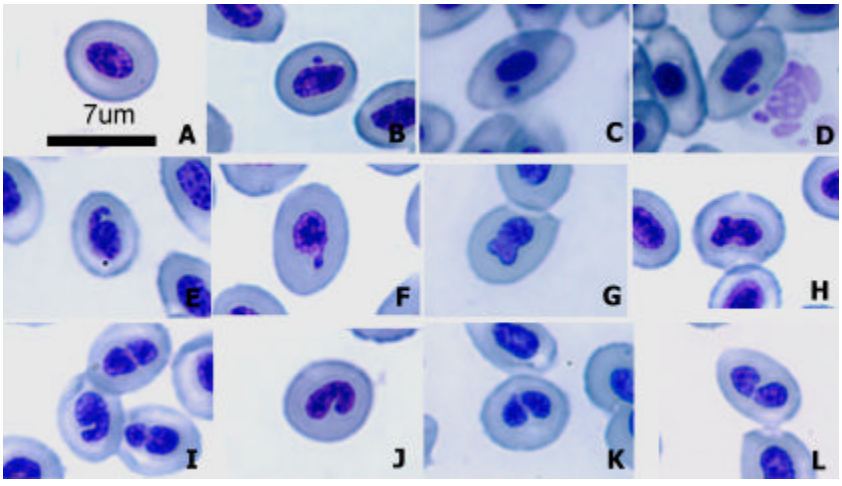

Fig. 1: Photomicrographs of normal nucleus (A); micronuclei (B-D) and nuclear abnormalities (E-L): blebbed (E-F); lobed (G-H); notched (I-J); binucleated nuclei (K-L) in peripheral blood erythrocytes

Table 1: The LC_{50} values of heavy metal exposure to each fish

		LC_{50} (mg L^{-1})			
Fish	Gr.	24 h	48 h	72 h	96 h
Nile tilapia	Cd	203.06	197.31	182.55	180.47
	Cu	210.27	213.34	193.30	185.75
	Pb	247.51	199.65	183.43	182.12
Butterfish	Cd	170.65	114.44	99.08	97.58
	Cu	129.72	127.12	108.36	104.78
	Pb	139.72	113.34	103.91	102.31
Barb	Cd	182.22	170.46	167.18	166.22
	Cu	173.58	180.86	169.49	167.07
	Pb	187.60	172.19	167.60	166.11

Table 2: Frequencies (%) of micronuclei in different fish species exposed to different time and treatments (mean±SD)

Micronuclei	Time (h)	Cont	Pb	Cu	Cd
Nile tilapia	24	0.033±0.058	0.467±0.115^{a}	0.267±0.058^{a,b}	0.200±0.000^{a,b}
	48	0.033±0.058	1.000±0.100^{a}	0.433±0.058^{a,b}	0.333±0.115^{a,b}
	72	0.033±0.058	0.700±0.100^{a}	0.300±0.000^{a,b}	0.233±0.058^{a,b}
	96	0.033±0.058	0.467±0.058^{a}	0.267±0.115^{a,b}	0.200±0.000^{a,b}
Butterfish	24	0.033±0.058	0.400±0.100^{a}	0.167±0.058^{b,*}	0.133±0.115^{b}
	48	0.033±0.058	0.733±0.058^{a,*}	0.200±0.000^{a,b,*}	0.200±0.000^{a,b}
	72	0.033±0.058	0.400±0.200^{a,*}	0.100±0.100^{b,*}	0.133±0.058^{b}
	96	0.033±0.058	0.333±0.058^{a}	0.067±0.058^{b,*}	0.133±0.058^{b}
Red-tailed	24	0.033±0.058	0.267±0.058^{a,*}	0.100±0.000^{b,*}	0.133±0.058^{a,b}
tinfoil barb	48	0.033±0.058	0.467±0.058$^{a,*,\#}$	0.167±0.058^{a,b,*}	0.167±0.058^{a,b,*}
	72	0.033±0.058	0.167±0.115*	0.067±0.058*	0.100±0.100
	96	0.033±0.058	0.133±0.115$^{*,\#}$	0.067±0.058*	0.100±0.100

Cont = Control group; Pb = Lead group; Cu = Copper group; Cd = Cadmium group. ^{a}The mean difference was significant in row when compared the control group ($p<0.05$). ^{b}The mean difference was significant in row when compared the lead group ($p<0.05$). *The mean difference was significant in column when compared the Nile tilapia group ($p<0.05$). $^{\#}$The mean difference was significant in column when compared the Butterfish group ($p<0.05$)

Table 3: Frequencies (%) of total nuclear abnormalities cells in different fish species different cells exposed to different time and treatments (mean±SD)

Nuclear abnormalities	Time (h)	Cont	Pb	Cu	Cd
Nile tilapia	24	0.333±0.058	1.067±0.115^{a}	0.933±0.058^{a}	0.667±0.058^{a,b,c}
	48	0.433±0.058	1.867±0.115^{a}	1.333±0.058^{a,b}	0.700±0.100^{a,b,c}
	72	0.400±0.100	1.467±0.153^{a}	0.833±0.058^{a,b}	0.600±0.000^{a,b,c}
	96	0.367±0.058	0.833±0.058^{a}	0.800±0.100^{a}	0.433±0.208^{b,c}
Butterfish	24	0.267±0.058	0.967±0.115^{a}	0.833±0.058^{a}	0.567±0.058^{a,b,c}
	48	0.333±0.058	1.767±0.115^{a}	1.233±0.058^{a,b}	0.600±0.100^{a,b,c}
	72	0.167±0.058*	1.367±0.153^{a}	0.733±0.058^{a,b}	0.500±0.000^{a,b,c}
	96	0.167±0.058*	0.733±0.058^{a}	0.700±0.100^{a}	0.433±0.058^{a,b,c}
Red-tailed tinfoil barb	24	0.167±0.058*	0.867±0.115^{a}	0.733±0.058^{a,*}	0.467±0.058^{a,b,c,*}
	48	0.333±0.058	1.667±0.115^{a}	1.133±0.058^{a,b,*}	0.500±0.100^{a,b,c,*}
	72	0.267±0.058	1.267±0.153^{a}	0.633±0.058^{a,b,*}	0.400±0.000$^{b,c,*,\#}$
	96	0.200±0.000*	0.633±0.058^{a,*}	0.600±0.100^{a,*}	0.333±0.058^{a,b,c}

Cont = Control group; Pb = Lead group; Cu = Copper group; Cd = Cadmium group. ^{a}The mean difference was significant in row when compared the control group ($p<0.05$) ^{b}The mean difference was significant in row when compared the lead group ($p<0.05$). ^{c}The mean difference was significant in row when compared the copper group ($p<0.05$). *The mean difference was significant in column when compared the Nile tilapia group ($p<0.05$). $^{\#}$The mean difference was significant in column when compared the Butterfish group ($p<0.05$)

DISCUSSION

The widely LC_{50} values of heavy metals have been reported depending on the species of fish and test conditions (WHO, 1992, 1995, 1998). Table 1 presents the 96-h LC_{50} values greater number in Nile tilapia following by barb and butterfish. Each fish was sensitive in the exposure to Cd followed by Pb and Cu.

In the present study, fish were not significantly different within species for length and weight; therefore, they can be considered homogeneous and differences of MN and NA counts between groups should not be attributed to intrinsic problems in the experimental design. The occurrence of MN and different types of NA has been adopted as a good substitute for the chromosomal assay. Based on the frequency of MN or NA, a monitoring system for potential genotoxicity of an agent has been proposed (Guha and Khuda-Bukhsh, 2002; Al-Sabti and Metcalfe, 1995). MN is cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosomes lagging behind in the anaphase stage of cell division. Their presence in cells is a refection of structural and/or numerical chromosomal aberrations arising during mitosis (Heddle *et al*., 1991). MN and NA tests in fish are generally performed in enucleated peripheral blood erythrocytes mainly due to its technical feasibility. In most studies short-term exposure period ranging between 24 and 96 h were reported to be sufficient to induce micronuclei and erythrocytes have been reported to be a sensitive biomarker of genotoxicity (Heddle *et al*., 1991; Ayllon and Garcia-Vazquez, 2000).

There are some assays in fish aimed to evaluate the differential sensitivity of diverse species to different chemicals (Ayllon and Gardia-Vazquez, 2000). But in most cases the method of exposure has been intraperitoneal injection, for the example, cadmium significantly induced micronuclear expression in European minnow, *Phoxinus phoxinus* and mollie, *Poecilia latipinna* (Ayllon and Garcia-Vazquez, 2000) and rainbow trout, *O. mykiss* (Castano *et al*., 1998). In the present study, the peak values for MN and NA in erythrocytes have been obtained after 48 h. Similar time-dependent effects were also previously reported in fish erythrocytes exposed to textile mill effluent (Cavas and Ergene-Gozukara, 2003) and cadmium chloride (Ayllon and Garcia-Vazquez, 2000).

Table 2 presents the frequencies of MN significant greater number in fish treated with Pb following by Cu and Cd. Although MN averages were always higher in Cu than Cd treatment, the apparent difference was not statistically significant in any time intervals. There are contradictory results about the genotoxicity of Cu and Cd in fish species. Sanchez-Galan *et al*. (2001) reported that the cadmium chloride injection induced the formation of MN in erythrocytes of *Anguilla anguilla* whereas copper sulphate caused no significant effect. In contrast, Bolognesi *et al*. (1999) reported that cadmium yielded negative results with the micronucleus test whereas copper induced MN in gill cells.

Although mechanism of Pb and Cu genotoxicity remains insufficiently understood, however, it is well known that heavy metals interferes the regular chromosome segregation during cell division mainly by inhibition of polymerization of actin tubules, an essential structure of the mitotic spindle (Miura and Imura, 1987). It was reported that the mechanism of Cd genotoxicity is mainly conditioned by single strand breaks in DNA through the direct cadmium-DNA interactions as well as by the action of incision nucleases and/or DNA-glycosylase during DNA repair (Privezentsev *et al*., 1996). Correspondingly, most of the toxic chemicals that produce genotoxic effects have been known to form reactive oxygen species as well as electrophilic free-radical metabolites that interact with DNA to cause disruptive changes. It has been suggested that during the heavy metal exposure, electrophilic ions and radicals are produced, interacting with nucleophilic sites in DNA and leading to breaks and other related damage in the latter.

Table 3 presents the frequencies of NA obtained in the experimental groups, as well as the summary of the statistical analysis. There was a general tendency of occurrence of notched and lobed types in higher frequencies in all the species. An analysis revealed spontaneous frequencies of nuclear abnormalities were found in the following order: NT>LB>BN>BL. Thus, our results seem to be in agreement with previous studies (Cavas and Ergene-Gozukara, 2003; Mallick and Khuda-Bukhsa, 2003).

In conclusion, all analyzed nucleolar parameters responded to different heavy metals in different fish species. The results presented in this study show that the MN and NA in Nile tilapia, *O. niloticus*, could be a bioassay of choice to biomonitor of water pollution. Otherwise, we also

propose that the nuclear abnormalities should be considered as indicators of structural genomic changes in addition to micronuclei. Thus the combination of both biomarkers seems to be useful when fish are used as experimental animal.

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REFERENCES

Al-Sabti, K. and C.D. Metcalfe, 1995. Fish micronuclei for assessing genotoxicity in water, Mutat. Res., 343: 121-135.

American Public Health Association (APHA), 2005. Standard methods for the examination of water and wastewater. 21st Edn. The American Public Health Association, American Water Works Association and Water Pollution Control Federation, Washington DC.

Ayllon, F. and E. Garcia-Vazquez, 2000. Induction of micronuclei and other nuclear abnormalities in European minnow *Phoxinus phoxinus* and mollie *Poecilia latipinna*: An assessment of the fish micronucleus test. Mutat. Res., 467: 177-186.

Bolognesi, C., E. Landini, P. Roggieri, R. Fabbri and A. Viarengo, 1999. Genotoxicity biomarkers in the assessment of heavy metal effects in mussels: Experimental studies. Environ. Mol. Mutagen, 33: 287-292.

Carrasco, K.R., K.L. Tilbury and M.S. Myers, 1990. Assessment of the piscine micronucleus test as an *in situ* biological indicator of chemical contaminant effects. Can. J. Fish Aquat. Sci., 47: 2123-2136.

Castano, A., G. Carbonell, M. Carballo, C. Fernandez, S. Boleas and J.V. Tarazoma, 1998. Sublethal effects of repeated intraperitoneal cadmium injection on rainbow trout. Ecotoxicol. Environ. Sef., 41: 29-35.

Cavas, T. and S. Ergene-Gozukara, 2003. Micronuclei, nuclear lesions and interphase silver-stained nucleolar organizer (AgNORs) as cyto-genotoxicity indicators in *Oreochromis niloticus* exposed to textile mill effluent. Mutat. Res., 538: 81-91.

De Flora, S., L. Vigano, F. D'Agostini, A. Camoirano, M. Bagnasco, C. Bennicelli, F. Melodia and A. Arillo, 1993. Multiple genotoxicity biomarkers in fish exposed *in situ* to polluted river water. Mutat. Res., 319: 167-177.

Duangduen, C., D. Supatimusro, S. Yodpeth, D. Navanukkroh, M. Suksawat and K. Rudisirisak, 1999. The Study of the Concentration of Some Heavy Metals in the Water and Sediment from the Yom River. Chemical Research Institute, Patum Thani, Thailand. (http://content.rit.ac.th/chem/research9.asp).

Fenech, M., W.P. Chang, M. Kirsch-Volders, N. Holland, S. Bonassi and E. Zeiger, 2003. HUMN project: Detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat. Res., 534: 65-75.

Finney, D.J., 1971. Probit analysis. 3rd Edn. London: Cambridge University Press.

GERBE, 1996. Programme de recherche: Toxicologie, ecotoxicologie des pesticides et des metaux lourds. Europol' Agro Universite de Reims Champagne-Ardenne, Reims, France, pp: 39.

Goering, P.L., M.P. Waalkers and C.D. Klaassen, 1995. Toxicology of Cadmium. In: Toxicology of Metals: Biochemical Aspects. Goyer, R.A. and M.G. Cherian (Eds.), Berlin: Springer, pp: 189-214.

Guha, B. and A.R. Khuda-Bukhsh, 2002. Efficacy of Vitamin C (L-Ascorbic acid) in reducing genotoxicity in fish (*Oreochromis mossambicus*) induced by ethyl methane sulphonate, Chemosphere 47: 49-56.

Heddle, J.A., M.C. Cimino, M. Hayashi, F. Romagna, M.D. Shelby, J.D. Tucker, P. Vanparys and J.T. MacGregor, 1991. Micronuclei as an index of cytogenetic damage: Past, present and future. Environ. Mol. Mutagen., 18: 277-291.

Hoofman, R.N. and W.K. Raat, 1982. Induction of nuclear anomalies (micronuclei) in the peripheral blood erythrocytes of the eastern mudminnow *Umbra pigmaea* by ethyl methane sulphonate. Mutat. Res., 104: 147-152.

Jiraungkoorskul, W., E.S. Upatham, M. Kruatrachue, S. Sahaphong, S. Vichasri-Grams and P. Pokethitiyook, 2002. Histopathological effects of Roundup, a Glyphosate Herbicide, to Nile tilapia (*Oreochromis niloticus*). Sci. Asia, 28: 121-127.

Jiraungkoorskul, W., E.S. Upatham, M. Kruatrachue, S. Sahaphong, S. Vichasri-Grams and P. Pokethitiyook, 2003. Biochemical and histopathological effects of glyphosate herbicide on Nile tilapia (*Oreochromis niloticus*). Environ. Toxicol., 18: 260-267.

Jiraungkoorskul, W., S. Sahaphong, N. Kangwanrangsan and M.H. Kim, 2006a. Histopathological study: The effect of ascorbic acid on cadmium exposure in fish (*Puntius altus*) J. Fisheries Aquat. Sci., 1: 191-199.

Jiraungkoorskul, W., S. Sahaphong and N. Kangwanrangsan, 2006b. Toxicity of copper in butterfish (*Poronotus triacanthus*): Tissues accumulation and ultrastructural changes. Environ. Toxicol., (In Press).

Jiraungkoorskul, W., S. Sahaphong, P. Kosai and M.H. Kim, 2007. Micronucleus test: the effect of ascorbic acid on cadmium exposure in fish (*Puntius altus*). Res. J. Environ. Toxicol., 1: 27-36.

Mallick, P. and A.R. Khuda-Bukhsa, 2003. Nuclear anomalies and blood protein variations in fish of the Hooghly-Matlah river system, India, as an indicator of genotoxicity in water. Bull. Environ. Contam. Toxicol., 70: 1071-1082.

Miura, K. and N. Imura, 1987. Mechanism of methyl mercury toxicity. CRC Crit. Rev. Toxicol., 18: 161-187.

Privezentsev, K.V., N.P. Sirota and A.I. Gaziev, 1996. The genotoxic effects of cadmium studied *in vivo*. Tsitol. Genet., 30: 45-51.

Sanchez-Galan, S., A.R. Linde, J.I. Izquierdo and E. Garcia-Vazquez, 1998. Micronuclei and fluctuating asymmetry in brown trout (*Salmo trutta*): Complementary methods biomonitor freshwaters ecosystems. Mutat. Res., 412: 218-225.

Sanchez-Galan S. A.R. Linde, F. Ayllon and E. Garcia-Vazquez, 2001. Induction of micronuclei in Eel (*Anguilla anguilla*) by heavy metals. Ecotoxicol. Environ. Sef., 49: 139-143.

Schlotfeldt, H.J., 1992. Current Practices of Chemotherapy in Fish Culture. In: Chemotherapy in Aquaculture: From Theory to Reality. Michel, C. and D.J. Alderman (Eds.), Paris: OIE., pp: 25-38.

Tonmanee, N., 2001. Lead contamination in Klity Creek, Klity Village, Kanjanaburi Province, Thailand. International Water Association: Specialist Group on Diffuse Pollution, 15: 1-12.

WHO., 1992. Environmental Health Criteria No. 134, Cadmium. International Programme on Chemical Safety (IPCS), World Health Organisation, Geneva.

WHO., 1995. Environmental Health Criteria No. 165, Lead, inorganic. International Programme on Chemical Safety (IPCS), World Health Organisation, Geneva.

WHO., 1998. Environmental Health Criteria No. 200, Copper. International Programme on Chemical Safety (IPCS), World Health Organisation, Geneva.