Primary cultured cells as sensitive in vitro model for assessment of toxicants—comparison to hepatocytes and gill epithelia

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Abstract

In an effort to develop cultured cell models for toxicity screening and environmental biomonitoring, we compared primary cultured gill epithelia and hepatocytes from freshwater tilapia (Oreochromis niloticus) to assess their sensitivity to AhR agonist toxicants. Epithelia were cultured on permeable supports (terephthalate membranes, “filters”) and bathed on the apical with waterborne toxicants (pseudo in vivo asymmetrical culture conditions). Hepatocytes were cultured in multi-well plates and exposed to toxicants in culture medium. Cytochrome P4501A (measured as 7-Ethoxyresorufin-O-deethylase, EROD) was selected as a biomarker. For cultured gill epithelia, the integrity of the epithelia remained unchanged on exposure to model toxicants, such as 1,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo(a)pyrene B[α]P, polychlorinated biphenyl (PCB) mixture (Aroclor 1254), and polybrominated diphenyl ether (PBDE) mixture (DE71). A good concentration-dependent response of EROD activity was clearly observed in both cultured gill epithelia and hepatocytes. The time-course response of EROD was measured as early as 3 h, and was maximal after 6 h of exposure to TCDD, B[α]P and Aroclor 1254. The estimated 6 h EC 50 for TCDD, B[α]P, and Aroclor 1254 was 1.2 × 10−9, 5.7 × 10−8 and 6.6 × 10−6 M. For the cultured hepatocytes, time-course study showed that a significant induction of EROD took place at 18 h, and the maximal induction of EROD was observed at 24 h after exposure. The estimated 24 h EC50 for TCDD, B[α]P, and Aroclor 1254 was 1.4 × 10−9, 8.1 × 10−8 and 7.3 × 10−6 M. There was no induction or inhibition of EROD in DE71 exposure to both gill epithelia and hepatocytes. The results show that cultured gill epithelia more rapidly induce EROD and are slightly more sensitive than cultured hepatocytes, and could be used as a rapid and sensitive tool for screening chemicals and monitoring environmental AhR agonist toxicants.

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Keywords: Primary cultured cells; Hepatocytes; Gill epithelia; EROD; Toxicity; Tilapia

1. Introduction

The increased use of synthesized chemical compounds in recent decades has brought with it the need to develop rapid, sensitive, reproducible, and cost-effective methods to assess toxicity before production. Environmental contaminants in the aquatic environment have necessitated the development of tools for environmental biomonitoring. Cells are a key level of organization for understanding mechanisms of toxicity. In coping with the new approach to risk assessment for regulatory tests and satisfying the social desire to reduce or replace the use of animals in testing (Castro et al., 2003; for a review see Schirmer, 2006), as alternative models, cultured cells are applied in mechanistic studies and toxicity identification in ecotoxicology. Hence, large numbers of chemicals can be screened quickly in multi-well microplates. This means that chemical screening tests can use few test substances and thus produce little waste. For environmental monitoring, this is particularly important, because extracted pollutants from environmental samples are usually in small amounts. Fish are the dominant vertebrate species for the regulatory evaluation of ecotoxicology, and they are also accorded the same legal protection as model mammals (for a review see, Schirmer, 2006). Fish cells have many functions that are similar to those of mammalian cells, but they also have many advantages over mammalian cells. For example, they can be cultured at room temperature (20–28 °C) and can be directly exposed to environmental samples with varying osmolarities. This means that they can greatly facilitate the collection of more useful in vitro data for toxicity tests. Indeed, over 150 cell lines have been established from fish. The advantages of these cell lines are that they are standardizable, easy to handle with relative low variability, more convenient, and less labor-

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ous. However, the limitations are that they are less differentiated and have lost most of their original genetical/biochemical characteristics. In contrast, primary cultured cells keep most of their original characters and could be used as a bridge between cell lines and in vivo systems. Moreover, primary cultured cells are considered to be more sensitive, with a high metabolic capacity compared with cell lines (Smeets et al., 1999; Chen and Bunce, 2003).

In fish, a wide range of primary cell cultures, including of the liver, gonads, skin epithelia, endocrine tissues, muscle cells, white blood cells, and gill epithelia have been developed. Fish liver represents the major target organ for the metabolism of most chemicals. Cultured hepatocytes have a three-dimensional cellular organization. Primary cultured hepatocytes maintain most of their original differentiated in vivo characteristics, and therefore facilitate the extrapolation of the results in vivo. Fish hepatocytes have been extensively used for ecotoxicological exposure assessment studies (Pesonen and Andersson, 1997; Segner, 1998; Sturm et al., 2001; Navas and Segner, 2000; Smeets et al., 2002; Kuiper et al., 2004; for a review, see Schirmer, 2006). Fish gills are continuously exposed to waterborne toxicants, comprise the first barrier against toxicants that are entering the fish body, and hence are a major organ for the uptake and depuration of many toxicants (Wood, 2001), including organic chemicals (McKim et al., 1985; Randall et al., 1998). They also perform gas exchange, ion transport, acid–base regulation, osmoregulation, nitrogenous waste excretion, and the excretion of metabolites from xenobiotic biotransformation, which explains their high sensitivity to waterborne toxicants relative to mammalian systems (for a review, see Wood, 2001). Pärt et al. (1993) initially developed primary cultured gill epithelia in culture plates from freshwater rainbow trout (Oncorhynchus mykiss). Since then, several gill epithelia of freshwater rainbow trout that have been grown on filter “inserts” have been developed as a surrogate in vitro primary cultured model for fish gills (Wood and Pärt, 1997; Fletcher et al., 2000; Wood et al., 2003). Among these, double-seeded insert (DSI) preparations that incorporate both pavement cells (respiratory cells) and mitochondria-rich cells (MRCs) can form two to four layers of epithelial cells with polarity. This preparation has the advantage of maintaining the electrophysiological and permeability characteristics of the gills. It allows the exposure of the apical surface to water/toxicants while maintaining blood-like culture media on the basolateral surface, thus exposing the apical surface to water/toxicants while maintaining blood-like culture media on the basolateral surface, thus simulating an in vivo “reconstructed gill” and its “asymmetrical” characteristics (Fletcher et al., 2000). This preparation has already been used for testing the presence or absence of inducing CYP1A of AhR agonists (Carlsson et al., 1999; Sandbacka et al., 1999; Carlsson and Pärt, 2001). A cultured gill epithelium model from the seawater-acclimated tilapia has also been developed for testing AhR agonists in seawater (Zhou et al., 2005).

Recently, polybrominated diphenyl ethers (PBDEs) have received significant attention because they are used as additives in flame retardants, and levels that have been detected in biota have doubled every 5 years (for a review see Law et al., 2006). Among the various congeners, BDE-47 is the predominated form in fish, followed by BDE-99, 100, 153, and 154. Due to their structural resemblance to PCBs, PBDEs may act as AhR agonists.

In the study reported herein, we developed cultured gill epithelia and hepatocytes from freshwater tilapia and selected model AhR agonists, such as B[a]P, TCDD, and PCB mixture (Aroclor 1254). The objectives were (1) to compare the sensitivity of cultured gill epithelia and hepatocytes to these AhR agonists, and to evaluate cultured primary cells as a tool for screening toxicants; and (2) to test the capacity to induce EROD with exposure to a PBDE mixture (DE71). DE71 was selected because it contains most of the congeners that are found in the environment and fish: that is, it contains primary tetra-, penta-, and hexa-brominated diphenyl ethers (such as DE-47, 99, 100, 153, 154). The major BDE congeners (percent by weight) in DE71, as identified by Rayne and Ikonomou (2002) include BDE-47 (30.8%), BDE-99 (48.1%), BDE-100 (8.8%), BDE-153 (6.6%), and BDE-154 (4.4%). Meanwhile, Aroclor 1254 contains the high levels of toxic coplanar PCBs and is composed of tetra- (11%), penta- (49%), hexa- (34%), and heptachlorobiphenyls (6%).

2. Materials and methods

2.1. Fish

Freshwater tilapia (O. niloticus) of 60–150 g each were obtained from a local fish farm that was far from any industrial areas. They were kept in 3001 fiberglass tanks with re-circulating water for 3 weeks (22 ± 0.5 °C, 12 light:12 h dark cycles). The recirculating water was UV-sterilized. The fish were fed once every other day with commercial food (J.W. Vitra).

2.2. Cell culture medium and chemicals

Leibiwiotlz L-15 culture medium with L-glutamine, penicillin–streptomycin, trypsin, fungizone, fetal bovine serum (FBS) was obtained from Gibco Life Technology (USA). Benzo[a]pyrene (B[a]P), 7-ethoxyresorufin, resorufin, NADPH, bovine serum albumin (BSA), and Bradford regent were obtained from Sigma. 1,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was obtained from Cambridge Isotope Laboratories (MA, USA). All of the chemicals that were used were of analytical grade. DE71 was obtained from Wellington Laboratories Inc. (Canada). Aroclor 1254 was obtained from Sigma–Aldrich.

2.3. Preparation of cultured branchial epithelia and hepatocytes

For gill epithelial cell culture, 60–80 g of fish was selected. Gill cells were isolated in a laminar flow hood using sterile techniques according to Wood and Pärt (1997). The double-seeded insert (DSI) epithelia were prepared following Kelly et al. (2000) and Zhou et al. (2005). Briefly, the fish were stunned by a blow to the head and then decapitated. Gill cells were excised from gill filaments by two consecutive cycles of tryptic digestion at room temperature (0.05% trypsin in phosphate-buffered saline
nylon mesh. The resulting cell suspension was transferred to softened liver tissue was agitated and filtered through 70-PBS that contained 0.1% collagenase (Sigma) on a shaker. The isolated hepatocytes were more than 90% viability by the trypan blue exclusion method. Cells were counted using a hemocytometer, and those with viability were used for the experiment. The isolated hepatocytes were re-suspended in Leibovitz’s L-15 medium (L-15, Gibco). After 24 h of exposure, the medium was changed and cultured for another 24 h for exposure.

2.4. Electrophysiological measurements

Transepithelial electrical resistance (TER) was monitored with STX-2 chopstick electrodes that were connected to an EVOM epithelial voltohmmeter (Millipore, USA). Corrections for blank TER of vacant inserts in each experimental condition were performed as described by Fletcher et al. (2000). TER was measured at the beginning and end of the experimental period. Reported TER was the mean values of the exposed epithelia (n = 4 inserts).

2.5. Time-course study of TER and EROD activity

Each of the four toxicants was dissolved in dimethylsulfoxide (DMSO) and diluted with filtered (0.2 μm, Millipore) freshwater. Epithelia were prepared from 6 individual fish. The epithelia were rinsed with 2 ml filtered water. 1.5 ml filtered water that contained TCDD, B[a]P, Aroclor 1254 or DE71 (with concentrations of 5 × 10^{-9}, 10^{-7}, 10^{-5}, and 10^{-3} M, respectively) was added to the apical side, and 2 ml of L15 culture medium (with 5% FBS) was added to the basolateral side. All of the cells were incubated at 25°C in air. After exposure, both apical and basolateral sides were rinsed twice with HEPES, then 1 ml reaction buffer was added to both sides. EROD activity was measured at 0, 3, 6, 12, and 24 h, and TER was also monitored.

For hepatocytes, chemicals were dissolved in DMSO and diluted with L15 medium, and 200 μl cultured medium that contained TCDD, B[a]P, Aroclor 1254 or DE71 (with concentrations of 10^{-7}, 10^{-6}, 2 × 10^{-5}, and 2 × 10^{-5} M, respectively) was added to each well. Hepatocytes were prepared from four individual fish. After exposure, the medium was removed and rinsed with HEPES for two times, then 250 μl reaction medium was added to each well and incubated for 30 min. EROD activity was measured at 0, 3, 6, 12, 18, 24, and 36 h. Both of the control groups of pill epithelia and hepatocytes received 0.1% DMSO.

2.6. Concentration-dependent EROD responses in epithelia and hepatocytes

Similar to the above, for gill epithelia, the apical sides were rinsed with 2 ml filtered water. Various concentrations of B[a]P (10^{-8}, 5 × 10^{-8}, 10^{-7}, 5 × 10^{-7}, 10^{-6} and 2 × 10^{-6} M), TCDD (5 × 10^{-10}, 10^{-9}, 5 × 10^{-9}, 10^{-8}, 5 × 10^{-8}, 10^{-7} and 5 × 10^{-7} M), Aroclor 1254 (10^{-7}, 5 × 10^{-7}, 10^{-6}, 5 × 10^{-5}, 10^{-5}, and 10^{-4} M) and DE71 (10^{-7}, 5 × 10^{-7}, 10^{-6}, 5 × 10^{-5}, 10^{-5}, and 10^{-4} M) were prepared and added to the apical sides for 6 h of exposure. L15 culture medium supplement with 5% FBS was maintained on basolateral sides. Apical sides of control epithelia received 0.1% dimethylsulfoxide (DMSO) in water. Cells from individual fish (n = 6) were used for each toxicant exposure. Stock solutions of the three AhR agonists were dissolved in DMSO and diluted with freshwater to achieve the desired concentration.

For hepatocytes, a 200 μl culture medium that contained various concentrations of B[a]P (10^{-8}, 5 × 10^{-8}, 10^{-7}, 2 × 10^{-7}, 4 × 10^{-7}, 10^{-6}, and 2 × 10^{-6} M), TCDD (10^{-10}, 5 × 10^{-10}, 10^{-9}, 8 × 10^{-9}, 5 × 10^{-9}, and 5 × 10^{-7} M), Aroclor 1254 (10^{-7}, 5 × 10^{-7}, 10^{-6}, 5 × 10^{-5}, 2 × 10^{-5}, and 5 × 10^{-4} M) was added to each well for 24 h of exposure. Hepatocytes were prepared from four individual fish for each toxicant exposure.
2.7. Measurement of EROD activity

For cultured gill epithelia, at the end of the exposure period, apical exposure medium and basolateral culture medium were aspirated. A 2 ml 0.1 M HEPES (pH 7.8) was added to both the apical and basolateral sides and then aspirated. The reaction buffer, a 1 ml mixture that contained 1 μM ethoxyresorufin, 1.2 mM NADPH, 5 mM MgSO₄ (pH 7.8), was added to both apical and basolateral compartments, and was incubated for 30 min at 25 °C. Aliquots of 0.2 ml of reaction mixtures from both apical and basolateral compartments were transferred to a 96-well Nunculone plate. Resorufin concentrations in both apical and basolateral compartments were measured by reading concentrations of resorufin that were formed at an excitation of 535 nm and emission of 590 nm with a microplate reader (molecular device, M₂). Total EROD activities were obtained by summing the mean values from both the apical and basolateral compartments. For cultured hepatocytes, at the end of the exposure period, the culture medium was aspirated and a 250 μl 0.1 M HEPES was added to each well and then aspirated. A 200 μl reaction buffer (as described above) was added to each well and incubated for 30 min at 25 °C.

2.8. Protein assay

After the EROD assay, the remaining reaction solution was aspirated and 0.2 ml of 0.3 M NaOH with 0.1% SDS was added to each insert and well. Protein was assayed by the Bradford method, using bovine serum albumin (Sigma) as a standard.

2.9. Statistical analysis

The results are presented as mean ± S.E. One-way analysis of variance (ANOVA) was used to test the null hypothesis that there was no significant difference in responses between the various concentrations of the same toxicant, and also between different times of the same concentration of individual toxicants. Whenever any significant difference was found, pairwise comparisons were made using a Dunnet’s test, to identify differences between mean values. The level of statistical significance for all analyses was set at 0.05. Non-linear regressions were conducted with Graph prism software, and EC₅₀ (defined as the concentration to induce half of the maximal EROD activity) was calculated, in which induction of EROD activity beyond the maximal level attainable was excluded.

3. Results

3.1. Growth pattern of the cultured hepatocytes and time-course change of TER in the cultured gill epithelia after apical water exposure

After 24 h of culturing, 70–80% of the hepatocytes reached confluence. After 48 h of culturing, they fused and adhered to the plate and showed hepatic cell cord structures, which is similar to in vivo morphology (Fig. 1A). For the cultured gill epithelia, after 4–5 days of culturing (Fig. 1B), the TER increased in a sigmoid way and reached a plateau phase and normally around 5000–8000 Ω cm² (data not shown). Upon exposure to apical water, TER increased dramatically within a few minutes, and reached a maximal level of over 12,000–15,000 Ω cm² at about 3 h of exposure. The TER then gradually declined to around 2000–3000 Ω cm² after 24 h of exposure (Fig. 2). However, there was no significant difference in the TER values among the control (DMSO) and toxicant exposure groups (Fig. 2).

3.2. Time-course induction of EROD

For the cultured gill epithelia, a significant induction of EROD was observed as early as 3 h in all exposure groups, and the maximal induction was observed at 6 h of exposure for TCDD, B[α]P, and Aroclor 1254 (6.2 ± 0.66, 5.5 ± 0.32 and 4.8 ± 0.11 pmol min⁻¹ mg⁻¹, respectively). The EROD activity then gradually decreased (Fig. 3A). There was also a significant difference in the EROD activity between the TCDD and Aroclor 1254 exposure groups at 6 and 12 h of exposure. For cultured hepatocytes, EROD was more rapidly induced in B[α]P and Aroclor 1254 at 12 h of exposure and EROD was induced at 18 h exposure for TCDD. However,
a maximal level of EROD activity was induced at 24 h for TCDD, B[a]P, and Aroclor 1254 (106.4 ± 11.6, 55.2 ± 2.5 and 43.5 ± 4.1 pmol min⁻¹ mg⁻¹, respectively), and then remained stable until 36 h (slightly decreased, but not significant different) (Fig. 3B). At 18 h of exposure, TCDD induced greater EROD activity than B[a]P and Aroclor 1254 treated groups. The significant difference in the EROD activity was observed among

Fig. 4. Concentration-dependent EROD induction in gill epithelia exposure to (A) TCDD; (B) B[a]P; (C) Aroclor 1254 for 6 h. Value represents the mean ± S.E. of four replicate inserts from six different fishes. Significance between control and exposure groups are indicated by *P < 0.05 and **P < 0.01.
TCDD, B[α]P and Aroclor 1254 exposure groups after 24 and 36 h of exposure.

3.3. Concentration-dependent EROD responses

The control epithelia showed very low EROD activity (<1 pmol min⁻¹ mg⁻¹), and EROD induction by the toxicants showed a good concentration-dependent response. The EROD activity increased with increases of the toxicant concentration, and reached a maximal activity then decreased with increasingly higher concentrations (Fig. 4). In cultures that were exposed to TCDD, the lowest concentration to induce EROD activity was observed at 10⁻⁷ M with a value of 4.8 ± 0.6 pmol min⁻¹ mg⁻¹, and a maximal EROD activity was observed with 10⁻² M, which was 10 times higher than the control (Fig. 4A, 9.7 ± 0.2 pmol min⁻¹ mg⁻¹). In cultures that were exposed to B[α]P, the lowest concentration to induce EROD activity was 5 × 10⁻⁸ M (2.7 ± 0.2 pmol min⁻¹ mg⁻¹), and a maximal EROD activity was observed with 10⁻⁶ M (Fig. 4B, 7.1 ± 0.5 pmol min⁻¹ mg⁻¹). In cultures that were exposed to Aroclor 1254, the lowest concentration of induction of EROD was at 5 × 10⁻⁷ M (2.24 ± 0.2 pmol min⁻¹ mg⁻¹), and a maximal induction of EROD activity was at 5 × 10⁻⁵ M (Fig. 4C, 5.1 ± 0.1 pmol min⁻¹ mg⁻¹). The estimated EC₅₀ was 1.18 × 10⁻⁹, 5.8 × 10⁻⁸ and 6.6 × 10⁻⁸ M for TCDD, B[α]P and Aroclor 1254, respectively. There was no induction or inhibition of EROD activity with exposure to DE71 (data not shown).

In the hepatocyte cultures, the control hepatocytes showed EROD activity around 7 pmol min⁻¹ mg⁻¹. The EROD induction by toxicants also showed a good dose-dependent response (Fig. 5). In cultures that were exposed to TCDD, the lowest observable effect concentration was at 10⁻⁵ M (70.6 ± 6.2 pmol min⁻¹ mg⁻¹), and a maximal induction was measured at 10⁻³ M (Fig. 5A, 146.9 ± 6.8 pmol min⁻¹ mg⁻¹). A higher concentration (5 × 10⁻⁷ M) inhibited the EROD activity (45.1 ± 6.0 pmol min⁻¹ mg⁻¹). In cultures that were exposed to B[α]P, the lowest observable effect concentration at 10⁻⁷ M (17.6 ± 1.1 pmol min⁻¹ mg⁻¹), and a maximal induction was measured at 10⁻⁴ M (31.9 ± 2.3 pmol min⁻¹ mg⁻¹). However, a slightly decrease in the EROD activity was observed with 2 × 10⁻⁶ M exposure (25.9 ± 2.8 pmol min⁻¹ mg⁻¹). In cultures that were exposed to Aroclor 1254, the lowest observable effect concentration was at 10⁻⁶ M (31.3 ± 1.8 pmol min⁻¹ mg⁻¹), and a maximal induction was measured at 2 × 10⁻⁵ M (Fig. 5C, 44.6 ± 6.4 pmol min⁻¹ mg⁻¹). At higher concentration (5 × 10⁻⁴ M) of Aroclor 1254 exposure, EROD activity decreased (30.2 ± 3.1 pmol min⁻¹ mg⁻¹). The estimated EC₅₀ was 1.3 × 10⁻⁹, 8.1 × 10⁻⁸, 7.3 × 10⁻⁶ M for TCDD, B[α]P, and Aroclor 1254, respectively. There was no induction or inhibition of EROD activity with exposure to DE71 (data not shown). The lowest concentration, the maximal concentration and EC₅₀ to induce EROD are shown in Table 1.

4. Discussion

Without the supplementation of FBS, the cultured hepatocytes could attach and reach 70–80% confluence after 24 h of culturing. The cells were exposed to toxicants after 48 h of culturing, because the hepatocytes fused and adhered to the culture plate, and this could reduce the loss of cells following treatment. For cultured gill epithelia, TER generally reached the plateau phase after 4–5 days of symmetrical culturing. Zhou et al. (2005) showed that TER reached the plateau phase in 5–6 days in cultured gill epithelia from seawater-adapted tilapia. However, the
TER from freshwater tilapia in the plateau phase value was comparable to that in seawater tilapia.

TER is an indicator of the tightness of epithelial cells (Wood et al., 2002). In the present study, the TER value is general around 5000–8000 Ω cm², which is believed to indicate “tight” epithelia. Importantly, TER remained unchanged upon exposure to toxicants among groups, which suggests that the integrity was not affected by toxicants. The rapid increase in TER upon apical side exposure to freshwater may have resulted from the close of the apical channel (Wood et al., 2002). TER declined gradually after long-term exposure (6–24 h), which was also reported by previous studies of freshwater rainbow trout gill epithelia. However, the TER value was around 2000–3000 Ω cm² after 24 h of exposure to water, which indicates that it is tolerant to water exposure and could be used for waterborne toxicant exposure.

The results also showed that EROD induction was more rapid in cultured gill epithelia (3 h) than in hepatocytes (12–18 h) after exposure to toxicants. Moreover, there was a rapidly maximal induction of EROD activity in cultured gill epithelia (6 h) than in hepatocytes (24 h). This result is consistent with earlier in vitro experiments on rainbow trout. Levine and Oris (1999) studied the time-course of CYP1A mRNA induction in rainbow trout gills and liver following exposure to B[a]P, and found that CYP1A mRNA was maximally induced at 6 and 12 h, respectively. Another study showed that EROD was induced after rainbow trout samples were exposed to a low concentration of B[a]P for 6 h, and EROD was induced in liver after 24 h of exposure (Jönsson, 2003). Jönsson et al. (2003) suggested that the slower induction of EROD in the liver than in the gills was possibly due to the slow distribution of toxicants to the liver after uptake in the gill. Carlsson et al. (1999) suggested that fish gills could significantly prevent the accumulation of pollutants through other areas of bodily uptake, such as in the liver. However, in the present in vitro study, EROD induction was more rapid in epithelia than in hepatocytes. Clearly, this is due to toxicants being more efficiently uptaken by the gills than by hepatocytes. McKim et al. (1985) demonstrated that the gill uptake of toxicants was controlled by aqueous diffusion rates rather than gill membrane permeability, and gave their support to the passive diffusion model for the uptake of organics at the gill–water interface. Randall et al. (1998) showed that fish gills are a major site for the uptake of organics, and that the flux rates of most lipid soluble toxicants across the gills are rapid. In the exposure of cultured hepatocytes, toxicants were exposed in cultured medium. Taken together, toxicants may more rapidly or more efficiently uptake in aquatic environments (in water) than in culture medium.

The EROD activity in hepatocytes is much higher than in gill epithelia in vitro, and this is in general agreement with the results of other studies in vivo: that is, the capacity to metabolize toxicants is much lower in the gills than in the liver (Quabius et al., 2002; Mdegela et al., 2006). It is well known that fish liver is a target organ of toxic chemicals and center for metabolism of them. Immunohistochemical studies have provided qualitative information in cell and tissue distribution of CYP1A in teleost fish and showed that the liver is the major organ of CYP1A activity in fish, while weak immunoreactivity present in respiratory cells in fish gill (see review, Sarasquete and Segner, 2000). A study also showed that EROD activity in cultured gill epithelial cells from rainbow trout was between 1.5 and 14% of the activity level observed in isolated hepatocytes depending on the substrates (Leguen et al., 2000). In the cultured gill epithelia from rainbow trout, two major cell types are respiratory cells (85%) and chloride-rich cells (15%) (Fletcher et al., 2000). In cultured gill epithelia from tilapia, the cultures also contain the tow cell types (Zhou et al., 2005). In cultured gill epithelia, the lowest concentrations of the induction of EROD for TCDD, B[a]P, and Aroclor 1254 were, respectively, 10⁻⁷, 10⁻⁹, and 5 × 10⁻⁷ M, while for cultured hepatocytes, the lowest concentrations were 10⁻⁷, 10⁻⁷, and 10⁻⁸ M, which suggests that gill epithelia is slightly more sensitive to B[a]P than hepatocytes, and that the sensitivity of the two cultures to TCDD and Aroclor 1254 is comparable. However, the EC₅₀ values in cultured gill epithelia (1.2 × 10⁻⁹, 5.7 × 10⁻⁸, and 6.6 × 10⁻⁶ M) were slightly more sensitive than in hepatocytes (1.4 × 10⁻⁹, 8.1 × 10⁻⁸, and 7.3 × 10⁻⁸ M). Using cell viability (EC₅₀) as the end point, an early study by Lilius et al. (1995) showed that freshly prepared gill cells in suspension are more sensitive to chemicals than hepatocytes. An in vivo study of rainbow trout that were treated with B[a]P, Jönsson (2003) showed that the induction of EROD in fish gills is more sensitive than in the liver. In spite of the lower role played by branchial cells in the biotransformation of toxicants, the gills are the first point of contact with waterborne toxicants, a major route of uptake for virtually all of them and the key organ for lethal damage for many of them (Castaño et al., 2003). Therefore, fish gill could play an important role in the enzymatic detoxification or prevent xenobiotics to enter the body through the gill. As stated above,

### Table 1

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<th>Hepatocytes</th>
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<td>6.6 × 10⁻⁶</td>
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LOEC: the lowest observed effect concentration; maximal: the highest induced concentration; EC₅₀: the concentration to induce half of the maximal EROD activity.

Concentrations shown in gill epithelia are from four replicate inserts of six individual fish and for hepatocytes are from four replicate wells of four individual fish. ANOVA and Dunnet’s post hoc test used for statistical analysis.
Table 2
Comparison between cultured gill epithelia and hepatocytes from tilapia (Oreochromis niloticus)

<table>
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<td>Induction of EROD</td>
<td>Weak</td>
<td>Strong</td>
</tr>
<tr>
<td>Sensitivity to AhR agonists</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Represent target organ</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

fish gills integrate many other physiological functions contacting with surrounding water (e.g., respiration, ion transport, nitrogenous excretion, acid–base regulation), all these could explain cultured fish gill epithelia are more sensitive to waterborne toxicants than hepatocytes. The differences between the cultured tow systems are shown in Table 2.

As expected, at higher concentration of toxicants, EROD activity goes down (Fig. 3A and B; Fig. 4), this observation is consistent with in vitro studies in primary cultured gill epithelia from freshwater rainbow trout (Carlsson et al., 1999; Carlsson and Pärt, 2001), seawater-adapted tilapia (Zhou et al., 2005), in mammalian cell lines (Hahn et al., 1996), and in vivo studies in fish (Schlezinger and Stegeman, 2001). Schlezinger and Stegeman (2001) suggested that one of the possible causes of EROD suppression by a higher dose of toxicants (such as PCB126) might be the suppression of CYP1A at a post-transcriptional level mediated through a down regulation of the CYP1A protein. Clearly, the measurement of levels of CYP1A protein and mRNA will provide more reliable estimates of AhR agonistic-inducing potency. It should be mentioned that the measured gill epithelia EROD activity in this study is much lower than those in cultured gill epithelia from seawater-adapted tilapia (Zhou et al., 2005). Hylland et al. (1998) studied the natural modulation of hepatic EROD in flounder (Platichthys flesus L.) and found that season contributed significantly to the model for EROD. Jönsson et al. (2003) showed that there was also a significant difference in the EROD activity that was induced by β-naphthoflavone (β-NF) in Atlantic salmon smolts (Salmon salar) that were held in seawater or freshwater. However, the reasons for this difference were unknown.

There was no EROD induction by DE71 in both cultured gill epithelia and hepatocytes in the present study, although a weak EROD induction was reported in BDE-77, 100, 153, and 183 in mammalian cell lines (Chen et al., 2001; Behmisch et al., 2003) and in primary cultured rat hepatocytes (Rattus norvegicus) (Chen et al., 2001; Chen and Bunce, 2003). Our results are consistent with previous experiments in primary cultured carp (Cyprinus carpio) hepatocytes (Kuiper et al., 2004) and monkey (Macaca fascicularis) hepatocytes (Peters et al., 2006). Moreover, Kuiper et al. (2004) found a significant reduction of TCDD-induced EROD activity in the presence of BDE-47, 99, and 153 in cultured carp hepatocytes. They also found in DE71 an even more potent inhibition of TCDD-induced EROD activity. More recently, Peters et al. (2006) showed no significant induction of EROD after exposure to BDE-47, 99, 100, and 153, whereas there was inhibition of TCDD-induced EROD activity in cultured monkey (M. fascicularis) hepatocytes. These results suggest that although the structure of PBDEs resembles that of PCBs, PBDEs are not able to elicit a AhR-mediated pathway in tilapia and previous reports of weak induction by some PBDEs might be due to the presence of other AhR-active compounds in these PBDE preparations (Peters et al., 2004, 2006). Hence, EROD is not suitable as a biomarker for toxicity screening tests and environmental risk assessment for PBDEs.

Recently, Schirmer (2006) proposed that a single culture has a limited number of target sites in comparison to a whole organism, and alone is not able to represent the diversity in the target tissue in whole fish. Higher concentrations of chemicals are required in the cell culture than in the fish to detect a toxic response. However, as stated previously, fish gills are major sites for the uptake of most waterborne toxicants, and fish liver is a major organ for metabolizing toxicants. Hence, cultured gill epithelia and hepatocytes should represent good in vitro models for screening chemical toxicity and for environmental monitoring. Sturm et al. (2001) compared the sensitivity of in vitro hepatocytes and in vivo liver in trout, and found that the in vivo exposure of juvenile trout to 0.27 μM prochloraz resulted in an induction of CYP1A and EROD after 7 and 14 days, whereas 0.027 μM prochloraz had no effects. This indicated that in vivo exposure is more sensitive than in vitro. The sensitivity of cultured gill epithelia and in vivo gill to toxicants may need further investigation.

In summary, following exposure to waterborne AhR agonists, cultured gill epithelial cells more rapidly show an induction in EROD than do hepatocytes. Gill epithelia are slightly more sensitive to AhR agonists than hepatocytes. There was no induction of EROD in both cultured gill epithelia and hepatocytes that were exposed to DE71, which suggests that there is no AhR-mediated pathway. The branchial epithelia are a unique organ that is in direct contact with waterborne toxicants, for which no corresponding mammalian cells are available. Furthermore, Gill cells tolerate various conditions, such as osmoraities and variations in temperature, and non-sterile environmental samples can be exposed, without alteration of their physico-chemical characteristics. They may therefore be suitable for screening wastewaters. Fish cells can be directly exposed to waterborne toxicants at temperatures that are more typical in the environment, as environmental temperature affects the toxicity of toxicants, and thus reflect the situation of fish that are exposed in the environment. In addition, primary cultured gill epithelia in “filter inserts” tolerate not only freshwater but also seawater (Zhou et al., 2005) and thus could be used to test the effects of various environmental factors (such as temperature and salinity) on toxicity. Lilius et al. (1995) reported that the use of freshly isolated cells in suspension is restricted to about 5 h, whereas gill epithelia on filters can tolerate freshwater on the apical surface for as long as 24 h, and can mimic asymmetrical conditions similar to those that occur in vivo. Hence, cultured gill epithelia in filters could
be used to test the toxicity of effluent waters and to screen for chemical toxicity.

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