Korean J. Limnol. 37 (3): 313~318 (2004)

Di-2-ethylhexyl Phthalate Induced Haematological Effects in Bagrid Catfish, *Pseudobagrus fulvidraco* After Short Term Exposure

Jee, Jung-Hoon, Yoo-Hwa Keum[1] and Ju-Chan Kang[1,*]

Institute of Fisheries Sciences, Pukyong National University, Busan 612-021, Korea
[1]Department of Aquatic Life Medicine, Pukyong National University, Busan 608-737, Korea

Di-2-ethylhexyl Phthalate (DEHP)에 노출된 동자개, *Pseudobagrus fulvidraco*의 혈액학적 영향. 지정훈 · 금유화[1] · 강주찬[1,*](부경대학교 수산과학연구소, [1]부경대학교 수산생명의학과)

Di-2-ethylhexyl phthalate (DEHP)는 내분비장애물질로 분류되어 있는 플라스틱 가소제로서 *in vitro*에서 혈구세포막에 영향을 주는 것으로 알려져 있다. 본 연구는 동자개, *Pseudobagrus fulvidraco*를 대상으로 DEHP 급성 노출에 따른 혈액학적 영향을 파악하기위하여 어체중 당 300 및 1,000 mg의 DEHP를 복강주사하였다. DEHP (1,000 mg b.w.$^{-1}$)에 노출된 동자개는 적혈구수와 혈색소 농도 및 적혈구 용적이 감소하는 경향을 나타내었다. 또한, 혈청 내 유기성분인 총단백질량, 콜레스테롤 수치는 300 및 1,000 mg 주사구에서 유의적으로 감소하였으며 지방산의 농도는 1,000 mg 주사구에서 대조구와 비교하여 유의적으로 감소하였다. 혈청 무기성분인 칼슘농도와 혈액 삼투압 농도는 1,000 mg DEHP 노출구에서 유의적인 감소가 관찰되었다.

Key words : Di-2-ethylhexyl phthalate (DEHP), haematological property, *Pseudobagrus fulvidraco*, serum chemistry, toxic responses

INTRODUCTION

Organisms on wild life are exposed to many chemicals in their environment. Especially, phthalate esters are going attract public attention about potential health risks in recent years (Watanuki *et al*., 2003; Casajuana and Lacorte, 2004).

Phthalate esters are plasticizers used in food handling and storage, and some of them are considered to be ubiquitous pollutants but present slight endocrine-disrupting properties (Céspedes *et al*., 2004). Phthalate are commonly used as plasticizers to give flexibility to plastics. 1/4 part of the phthalate is constitute by di-ethyl hexyl phthalate (DEHP). Phthalates have received a great amount of public attention from various aspects since some of them are suspected of possessing endocrine disrupting properties either by acting as hormone mimic or antagonists, or by more indirect mechanisms (Tyler *et al*., 1998).

Di-2-ethylhexyl phthalate (DEHP) derived from phthalic acid is one of the most important plasticizers and it has become an ubiquitous contaminant. Because it is not permanently bounded to the plastic matrix, DEHP can leach and contaminate its surrounding environment. Thus, it has been identified in many ecological systems. The toxicity and many different biological interactions of DEHP have been previously described (Thomas, 1973; Daniel and Bratt, 1978). Reported levels of DEHP in water samples from rivers in the UK, Sweden, USA and the Netherlands

* Corresponding Author: Tel: +82-51-620-6146, Fax: +82-51-628-7430, E-mail address: jckang@pknu.ac.kr

are all within the range 0.3~1.6 μgL^{-1} with levels in coastal, marine and estuarine waters between <2 ng L^{-1} and 335 ng L^{-1}. contaminate sediment collected from the Mersey Estuary contained up to 1,700 mg kg^{-1} of DEHP. In Korea, the concentration in surface water from Seomjin River were in the ranges of 25.6~116.1 ng L^{-1} for DEHP. In sediments DEHP ranged 46.3~156.3 ng g^{-1} (Kim *et al.*, 2004).

The toxic effects of DEHP on fish have been studied to some extent. Mayer and Sanders (1973) studied the effects of DEHP on the reproduction of zebra fish (*Brachydanio rerio*) and guppies (*Lebistes reticulatus*). Defoe *et al.* (1990) reported a significant reduction in the growth of Japanese medaka (*Oryzias latipes*) exposed to DEHP.

Blood is a physiological reflector of the whole body, so blood parameters are important in diagnosing the structure and functional status of the fish exposed to the toxicant (Jee *et al.*, 2004). In the present study the effect of DEHP on the haematological property, serum organic and inorganic chemistry in the Bagrid catfish (*Pseudobagrus fulvidraco*), which is often very abundant in lakes, reservoirs and even in rivers, and despite its small size, is important in the aquaculture in Korea (Lee and Kim, 1990; Kim and Park, 2002).

MATERIALS AND METHODS

1. Materials

All chemicals were of analytical grade. Di(2-ethylhexyl)phthalate ($C_{24}H_{38}O_4$, CAS registry number 117-81-3) was purchased from Sigma (St. Louis, MO, USA).

2. Fish and rearing conditions

Healthy, cultured Bagrid catfish (*Pseudobagrus fulvidraco*) were obtained from an Inland Fisheries Research Institute in Chung Cheong, Korea. Prior to exposure, fish were held three weeks for acclimatization and evaluation of over-all fish health under the laboratory condition in 12 : 12 h light/dark cycle for further studies. During acclimation fish were fed with basal diet twice daily. After acclimatization, fish (mean length, 17.10±0.11 cm, body weight 52.50±0.90 g) were selected for the experiments.

Table 1. Water quality during the experimental period

Parameters	Values
Temperature (°C)	22.3±0.8
pH	7.2±0.5
NH_4-N (μg-at N L^{-1})	8.5±0.6
NO_2-N (μg-at N L^{-1})	1.5±0.2
NO_3-N (μg-at N L^{-1})	7.5±0.7
PO_4-P (μg-at P L^{-1})	4.6±0.4
SS (mg L^{-1})	3.7±0.3
Dissolved oxygen (mg L^{-1})	6.7±0.5
Hardness (mg L^{-1})	231.7±5.7

Values indicate mean±S.E. (n=8)

3. Exposure

The exposure took place in 80 L aquaria containing 7 fish each under flow-through conditions (Table 1). Each tank received a flow of 9 L h^{-1} with continous aeration. Fish were administered two concentrations of DEHP (300, 1,000 mg DEHP kg body weight^{-1}) by thrice intraperitoneal injection during 3 days. A 50-μL volume was injected in to the peritoneal cavity of each fish at the ventral surface midline by using a 1-mL tuberculin syringe. The control group was subjected to the same regime injected an equal values of sunflower seed oil only.

4. Blood sample and haematological assay

24 hours after last injection of DEHP, fish were anesthetized with 3-aminobenzoic acid ethyl ester methanesulfonate. Blood samples were taken from each fish by puncture of the caudal vessel using heparinized syringes for hematological test. For serum analysis blood was collected by unheparinized syringe. Blood was allowed to coagulate at room temperature for 2 hr and serum was obtained by centrifugation of an amount of blood i.e. approximately 1.5 mL, at 3,000×g for 8 min at 4°C (MIKRO 22R, Hettich, Germany) and then stored at −80°C until analyzed. Hematocrit (Ht) was determined by the microhematocrit technique using capillary tubes by centrifugation at 12,000 rpm for 5 min. Hemoglobin (Hb) was determined spectrophotometrically (540 nm) using the cyanomethemoglobin method (Drabkin and Austin, 1935). Hb concentration was expressed as g dL^{-1} of sample. Red blood cell (RBC) counts were estimated using a

Neubauer hemocytometer (Hesser, 1960). Haematological analyses were done by following techniques described by Tvedten (1989) and Campbell and Murru (1990). The mean corpuscular haemoglobin MCH (pg), the mean corpuscular haemoglobin concentration MCHC (%) and the mean corpuscular volume MCV (μm^3) were calculated using the following formulae.

MCV = (Packed cell volume as percentage/ RBC in millions) × 10 μm^3
MCH = (Hb in grams/RBC in millions) × 10 pg
MCHC = (Hb in grams/Packed cell volume) × 100 g per 100 mL

5. Serum chemistry and osmolality analysis

Serum total protein (colorimetric method) was determined on serum using Sigma Diagnostic Kit. Serum samples were analyzed for calcium (SIGMA Diagnostics kit 588, colorimetric method), magnesium (SIGMA Diagnostics kit 595, colorimetric method), and phosphorus (SIGMA Diagnostics kit 360-UV, colorimetric method). Blood glucose (glucose oxidase/peroxidase method), triglycerides (colorimetric method) and total cholesterol (colorimetric method) were determined on serum using Sigma Diagnostic Kit. Serum osmolality was measured directly by using an advanced micro-osmometer (Model 3300, Norwood, MA).

6. Statistical analysis

Statistical analysis was performed using SPSS/PC+ statistical package. Significant differences between groups were determined using one-way ANOVAs and Duncan's test for multiple comparisons or the Student's t-test for two groups (Duncan, 1955). The level of significance was established at $P < 0.05$ or $P < 0.01$.

RESULTS

1. Responses in Haematological Property

It has been observed that higher concentration of DEHP causes a decrease in RBCs count, Hb concentration and Ht level. The predominant haematological finding was a highly significant decrease of the total number of red blood cell and hemoglobin concentration in fish exposed to 1,000 mg DEHP group thereby indicating a severe anaemia ($P < 0.01$). The hematocrit value was significantly decreased in the 1,000 mg DEHP-treated group compared to that of control group ($P < 0.05$). With regard to the mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular volume (MCV), no significant differences between treatment and control groups were observed (Table 2).

Table 2. Haematological property of Bagrid catfish, *Pseudobagrus fulvidraco* treated with DEHP

Parameters	Groups		
	Control	DEHP (mg DEHP kg b.w.$^{-1}$)	
		300	1,000
RBC count ($\times 10^4$ mm^{-3})	185.2±2.9^a	173.8±2.9^{ab}	163.4±6.1^b
Hb (g dL^{-1})	8.0±0.2^a	7.6±0.2^a	6.9±0.2^b
Ht (%)	28.4±0.7^a	27.4±0.5^a	24.8±0.9^b
MCH (pg)	43.4±1.2	43.6±1.0	42.3±0.4
MCHC (%)	28.3±0.2	27.6±0.3	27.9±0.3
MCV (μm^3)	153.5±4.5	157.7±1.9	151.9±2.4

Value are mean±S.E. (n = 7). Values with different superscript are significantly different ($P < 0.05$) as determined as Duncan's multiple range test. RBC: red blood cell, Hb: hemoglobin, Ht: hematocrit, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, MCV: mean corpuscular volume.

Table 3. Serum organic chemistry of Bagrid catfish, *Pseudobagrus fulvidraco* treated with DEHP

Parameters	Groups		
	Control	DEHP (mg DEHP kg b.w.$^{-1}$)	
		300	1,000
TP (g dL^{-1})	5.2±0.1^a	4.1±0.2^b	3.9±0.2^b
GLC (mg dL^{-1})	55.7±5.2	67.3±10.8	67.8±10.4
CHOLE (mg dL^{-1})	278.6±11.6^a	216.5±19.2^b	213.3±7.9^b
TG (mg dL^{-1})	86.5±12.4^a	64.1±14.6^{ab}	41.2±10.2^b

Value are mean±S.E. (n = 7). Values with different superscript are significantly different ($P < 0.05$) as determined as Duncan's multiple range test. TP: total protein, GLC: glucose, CHOLE: cholesterol, TG: triglyceride.

2. Serum organic chemistry

As seen in Table 3, serum total protein and cholesterol levels showed significant decrease in DEHP treatment groups as compared to the control ($P < 0.01$). There was no significant difference in serum glucose concentration in treatment groups as compared to control group ($P > 0.05$). Serum triglyceride value was significantly

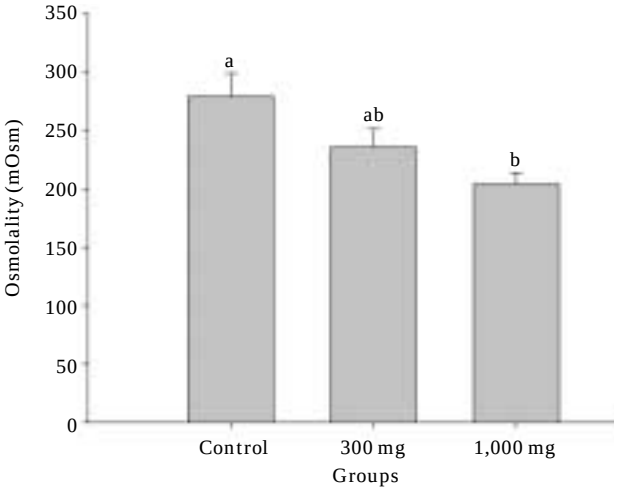

Fig. 1. Serum osmolality levels of Bagrid catfish, *Pseudobagrus fulvidraco* treated with DEHP. Vertical bars indicate standard error (Mean ± S.E., n = 7). Value with different letter are significantly different ($P < 0.05$) as determined as Duncan's multiple range test.

Table 4. Serum inorganic chemistry of Bagrid catfish, *Pseudobagrus fulvidraco* treated with DEHP

Parameters	Groups		
	Control	DEHP (mg DEHP kg b.w.$^{-1}$)	
		300	1,000
Mg (mg dL^{-1})	2.6 ± 0.2	2.7 ± 0.2	2.4 ± 0.1
Ca (mg dL^{-1})	8.4 ± 0.2^{a}	7.0 ± 0.6^{ab}	6.1 ± 0.6^{b}
P (mg dL^{-1})	10.7 ± 0.2	10.7 ± 0.4	10.6 ± 0.5

Value are mean ± S.E. (n = 7). Values with different superscript are significantly different ($P < 0.05$) as determined as Duncan's multiple range test. Mg: magnesium, Ca: calcium, P: inorganic phosphorus.

decrease in the 1,000 mg DEHP group ($P < 0.05$), where in fish administrated to 300 mg DEHP group, it was only slightly affected ($P > 0.05$).

3. Serum inorganic chemistry and osmolality

Serum osmolality levels were significantly lower ($P < 0.05$) in 1,000 mg DEHP treated fish as compared with control (Fig. 1). Significant decrease serum calcium level was observed at 1,000 mg DEHP exposed group. Magnesium and phosphorus indices revealed a marginal or no deviation from control values (Table 4).

DISCUSSION

The treatment with di-2-ethylhexyl phthalate (DEHP) in the present investigation showed remarkable haematological stress markers at higher level of DEHP. The decrease in RBC count, hemoglobin concentration and hematocrit level observed in this study may be due to the disruptive action of the DEHP. Alterations in the haematological parameters were brought about by DEHP as a hemolytic condition due to increased lipid peroxidation of RBC. Our results are in line with those found by Arun *et al.* (1999) in RBC *in vitro*. According to Deepa Devi *et al.* (1998), oxidative damage to the cell membrane leaded to lysis of the RBC. Our studies provide evidence that DEHP effect on erythrocyte hemolysis. Decline in RBC count, hemoglobin concentrations and hematocrit presumably reflect erythrocyte hemolysis and/or irreparable damage of gill morphology and function. The decrease in hemoglobin concentration may be due to either an increase in the rate at which hemoglobin is destroyed or a decrease in the rate of hemoglobin synthesis.

Fish under stress mobilize protein to meet the energy requirements which is needed to sustain increased physical activity (Sievers *et al.*, 1995). Decreased protein, triglyceride and cholesterol levels in the present study after DEHP exposure may be attributed to stress-mediated mobilization of these compounds to fulfill an increased demand for energy by the fish, to cope with detrimental conditions imposed by the toxicant. Reports on the reduction of these organic compounds in serum of vertebrates after exposure to DEHP suggest that these compounds are mobilized for energy production (Ranjitsingh and Badejo, 1996; Sonde *et al.*, 2000). In this study, serum organic compounds (total protein, cholesterol and triglyceride) were significantly decreased in DEHP administrated fish as compared to control group. DEHP can cause functional hepatic damage, as reflected by morphological changes, alterations in energy-linked enzyme activity, and changes in lipid and carbohydrate metabolism. The most striking effect of DEHP in rat is proliferation of hepatic peroxisomes (Seth, 1982). He reported decreased levels of serum triglyceride and cholesterol along with histological (microscopical) changes in the liver of rat after

short-term dietary exposure to DEHP. Indeed, Morton (1979) found significantly decreased serum triglyceride levels in rat exposed to DEHP.

With regard to the serum inorganic parameters, the most marked difference between the DEHP exposed fish and control group was lower serum calcium and osmolality level. Acute exposures to toxicant caused gill epithelial sloughing and mucosal cell changes in various fish species (Gardner, 1975; Hawker, 1977). Lipophilic materials are taken up in fish partly by the gills (McKeown and March, 1978). Lipophilic substances are expected to accumulate in the cell membranes where they may affect cell membrane permeability and membrane-located transport mechanisms (Payne *et al*., 1978). Thus DEHP may interfere with both the osmoregulatory function of the gill and volume regulation in cells in fish. These osmoregulatory disturbance are a rapid facet of the fish stress response; but the osmoregulatory process affected by toxic stress in bagrid catfish and other freshwater teleost species require more investigation. Consequently, stress markers such as haematological property, serum organic chemistry, serum osmolality and its ionic concentration are also susceptible to effect due to changes in hematological properties in Bagrid Catfish (*Pseudobagrus fulvidraco*) exposed to single phthalate, DEHP.

ABSTRACT

Di-2-ethylhexyl phthalate (DEHP) is a widely used plasticizer known to be a suspected xenoestrogen and a causative agent of oxidative damage to the RBC cell membrane *in vitro*. We evaluated the toxic effects of a scarcely documented aquatic environmental pollutant, DEHP, on selected haematological endpoints in the bagrid catfish, *Pseudobagrus fulvidraco*. Bagrid catfish were exposed to DEHP (300, 1,000 mg DEHP kg body weight^{-1}) through thrice intraperitoneal injection and effects were assessed in blood of the exposed organisms. Haematological property, serum organic and inorganic chemistry were monitored in blood of Bagrid catfish. DEHP exposed-fish showed erythropenia; low hematocrit (Ht) and hemoglobin (Hb) content and red blood cell count showed a significantly higher than in that of the control group. The treatment group showed a significantly lower concentration of serum total protein, cholesterol and triglyceride compared with those in the control group. The value of calcium and osmolality were significantly decreased in the DEHP treatment group, compared with the control group.

REFERENCES

Arun, P., K.G. Padmakumaran Nair, V. Manojkumar, K.V. Deepadevi, L.R. Lakshmi and P.A. Kurup. 1999. Decreased hemolysis and lipid peroxidation in blood during storage in the presence of nicotinic acid. *Vox Sang*., **76**(4): 220-225.

Campbell, T. and F. Murru. 1990. An introduction to fish hematology. *Com. Cont. Educ. Pract. Vet*., **12**: 525-532.

Casajuana, N. and S. Lacorte. 2004. New Methodology for the Determination of phthalate Esters, bisphenol A, bisphenol A diglycidyl ether, and nonylphenol in commercial whole milk samples. *J. Agric. Food Chem*., **52**: 3702-3707.

Céspedes, R., M. Petrovic, D. Raldúa, U. Saura, B. Piña, S. Lacorte, P. Viana and D. Barceló. 2004. Integrated Procedure for Determination of Endocrine-Disrupting Activity in Surface Waters and Sediments by Use of the Biological Technique Recombinant Yeast Assay and Chemical Analysis by LC-ESIMS. *Anal. Bioanal. Chem*., **378**: 697-708.

Daniel, J.W. and H. Bratt. 1974. The absorption, metabolism and tissue distribution of di (2-ethylhexyl) phthalate in rats. *Toxicology*, **2**: 51-65.

Deepa Devi K.V., V. Manoj Kumar, P. Arun, A. Santhosh, K.G. Padmakumaran Nair, L.R. Lakshmi and P.A. Kurup. 1998. Increased lipid peroxidation of erythrocytes in blood stored in polyvinyl chloride blood storage bags plasticized with di-[2-ethyl hexyl] phthalate and effect of antioxidants. *Vox Sang*., **75**: 198-204.

Defoe, D.L., G.W. Holcombe, D.E. Hammermeister and K.E. Biesinger. 1990. Solubility and toxicity of eight phthalate esters to four aquatic organisms. *Environ. Toxicol. Chem*., **9**: 623-636.

Duncan, D.B. 1955. Multiple-range and multiple F tests. *Biometrics*, **11**: 1-42.

Drabkin, D.L. and J.H. Austin. 1935. Spectrophometric studies. II. Preparations from washed blood cells, nitric oxide hemoglobin and sulhemoglobin. *J Biol. Chem*., **122**: 51-56.

Gardner, G.R. 1975. Chemically induced lesions in estuarine or marine teleost. p. 657-693. *In*: The pathology of fishes (W.E., Ribelin and G. Migaki, eds.). University of Wisconsin Press, Madison, Wisconsin.

Hawker, J.W. 1977. The effects of petroleum hydrocarbon exposure in the structure of fish tissues. p. 115-128. *In*: Fate and effects of petroleum hydrocarbons in marine organisms and ecosystems (D. A., Wolfe, ed.). Pergamon Press, New York.

Hesser, E.F. 1960. Methods for routine fish hematology. *Prog. Fish-Cult*., **22**(4): 164-171.

Jee, J.H., S.G. Kim and J.C. Kang. 2004. Effects of phenanthrene on growth and basic physiological functions of the olive flounder, *Paralichthys olivaceus. J. Exp. Mar. Biol. Ecol*., **304**: 123-136.

Kim, I.S. and J.Y. Park. 2002. Freshwater fishes of Korea. Kyo-Hak Publishing, Seoul.

Kim, M., D. Li, W.J. Shin and J.R. Oh. 2004. Distribution of phthalate compounds in Gwangyang bay and Seomjin river estuary. Korean *J. Environ. Biol*., **22**(S): 47-53.

Lee, C.L. and I.S. Kim. 1990. A Taxonomic revision of the family Bagridae (Pisces, Siluriformes) form Korea. *Korean J. Ichthyol*., **2**(2): 117-137.

Mayer, F.L. and H.O. Sanders. 1973. Toxicology of phthalic acid esters in aquatic organisms. *Environ. Health Perspect*., **3**: 153-157.

McKeown, B.A. and G.L. March. 1978. The effects of Bunker C oil and oil dispersant: Part 2. Effects on the accumulation of chloride-labelled Bunker C oil in various fish tissues. *Mar. Environ. Res*., **1**: 119-124.

Morton, S.J. 1979. The hepatic effects of dietary di-2-ethylhexyl phthalate. Ann Arbor, MI, Johns Hopkins University.

Payne, J.F., J.W. Kiceniuk, W.R. Squires and G.L. Fletcher. 1978. Pathological changes in a marine fish after a six month exposure to petroleum. *J. Fish. Res. Board Can*., **35**: 665-667.

Ranjitsingh, A.J.A. and M.A. Badejo. 1996. Effect of organo-phosphate pesticide (phosphamidon) on aquatic snail *Indoplanorbis exustus. J. Ecotoxicol. Environ. Monitoring*, **6**: 127-130.

Seth, P.K. 1982. Hepatic effects of phthalate esters. *Environ. Health Perspect*., **45**: 27-34.

Sievers, G., P. Palacios. R. Inostroza and H. Doelz. 1995. Evaluation of the toxicity of 8 insecticides in *Salmo salar* and the in vitro effects against the isopode parasite, *Ceratothoa gaudichaudii. Aquaculture*, **134**: 9-16.

Sonde, V., A. D'souza, R. Tarapore, L. Pereira, M.P. Khare, P. Sinkar, S. Krishnan and C.V. Rao. 2000. Simultaneous administration of diethylphthalate and ethyl alcohol and its toxicity in male Sprague-Dawley rats. *Toxicology*. **147**(1): 23-31.

Thomas, G.H. 1973. Quantitative determination and confirmation of identity of trace amounts of dialkylphthalates in environmental samples. *Environ. Health Perspect*., **3**: 23-28.

Tvedten, H. 1989. The complete blood count and bone marrow examination: General comments and selected techniques. p. 11-30. *In*: Small Animal Clinical Diagnosis by laboratory Methods (M.D. Willard, H. Tvedten & G.H. Turnwald, EDS.). W.B. Saunders, Philadelphia.

Tyler, C.R., S. Jobling and J.P. Sumpter. 1998. Endocrine disruption in wildlife: A critical review of the evidence. *Cirt. Rev. Toxicol*., **28**: 319-361.

Watanuki, H., Y. Gushiken and M. Sakai. 2003. *In vitro* modulation of common carp (*Cyprinus carpio* L.) phagocytic cells by Di-n-butyl phthalate and Di-2-ethylhexyl phthalate. *Aquat. Toxicol*., **63**: 119-126.

(Manuscript received 2 July 2004,
Revision accepted28 August 2004)