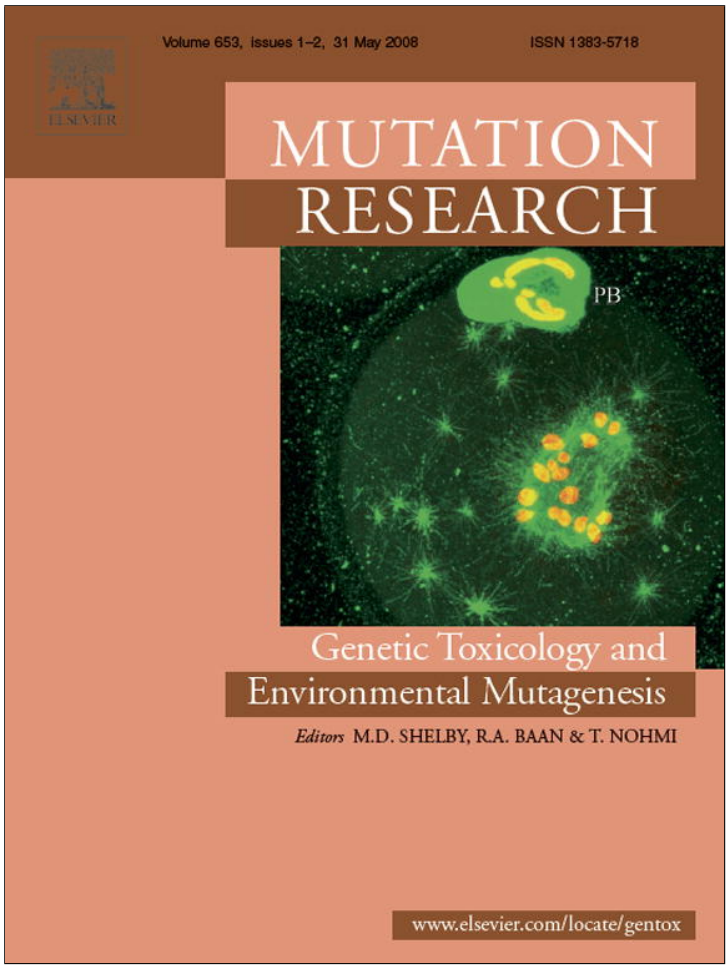

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Genotoxic evaluation of the GH transgenic *Synechocystis* using mice and turbot (*Scophthalmus maximus* L.)

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ABSTRACT

In the present paper, the genotoxicity of transgenic *Synechocystis* sp. PCC 6803 containing flounder (*Paralichthys olivaceus*) growth hormone gene has been assessed using micronucleus tests in mice and fish. No micronucleus inductions in bone marrow erythrocyte of mice were observed at three doses of transgenic *Synechocystis* (2.0, 5.0, and 10.0 g/kg). The frequencies of micronucleus and other nuclear abnormalities in turbot (*Scophthalmus maximus* L.) fed with 0.2%, 0.5% and 1.0% transgenic *Synechocystis* were also not significantly different ($P > 0.05$) from controls. In contrast, cyclophosphamide, used in detecting the sensitivity of the biological assays, significantly increased the frequencies of micronucleus and other nuclear abnormalities in mice and turbot. Results indicate that the flounder GH transgenic *Synechocystis* sp. PCC 6803 seems to have no genotoxic effects on fish.

1. Introduction

Recombinant growth hormone (rGH) was proved to have the ability to promote the growth of fish [1–4] and thus increasing attention has been paid on its potential usage in fish farming [5–7]. To date, several expression systems such as *Escherichia coli* and *Pichia pastoris* have been developed to produce rGH. The complicated procedure for purification restricts their application in commercial aquaculture. In order to develop a cost effective way of manufacturing biologically active rGH for its eventual use in aquaculture, we constructed transgenic *Synechocystis* sp. PCC 6803 containing flounder (*Paralichthys olivaceus*) GH gene. The aim is to promote fish growth by using it directly as feed additive and omit purification steps, then make the production economically viable. If the transgenic alga could be verified to be nontoxic and can promote fish growth, it would bring great benefit to fish farmers.

Synechocystis sp. PCC 6803 is basically considered as a model cyanobacterium adapted to wide range of ecological conditions [8]. The species possesses a short growth cycle and is easy to be cultured at low cost. However, investigations on the feasibility of *Synechocystis* as a feed supplement for fish are yet to be conducted. Therefore, studies on the safety of the GH transgenic *Synechocystis* with regard to fish health is of great concern.

Fish genotoxicity is widely discussed for the possible risk if exposure to humans through the food chain. Among the techniques available to detect the genotoxic effects, the micronucleus (MN) assay has extensively been employed in assessing the genotoxicity of many compounds under laboratory conditions [9,10] and for in situ biomonitoring of environmental mutagenesis [11,12]. Its wider application was supported by the sensitivity, reliability and the simplicity of use. For the MN assay, mice erythrocyte in bone marrow is a classical test-system. On the other hand, several studies have showed that fish micronucleus tests are sensitive enough to detect genotoxic compounds [9,10,13]. In addition, it was reported that abnormal nuclear morphology is also an indicator of genotoxic damage in fish [14–16]. In the present study, we evaluated the genotoxic potential of transgenic *Synechocystis* sp. PCC 6803 containing flounder GH gene through micronucleus and other nuclear abnormalities assay in mice and turbot (*Scophthalmus maximus* L.). The aim of this investigation was to verify the safety of GH transgenic *Synechocystis* as feed additive for fish.

2. Materials and methods

2.1. Culture of Synechocystis sp. PCC 6803

Non-transgenic and transgenic *Synechocystis* sp. PCC 6803 were cultured in liquid BG-11 medium with continuous air bubbling at 30 °C. Cultures were illuminated with fluorescent lamps. For the transgenic alga, medium was supple-

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mented with 50 μg/ml kanamycin sulphate and at the exponential growth phase (OD_{730} = 0.8 – 1.0), cells were harvested and inoculated into medium without ferric ammonium citrate to trigger the expression of the GH gene of flounder. Algae were harvested by centrifugation at 12,000 × *g* for 3 min, lyophilized and stored at −20 °C until use.

2.2. Mouse micronucleus test

Mice, obtained from the Center of Experimental Animal, Qingdao, China, were acclimatized to the laboratory conditions for one week prior to the experiment. All experiments were performed accordance with Welfare Committee of the Centre of Experimental Animal, Qingdao, China, and this project was also approved by the committee. Males and females, body weight 28 ± 2 g, were fed with mice feed (purchased from the Center of Experimental Animal, Qingdao, China) and were provided filtered water ad libitum. Mice were housed and randomly divided into 6 groups, 10 animals (5 males and 5 females) in each group. Each group mice in the three experimental groups were fed with transgenic three dose *Synechocystis* (2.0, 5.0 and 10.0 g/kg) twice within a 24 h interval by gavage. Transgenic alga liquid stock was prepared by dissolving alga powder in 0.5 ml distilled water. The negative control was the mice received distilled water (0.5 ml), and the positive control was the mice injected with cyclophosphamide at a dose of 40 mg/kg. The non-transgenic alga control received 10 g/kg non-transgenic *Synechocystis*. The bone marrow erythrocyte slides were fixed with methanol, stained with acridine orange and observed by fluorescent microscopy. 1000 cells per animal were counted and the frequency of MN was expressed per 1000 cells (‰).

2.3. Fish micronucleus test

Juvenile turbot (body weight 1.4 ± 0.3 g, body length 4.8 ± 0.3 cm) obtained from Xuejiadao Island Experimental Station, Institute of Oceanology, Chinese Academy of Sciences, were acclimatized in the laboratory for two weeks. During the period, fish were fed with commercial diet obtained from Qingdao Jinhaili Fishery Science Co., Ltd. A total of 125 fish were equally distributed into five 501 aquaria (25 fish/aquarium), which received continuously aerated seawater at 17–19 °C. They were maintained under natural photoperiod. Water in each aquarium was renewed daily. Fish fed with commercial diet (free from algae) and diet containing 1.0% non-transgenic alga were considered as the control groups. Fish in experimental groups were fed with commercial diet supplemented with 0.2%, 0.5% and 1.0% transgenic *Synechocystis* sp. PCC 6803. A mixture of commercial feed and transgenic or non-transgenic alga powder with appropriate amount of distilled water was used to prepare the pellets. The mixture was compressed with a laboratory-scale pellet mill equipped with 4.0 mm die, dried at room temperature and stored at −20 °C until use. Fish in each aquarium were hand fed three times a day to apparent satiation. After 8 weeks, 10 fish in each group were randomly selected for the micronucleus and abnormal nuclear examinations.

Cyclophosphamide, a well-known genotoxic compound, was employed in detecting the sensitivity of the biological assay. One day prior to the end of the trial, 30 mg/kg cyclophosphamide were intraperitoneally injected to each of the 10 fish in the control group fed with commercial diet. Blood samples were collected 24 h after the injection and slides were prepared for examination.

Peripheral blood samples from the caudal vein of each fish were collected and smeared immediately onto cleaned slides. After air-drying for 12 h, slides were fixed in absolute methanol for 15 min, followed by staining with aqueous Giemsa (15%) for 10 min.

Micronucleus and other nuclear abnormalities in erythrocytes were detected under 1000× oil-immersion lens. The criteria for the identification of MN were based on the methods description by Grisolia [9]: (a) MN should be smaller than one-third of the main nuclei, and (b) MN must not be refractive and should be the same color and intensity as the main nuclei. A total of 3000 erythrocytes were examined per fish in three coded slides (1000 cells per slide). All slides were scored by a single observer. The frequency of MN and other nuclear abnormalities were expressed per 1000 cells (‰).

2.4. Statistical analysis

The Kruskal–Wallis and Mann–Whitney tests with the confidence level of $P < 0.05$ were used in determining the significance between experimental and control groups.

3. Results

A representative fluorescent photomicrograph of micronucleated bone marrow erythrocyte of mice is shown in Fig. 1. We scored only MN that was clearly delineated and typically shaped. Micronucleus frequency of bone marrow erythrocyte of mice is summarized in Table 1. Cyclophosphamide, the positive control, caused a significant increase in the frequency of micronucleated cells ($P < 0.05$), but

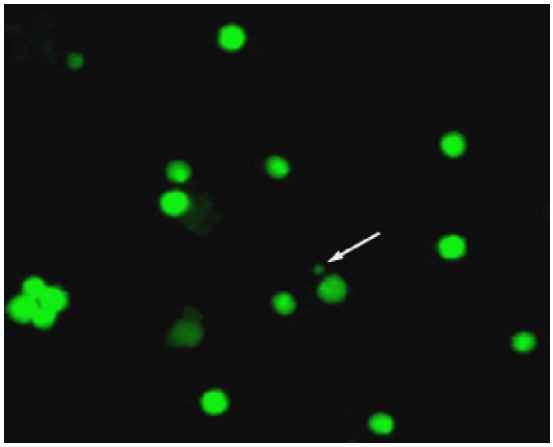

Fig. 1. Micronucleus of bone marrow erythrocyte of mice stained by acridine orange.

Table 1
Effect of transgenic *Synechocystis* sp. PCC 6803 containing flounder GH gene on the frequency of micronucleus of bone marrow erythrocyte in mice (mean ± S.D., $n = 10$)

Treatment	Number of cells	Number of MN	Frequency of MN (‰)
Commercial feed control	10,000	18	1.80 ± 1.04[a]
Non-transgenic alga control	10,000	16	1.60 ± 0.88[a]
Cyclophosphamide	10,000	221	22.10 ± 4.72[b]
2.0 g/kg transgenic alga	10,000	22	2.20 ± 0.81[a]
5.0 g/kg transgenic alga	10,000	20	2.00 ± 1.10[a]
10.0 g/kg transgenic alga	10,000	17	1.70 ± 0.96[a]

1000 cells were counted per each animal. Within the columns, values with different letters are significantly different ($P < 0.05$).

administrated with transgenic *Synechocystis* caused no MN induction compared to the controls ($P > 0.05$).

MN and other nuclear abnormalities (including binucleus, lobed nucleus and notched nucleus) in the peripheral blood erythrocytes of turbot were observed in all the groups (Fig. 2). The results of MN and other nuclear abnormalities are given in Figs. 3–6. No significant differences ($P > 0.05$) in the frequencies of micronucleus and nuclear abnormalities were found among the two control groups and the fish fed with transgenic *Synechocystis* sp. PCC 6803 (Fig. 3). However, MN and the nuclear abnormalities frequency of the fish treated with cyclophosphamide were significantly higher than those of the two control groups and the other administrated transgenic *Synechocystis* groups ($P < 0.05$). The frequency of the binucleated cells in non-transgenic alga control was significantly lower ($P < 0.05$) than that of the commercial feed control group (Fig. 4), but not significantly different from the three treated groups. Values of lobed nucleus cell and notched nucleus cell frequencies were found to be similar to all the five groups ($P > 0.05$).

4. Discussion

Growth-promoting effects of rGH have been well documented in many teleost species [17]. Recently, increasing commercial production demands have generated considerable interest regarding the use of rGH in increasing yield in aquaculture. However, its use has been impeded by lack of method of economical mass production and practical administration. Based on these grounds, we constructed transgenic *Synechocystis* sp. PCC 6803 containing flounder (*P. olivaceus*) GH gene, which could be used directly as feed additive. However, the safety needed to be studied before applying it to fish aquaculture.

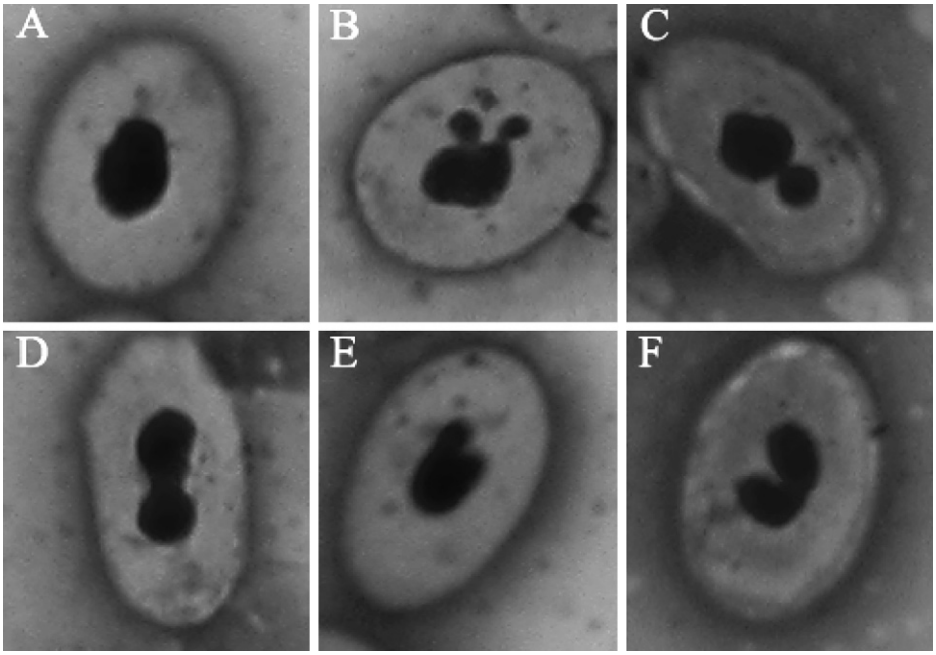

Fig. 2. Erythrocytes of turbot with normal nucleus (A), micronucleus (B and C), binucleus (D), lobed nucleus (E) and notched nucleus (F). Giemsa-stained peripheral blood smear. Micronucleus: smaller than one-third of the main nuclei and with a round or ovoid shape; binucleus: two nuclei with the same volume, isolated or connected by little part; lobed nucleus: evagination of the nuclear envelop that seem to contain euchromatin; notched nucleus: an appreciable depth into nucleus that dose not contain nuclear material.

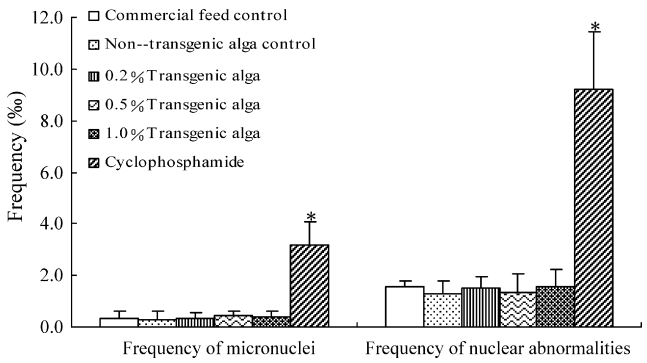

Fig. 3. Frequency distribution of micronucleus and nuclear abnormalities in peripheral blood erythrocytes of turbot fed with transgenic *Synechocystis* sp. PCC 6803 containing flounder GH gene for 8 weeks (mean ± S.D., $n = 10$). A total of 30,000 erythrocytes were tested in each group of fish (3000 erythrocytes/per fish); Frequency of nuclear abnormalities: the sum of frequencies of binucleus, lobed and notched nucleus; '*' represents significant difference ($P < 0.05$).

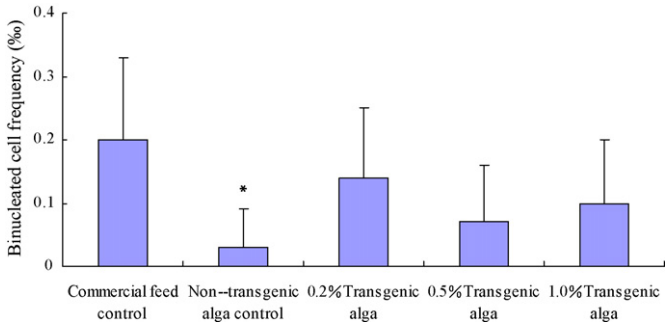

Fig. 4. Binucleated cell frequency in peripheral blood erythrocytes of turbot fed with transgenic *Synechocystis* sp. PCC 6803 containing flounder GH gene for 8 weeks (mean ± S.D., $n = 10$). A total of 30,000 erythrocytes were tested in each group of fish (3000 erythrocytes/per fish); '*' represents significant difference compared to commercial feed control ($P < 0.05$).

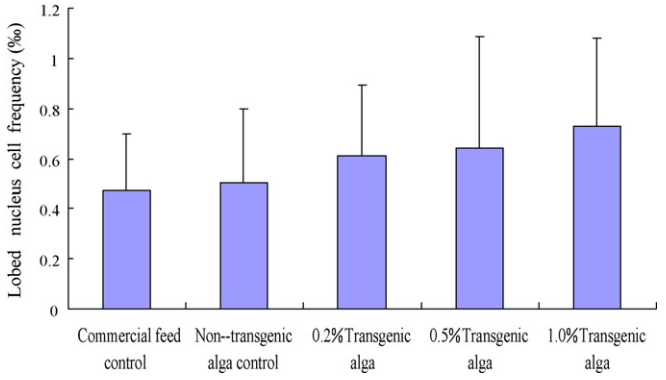

Fig. 5. Lobed cell frequency in peripheral blood erythrocytes of turbot fed with transgenic *Synechocystis* sp. PCC 6803 containing flounder GH gene for 8 weeks (mean ± S.D., $n = 10$). A total of 30,000 erythrocytes were tested in each group of fish (3000 erythrocytes/per fish); there were no significant differences among groups ($P > 0.05$).

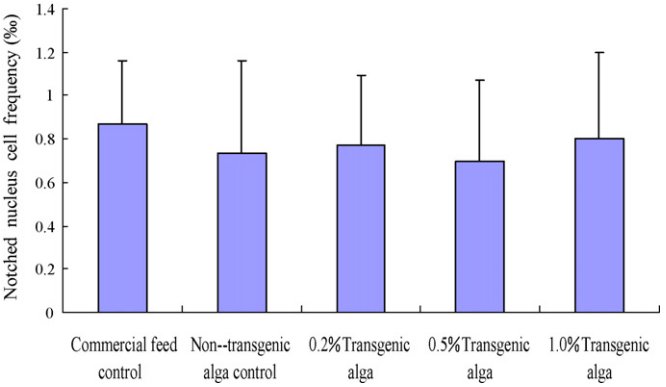

Fig. 6. Notched cell frequency in peripheral blood erythrocytes of turbot fed with transgenic *Synechocystis* sp. PCC 6803 containing flounder GH gene for 8 weeks (mean ± S.D., $n = 10$). A total of 30,000 erythrocytes were tested in each group of fish (3000 erythrocytes/per fish); there were no significant differences among groups ($P > 0.05$).

The count of MN has been used as an index of chromosome breaks and mitotic spindle apparatus dysfunction for over 20 years. Although originally developed for its application in mouse, MN test was subsequently modified by Hooftman and de Raat [18] for the use in fish. To date, it has been widely employed in assessing the biological impact of water pollution and the genotoxicity of chemical compounds in fish [19,20] and other aquatic organisms such as sea urchin [21], bivalves [22] and whales [23].

According to the present results, mice have not found MN expression, which induced by transgenic *Synechocystis* sp. PCC 6803 containing flounder GH gene. Juvenile turbot fed with the transgenic alga for 8 weeks also showed any effect on the frequencies of MN and other nuclear abnormalities in contrast to those fed with commercial feed and non-transgenic alga. However, cyclophosphamide, a well-known genotoxic compound has induced the formation of MN and other nuclear abnormalities both in mice and in juvenile turbot, which suggest that the two species are sensitive to the genotoxic compound. Based on these findings, it could be concluded that transgenic *Synechocystis* sp. PCC 6803 containing flounder GH gene is not a genotoxic agent for fish and mice. Because no report on the genotoxic effect of *Synechocystis* sp. PCC 6803 on animal was found up to now, this study appears to be the first investigation on the genotoxicity of *Synechocystis* sp. PCC 6803.

Blood physiological and chemical parameters are commonly used diagnostic tools in fish toxicology [24]. Results of red and white blood cell counts, hemoglobin content, volume of erythrocytes, serum enzyme activities and metabolite concentrations of juvenile turbot used in the present study and those cultured in an experimental station of Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (weight 15 ± 2.1 g, fed with 1% transgenic *Synechocystis* for 40 days) demonstrated that the transgenic alga had no adverse effect on these blood parameters [25,26]. These findings together with the results in MN tests in the present study suggest that the GH transgenic *Synechocystis* sp. PCC 6803 seems not to be a genotoxin for fish.

5. Conflict of interest

Authors declare that they have no financial relationship with a commercial entity that has an interest in the subject of this manuscript. Submitting this article in this journal is the collective decision of all the authors. Furthermore, the present paper is not under consideration as a whole or in part by another journal or elsewhere in any language.

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References

[1] H.J. Tsai, M.H. Hsih, J.C. Kuo, *Escherichia coli*-produced fish growth hormone as a feed additive to enhance the growth of juvenile black seabream (*Acanthopagrus schlegeli*), J. Appl. Ichthyol. 13 (1997) 79–82.
[2] H.S. Jeh, C.H. Kim, H.K. Lee, K. Han, Recombinant flounder growth hormone from *Escherichia coli*: overexpression, efficient recovery, and growth-promoting effect on juvenile *Paralichthys olivaceus* by oral administration, J. Biotech. 60 (1998) 183–193.
[3] T.A. Leedom, K. Uchida, T. Yada, N.H. Richman III, J.C. Byatt, R.J. Collier, T. Hirano, E.G. Grau, Recombinant bovine growth hormone treatment of tilapia: growth response, metabolic clearance, receptor binding and immunoglobulin production, Aquaculture 207 (2002) 359–380.
[4] B.C. Peterson, B.C. Small, B.G. Bosworth, Effects of bovine growth hormone (Posilac®) on growth performance, body composition, and IGFBPs in two strains of channel catfish, Aquaculture 232 (2004) 651–663.
[5] D.H. Kim, Y.T. Kim, J.J. Cho, J.H. Bae, S.B. Hur, I. Hwang, T.J. Choi, Stable integration and functional expression of flounder growth hormone gene in transformed microalga, *Chlorella ellipsoidea*, Mar. Biotechnol. 4 (2002) 63–73.
[6] B. Promdonkoy, S. Warit, S. Panyim, Production of a biologically active growth hormone from giant catfish (*Pangasianodon gigas*) in *Escherichia coli*, Biotechnol. Lett. 26 (2004) 649–653.
[7] R.Zh. Chen, F. Yang, X. Xu, Study on the growth-promoting effect of recombinant yeast containing fish growth hormone on sea water fish, Fish. Res. 7 (2005) 49–51 (in Chinese).
[8] N.T. Marsac, J. Houmard, Adaptation of cyanobacteria to environment stimuli: new steps towards molecular mechanisms, FEMS Microbiol. Lett. 104 (1993) 119–190.
[9] C.K. Grisolia, A comparison between mouse and fish micronucleus test using cyclophosphamide, mitomycin C and various pesticides, Mutat. Res. 518 (2002) 145–150.
[10] M.A. Campana, A.M. Panzeri, V.J. Moreno, F.N. Dulout, Genotoxic evaluation of the pyrethroid lambda-cyhalothrin using the micronucleus test in erythrocytes of the fish *Cheirodon interruptus interruptus*, Mutat. Res. 438 (1999) 155–161.
[11] C.K. Grisolia, F.L.R.M. Starling, Micronuclei monitoring of fishes from lake Paranoá, under influence of sewage treatment plant discharges, Mutat. Res. 491 (2001) 39–44.
[12] P. Van Ngan, V. Gomes, M.J.A.C.R. Passos, K.A. Ussami, D.Y.F. Campos, A.J.daS. Rocha, B.A. Pereira, Biomonitoring of the genotoxic potential (micronucleus and erythrocyte nuclear abnormalities assay) of the Admiralty Bay water surrounding the Brazilian Antarctic Research Station "Comandante Ferraz" King George Island, Polar Biol. 30 (2007) 209–217.
[13] F. Ayllon, E. Garcia-Vazquez, Induction of micronuclei and other nuclear abnormalities in European minnow *Phoxinus phoxinus* and mollie *Pocelia latipinna*: an assessment of the fish micronucleus test, Mutat. Res. 467 (2000) 177–186.
[14] M. Pachecco, M.A. Santos, Induction of liver EROD and erythrocytic nuclear abnormalities by cyclophosphamide and PAHs in *Anguilla Anguilla* L., Ecotoxicol. Environ. Saf. 40 (1998) 71–83.
[15] V. Bombail, D. Aw, E. Gordon, J. Barry, Application of the comet and micronucleus assays to butterfish (*Pholis gunnelus*) erythrocytes from the Firth of Forth, Scotland, Chemosphere 44 (2001) 283–392.
[16] C. Gravato, M.A. Santos, β-Naphthoflavone liver EROD and erythrocytic nuclear abnormality induction in juvenile *Dicentrarchus labrax* L., Ecotoxicol. Environ. Saf. 52 (2002) 69–74.
[17] E. McLean, R.H. Devlin, Application of biotechnology to enhance growth of salmonids and other fish, in: M. Fingerman, R. Nagabhushanam (Eds.), Recent Advances in Marine Biotechnology, Science Publishers, Enfield, NH, USA, 2000, pp. 17–55.
[18] R.N. Hooftman, W.K. de Raat, Induction of nuclear anomalies (micronuclei) in the peripheral blood erythrocytes of the eastern mudminnow *Umbra pygmea* by ethyl methanesulphonate, Mutat. Res. 104 (1982) 147–152.
[19] K. Al-Sabti, C.D. Metcalfe, Fish micronuclei for assessing genotoxicity in water, Mutat. Res. 343 (1995) 121–135.
[20] M.V.M. Ferraro, A.S. Fenocchio, M.S. Mantovani, C.deO. Ribeiro, M.M. Cestari, Mutagenic effects of tributyltin and inorganic lead (Pb II) on the fish *H. malabaricus* as evaluated using the comet assay and the piscine micronucleus and chromosome aberration tests, Genet. Mol. Biol. 27 (2004) 103–107.
[21] K. Saotome, T. Sofuni, M. Hayashi, A micronucleus assay in sea urchin embryos, Mutat. Res. 446 (1999) 121–127.
[22] J. Mersch, M.-N. Beauvais, P. Nagel, Induction of micronuclei in haemocytes and gill cells of zebra mussels, *Dreissena polymorpha*, exposed to clastogens, Mutat. Res. 371 (1996) 47–55.
[23] J.M. Gauthier, H. Dubeau, E. Rassart, Mercury induced micronuclei in skin fibroblasts of beluga whales, Environ. Toxicol. Chem. 17 (1998) 2487–2493.
[24] L.C. Folmar, G.R. Gardner, J. Hickey, S. Bonomelli, T. Moody, Serum chemistry and histopathological evaluations of brown bullheads (*Ameiurus nebulosus*) from the Buffalo and Niagara rivers, New York, Arch. Environ. Contam. Toxicol. 25 (1993) 298–303.
[25] Sh.M. Liu, Study on the growth-promoting effect on fish of transgenic Synechocystis PCC 6803 containing flounder growth hormone gene and its safety evaluation, Doctor Thesis, Ocean University of China, 2007, pp. 72–90 (in Chinese).
[26] Sh.M. Liu, X.N. Zang, B. Liu, X.Ch. Zhang, K.K.I.U. Arunakumara, X.Q. Zhang, B. Liang, Effect of GH transgenic *Synechocystis* on growth, feed efficiency, muscle composition, haematology and histology of turbot (*Scophthalmus maximus* L.), Aquaculture Res. 38 (2007) 1283–1292.