Science of the Total Environment 705 (2020) 135775

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Screening toxicological effects of different contaminants using hepatic homogenates-based ethoxyresorufin-O-deethylase in vitro

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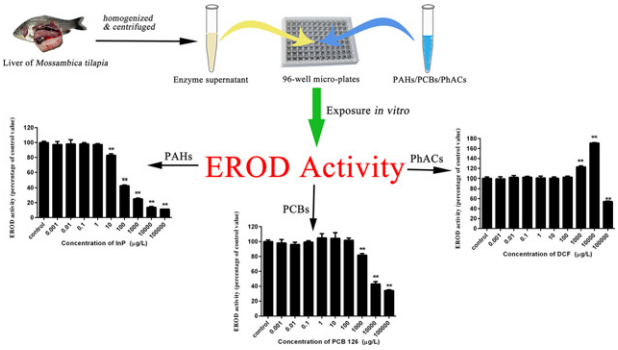
HIGHLIGHTS

- Feasibility of in vitro method of liver homogenate-based EROD was demonstrated.
- PAHs and PCBs inhibited EROD in a dose-dependent manner.
- PAHs showed more inhibition effects as the number of benzene rings increased.
- PhACs exhibited both induction and inhibition effects on EROD activity.
- EROD in vitro could be an effective supplement way of toxicant evaluation

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 5 September 2019
Received in revised form 23 November 2019
Accepted 24 November 2019
Available online 27 November 2019

Editor: Henner Hollert

Keywords:
In vitro
Liver homogenates
Toxicity screening
PhACs
PAHs
PCBs
EROD activity

ABSTRACT

In this paper, we demonstrated the potential of an in vitro method of liver homogenate-based ethoxyresorufin-O-deethylase (EROD) to determine the toxicological effects of multiple kinds of contaminants. We evaluated the in vitro impact of nine pharmaceutically active compounds (PhACs), 13 polycyclic aromatic hydrocarbons (PAHs), and three polychlorinated biphenyls (PCBs). There were different responses of EROD to these contaminants. The response of EROD to PhACs was quite complex, exhibiting both induction and inhibition effects. PAHs and PCBs elicited a strong inhibitory response on EROD activity at high concentrations in a dose-dependent manner. PAHs showed more inhibitory effects as the number of benzene rings increased. Our in vitro bioassay seems to be a potential method for toxicological screening of multiple types of contaminants.

1. Introduction

The environment is burdened by xenobiotic substances released by domestic sewage and industrial effluents. During the past hundred years, a large number of chemicals (e.g., pharmaceutically active

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https://doi.org/10.1016/j.scitotenv.2019.135775
0048-9697/

compounds (PhACs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), metals, etc.) have been introduced into the environment, leading to adverse effects on ecosystems because of their potential toxicity.

PhACs are emergent pollutants that have been recently in the spotlight. PhACs are a group of biologically active chemical substances that include prescription, non-prescription, and over-the-counter therapeutic drugs, and veterinary medicines (Mandaric et al., 2018). PhACs are released into the environment through improper disposal of expired and unused pharmaceutical products, through excretions from humans and animals, through runoffs from agricultural and livestock operations, through effluents and waste from hospitals, through urban communities, and through pharmaceutical manufacturing plants (Praveena et al., 2018). Some PhACs have effects at low concentrations, and might accumulate in organisms and in the environment. The occurrence of these contaminants may pose a serious threat to non-target organisms and to the environment. For instance, there is evidence that some PhACs, such as the anticancer drugs cisplatin, lead to changes in the antioxidant capacity and oxidative stress of organisms, causing DNA damage and neurotoxicity in mussels at a concentration of 100 ng/L; this directly affects health and indirectly affects the food chains and the ecosystems (Trombini et al., 2016).

PAHs and PCBs are two kinds of persistent organic pollutants. PAHs are hydrophobic hydrocarbons containing two or more benzene rings, which come from the incomplete combustion of fossil fuels, garden residues or tree pruning, waste, oil spills, and petroleum-based products; they are carcinogenic and exist ubiquitously in the environment (Kamal et al., 2015). Studies have demonstrated a link between PAHs exposure and mutagenesis, carcinogenesis, immunosuppression, and adverse developmental and reproductive effects (Rengarajan et al., 2015). PCBs are anthropogenic organic chemicals composed of a biphenyl ring connected to chlorine atoms (Zhao et al., 2019). Due to their high stability, PCBs are recalcitrant and distributed globally; they are widely used for industrial applications (e.g., as joint sealant, lubricants, plasticizers, dielectric fluids, etc.) (Vitale et al., 2018). PCBs accumulate in organisms causing teratogenesis, carcinogenesis, and endocrine disruption (Wimmerova et al., 2015).

Environmental pollution has become a serious issue due to the massive release of various contaminants into the environment, and the potential toxic effects of these pollutants should be studied.

EROD activity has been recognized as a specific biomarker of exposure to dioxin-like and other structurally relevant chemicals (Kammann et al., 2005). In vivo EROD assays, as a traditional toxicology approach, have been used to study many chemicals, including PAHs, PCBs, metals, and so on (Whyte et al., 2000). In vivo assays could provide basic toxicological information about contaminants. However, there are some limitations of in vivo approaches, as they are time-consuming and require animals, limiting the number and speed of chemicals being tested, and rise ethical and economic concerns (Stefan et al., 2013). In vivo assays can be affected by many abiotic and biotic elements, such as temperature, species, life stage, reproductive status, etc. (Au and Wu, 2001). Hence, in vitro techniques are proposed as a complement of in vivo studies (Espinosa et al., 2018; Wang et al., 2018).

In vitro approaches provide direct information on the primary pathways impacted by a specific substance, and conduce the priority of contaminants for risk monitoring (Pagé-Larivière et al., 2018). Moreover, in vitro systems allow possible development of high-throughput screening for potential environmental pollutants, and offer reproducible consequences based on a standardized and well-characterized environment (Langan et al., 2018).

In recent decades, cell-based in vitro EROD has been used to predict toxicity and measure a given response by quantifying the induction of the CYP450 monooxygenase system (Behnisch et al., 2002; Billiard et al., 2004; Eichbaum et al., 2014; Tavakoly Sany et al., 2016). In addition, some researchers have used hepatic microsomes to determine the effects of chemicals on EROD activity (Ribalta and Sole, 2014; Sakalli et al., 2018). However, the study of the toxic effects of multiple kinds of chemicals is limited when using enzyme-based EROD assays (Wang et al., 2018).

In this work, our main aim was to demonstrate the feasibility of in vitro EROD for screening the toxicological effects of multiple kinds of contaminants. We used hepatic homogenates of *Mossambica tilapia* as exposure model. The in vitro effects of nine PhACs, 13 PAHs, and three PCBs (the chemical structures and other details are shown in Tables S1–S3, Supplementary material) on EROD activity were explored. EROD activity was detected using a fluorescence plate-reader, which is 10–15 times faster compared to the traditional method of Burke (Burke and Mayer, 1974) and can test numerous samples simultaneously.

2. Materials and methods

2.1. Chemicals and reagents

Ofloxacin (OFX, CAS No. 82419-36-1), erythromycin (EM, CAS No. 114-07-8), trimethoprim (TMP, CAS No. 738-70-5), sulfamethoxazole (SMZ, CAS No. 723-46-6), 4-acetaminophen (AMP, CAS No. 103-90-2), diclofenac (DCF, CAS No. 15307-86-5), ibuprofen (IBF, CAS No. 15687-27-1), carbamazepine (CBZ, CAS No. 298-46-4), propranolol hydrochloride (PR, CAS No. 318-98-9) were purchased from J&K Scientific (Beijing, China). Naphthalene (Nap, CAS No. 91-20-3), acenaphthene (Ace, CAS No. 83-32-9), fluorene (Flu, CAS No. 86-73-7), anthracene (Ant, CAS No. 120-12-7), phenanthrene (Phe, CAS No. 85-01-8), pyrene (Pyr, CAS No. 129-00-0), fluoranthene (FluA, CAS No. 206-44-0), chrysene (Chr, CAS No. 218-01-9), benzo[*k*]fluoranthene (BkF, CAS No. 207-08-9), benzo[*a*]pyrene (BaP, CAS No. 50-32-8), benzo[*b*]fluoranthene (BbF, CAS No. 205-99-2), indeno[1,2,3-*cd*]pyrene (InP, CAS No. 193-39-5), benzo[*g*,*h*,*i*]perylene (BghiP, CAS No. 191-24-2), 3,3′,4,4′-tetrachlorobiphenyl (PCB-77, CAS No. 32598-13-3), 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126, CAS No. 57465-28-8), 2,2′,3,4,4′,5,5′-heptachlorobiphenyl (PCB-180, CAS No. 35065-29-3), and 7-ethoxyresorufin (ERF, CAS No. 5725-91-7) were purchased from Sigma (St. Louis, MO, USA). NADPH and dimethyl sulfoxide (DMSO) were obtained from Solarbio (Beijing, China).

Stock solutions of PAHs, PCBs, and PhACs were prepared in DMSO, and then diluted to 1 ng/L to 100 mg/L. Chemicals and reagents were kept at 4 °C until use.

The concentrations of the pollutants used in this study were based on our preliminary results and on the previous literature. Organisms were exposed to a wide concentration range of contaminants. For instance, total PAH range from 20.09 ± 0.68 to 105.77 ± 42.58 μg/kg, PCB range from 33.19 ± 6.25 to 126.28 ± 7.37 μg/kg, and metals range from 107.83 ± 1.83 to 187.21 ± 2.00 mg/kg in muscle tissue of grass goby fish specimens (*Zosterisessor ophiocephalus*), obtained from the Bizerte lagoon on the north coast of Tunisia (Barhoumi et al., 2014). Concentrations up to 1047.8 μg/kg of total PAHs were reported in the surface sediments of Taihu Lake (Wang et al., 2011). Concentrations of PCBs in fish range from μg/kg to mg/kg levels (Janz et al., 1992). PhACs (analgesic and anti-inflammatory drugs like ibuprofen and naproxen) have been frequently found at μg/L levels in environmental samples (Metcalfe et al., 2003). Although the environmentally relevant concentration of PhACs is low, long-term exposure to PhACs may induce a high concentration of PhACs. For instance the presence of roxithromycin in animals from Baiyangdian Lake has been reported at concentrations up to 1076 μg/kg (dry weight) (Li et al., 2012). Accordingly, scientists explored the toxic effect of contaminants at high concentrations (Germer et al., 2006; Cao et al., 2012). Considering these factors, the concentrations of the tested chemicals was 1 ng/L to 100 mg/L.

2.2. Hepatic homogenates preparation

Juvenile tilapia (*Oreochromis mossambicus*) 7–10 cm long, weighing 10–20 g, were supplied by a local fish farm, in Xiamen, China. Seven days before experiments, fish were acclimatized in tanks containing 60 L of aerated, sand-filtered seawater, with a natural photoperiod. Fish livers were collected, washed in pre-cooled KCl solution (0.15%), weighed, and homogenized with five volumes of pre-cooled phosphate buffer (PBS, pH 7.60) using an automatic homogenizer. These procedures were accomplished in <3 min, to minimize changes in enzyme activity. Homogenates were centrifuged at 12,000 ×*g* at 4 °C for 20 min. The supernatant was collected, divided into aliquots, and maintained at −80 °C until use.

2.3. Exposure to contaminants in vitro

Enzyme solution (10 μL) and PBS (189 μL) were mixed with the test compounds PAHs, PCBs, and PhACs (1 μL at a final concentrations of 1 ng/L–100 mg/L) for 1 h at room temperature (20 °C). DMSO (0.5%) was the solvent control for PAHs, PCBs, and PhACs. PBS (pH 7.60) was the blank control. The changes in enzyme activity after DMSO and blank control treatments were negligible; hence, data on the blank control is not presented. Controls were also pre-treated for 1 h under the same conditions. Each treatment was conducted in triplicate.

2.4. EROD assay

In order to obtain the optimum conditions for determining EROD activity in vitro, four parameters were tested: dose of enzyme supernatant (5–50 μL), concentration of ERF (0.05–1.25 mmol/L) and NADPH (0.05–1.0 mmol/L), and pH of the PBS solution (6.8–8.0). Results showed that EROD activity increased with the dose of enzyme supernatant and the concentration of ERF and NADPH (Figs. S1–S4, Supplementary materials), finally reaching an equilibrium. There was no significant change in the EROD activity at different pH values. The highest EROD activity was observed at 30 μL of enzyme supernatant, 0.5 mmol/L ERF, 0.4 mmol/L NADPH, and pH 7.4. However, under these conditions the speed of the enzymatic reaction was so fast that the experimental error would be high. Moreover, the high concentration of ERF would cause fluorescence values to exceed the detection range of fluorescence. In consideration of economic efficiency, experimental stability, and accuracy, 10 μL enzyme supernatant, 0.4 mmol/L ERF, 0.25 mmol/L NADPH, and pH 7.6 were selected as experimental parameters.

EROD activity was measured according to the plate-reader approach of Eggens (Eggens and Galgani, 1992). Test wells were filled with 200 μL of the pre-incubation solution (enzyme + PBS + tested pollutants, pH 7.60) and 10 μL ERF solution (0.4 mmol/L). Finally, the reaction started by the addition of 40 μL NADPH (0.25 mmol/L). EROD activity was determined by the

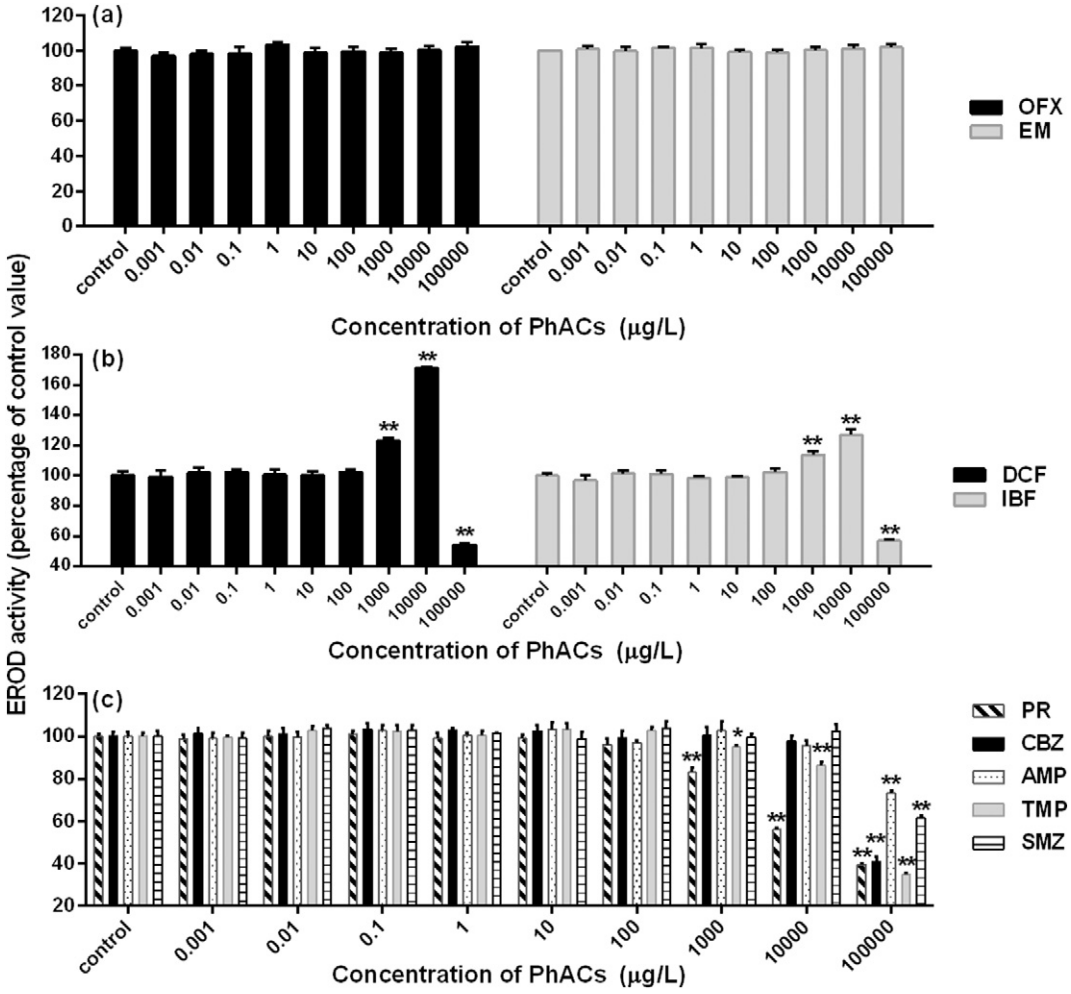

Fig. 1. Effects of 9 PhACs on EROD activity in the liver of *Mossambica tilapia* in vitro. (a: effects of OFX, EM on EROD activity, b: effects of DCF, IBF on EROD activity, c: effects of PR, CBZ, AMP, TMP, SMZ on EROD activity). Symbols (*) and (**) represent statistical significance relative to solvent control (DMSO): $^{*}P < 0.05$ and $^{**}P < 0.01$.

production of fluorescent resorufin; fluorescence was recorded every 20 s for 20 min using a SpectraMax M2/M2e plate-reader (Molecular Devices, San Francisco, CA, USA) at λex = 532 nm and λem = 580 nm. EROD activity was expressed as relative values (experiment groups/control groups), for the purposes of correcting differences in basal activity.

2.5. Statistical analysis

Data were expressed as the mean ± standard deviation. Statistical analysis was conducted by Prism 6 (GraphPad software Inc., La Jolla, CA, USA), and Excel 2016 (Microsoft Inc., Redmond, WA, USA). A one-way ANOVA followed by Dunnett's test was used to evaluate differences

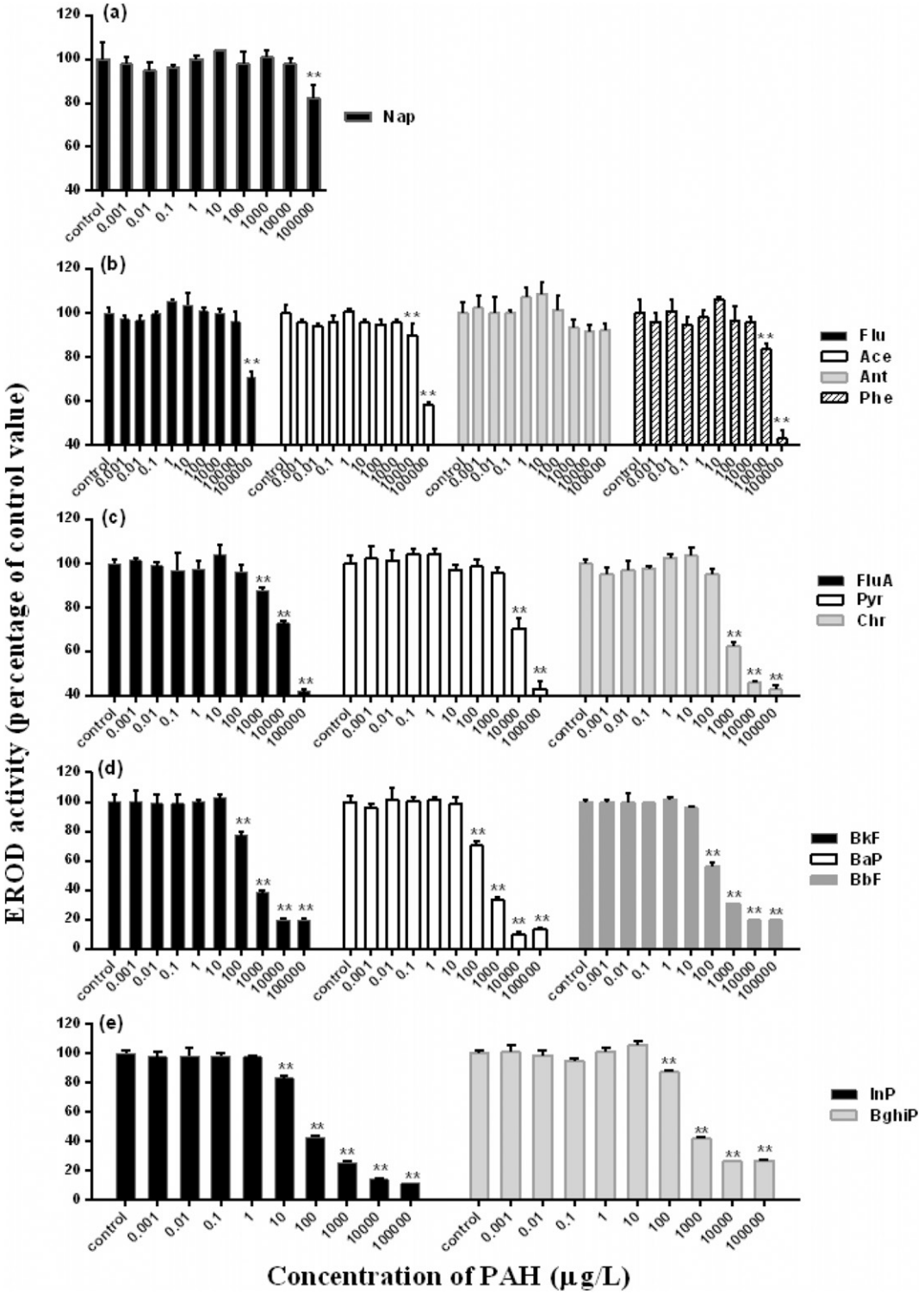

Fig. 2. Effects of 13 PAHs on EROD activity in the liver of *Mossambica tilapia* in vitro. (a: effects of 2 rings PAHs on EROD activity, b: effects of 3 rings PAHs on EROD activity, c: effects of 4 rings PAHs on EROD activity, d: effects of 5 rings PAHs on EROD, e: activity effects of 6 rings PAHs on EROD activity). Symbols (*) and (**) represent statistical significance relative to solvent control (DMSO): $^{*}P < 0.05$ and $^{**}P < 0.01$.

between the control and treatments. All differences were considered significant at $P < 0.05$, and markedly significant at $P < 0.01$.

3. Results

3.1. EROD response to PhACs in vitro

Changes in EROD exposed to 9 PhACs, including OFX, EM, TMP, SMZ, AMP, DCF, IBF, CBZ, and PR are shown in Fig. 1. For DCF and IBF, EROD activity increased and then decreased rapidly. After treatment with DCF or IBF (1–10 mg/L), EROD activity increased markedly. Induction of EROD activity was highest at 10 mg/L (171% for DCF and 127% for IBF, respectively). Following DCF and IBF exposure at 100 mg/L, EROD activity decreased, with maximal inhibition rates of 46% and 43%, respectively. PR, CBZ, AMP, TMP, and SMZ inhibited EROD activity at 1–100 mg/L. The lowest observed effect concentrations (LOEC) of PR, TMP, CBZ, AMP, SMZ was 1, 1, 100, 100,100 mg/L, respectively; with inhibition rates of 17%, 5%, 59%, 27%, and 39%. There were slight changes in EROD activity after OFX and EM treatment, but the changes were not significant ($P > 0.05$).

3.2. EROD response to PAHs in vitro

Changes in EROD activity caused by PAHs containing 2–6 rings are shown in Fig. 2. All the selected PAHs showed inhibitory effects on EROD activity at high concentrations. PAHs containing the same number of rings exerted similar effects on EROD activity, with similar values of LOECs and inhibition rates. LOECs and inhibition rates of PAHs are summarized in Table 1. The LOECs of PAHs with 2–6 rings were 100, 10, 1, 0.1, 0.01 mg/L, respectively. Accordingly, the LOEC of PAHs decreased as the number of rings increased. Besides, the higher the number of rings, the higher the inhibitory effects on EROD activity. For example, the inhibitory effects of PAHs containing 2–6 rings at 10 mg/L were 2% (Nap, 2 ring), 16% (Phe, 3 ring), 54% (Chr, 4 ring), 90% (BaP, 5 ring), and 86% (InP, 6 ring). In addition, for PAHs with 4, 5, and six rings, EROD activity decreased in a dose-dependent manner at 10 μg/L–100 mg/L.

3.3. EROD response to PCBs in vitro

The effects of PCB-77, PCB-126, and PCB-180 on EROD activity are shown in Fig. 3. The response of EROD activity after exposure to PCB-77 or PCB-126 was similar. There was a remarkable inhibition of EROD activity at high concentrations (≧1000 μg/L). A dose-response effect on EROD activity was found from 10 μg/L to 100 mg/L for PCB-77 and PCB-126. And, the lowest concentrations of PCB-77, PCB-126, and PCB-180 that markedly decreased EROD activities were 1, 1, and 100 mg/L, respectively.

4. Discussion

With the increasing number of contaminants released into the environment, more and more compounds require toxicity testing and their environmental risk evaluated. Unfortunately, the in vivo and cell-based in vitro approaches cannot meet the increasing demand as they are time-consuming and/or animal-consuming. In light of the demand for quick and simple toxicity assessment, the hepatic homogenates-based in vitro EROD assay was evaluated in this study to identify the adverse impacts of a wide range of substances.

In this investigation, the in vitro effects of nine PhACs, 13 PAHs, and three PCBs on EROD activity in *Mossambica tilapia* were explored using a liver homogenate model. EROD activity was influenced by nearly all the tested contaminants. As far as we know, there are a few studies about the in vitro effects of toxic substances on fish hepatic EROD activity (Sakalli et al., 2018). Sakalli et al. used rainbow trout hepatic microsomes as in vitro model. Hepatic microsomes were exposed to 4 phytochemicals (including diosmin, naringenin, quercetin and idole-3-carbinol) and two pharmaceutical drugs (clotrimazole and dexamethasone). Naringenin, diosmin and clotrimazole inhibited the EROD activity while quercetin, idole-3-carbinol and dexamethasone did not inhibit the EROD activity (Sakalli et al., 2018). In our lab, hepatic homogenates were also used as an in vitro model to determine the effects of different kinds of pollutants including four metals and four brominated flame retardants (in our published paper, Wang et al., 2018), nine PhACs, 13 PAHs, and three PCBs (in this study) on EROD activity. The results demonstrated that some chemicals inhibited EROD activity while others induced EROD activity. To the best of our knowledge, our current research is the first report summarizing the in vitro responses of EROD activity to multiple classes of pollutants. Our results together with other studies (Sakalli et al., 2018) provide evidence that this enzyme-based in vitro EROD bioassay is a promising tool for toxicology screening, and is a convenient way to evaluate the toxicological potential of contaminants.

In general, in vivo EROD is a common phase I biomarker that reflects CYP1A (cytochrome P450 gene subfamily) induction, often by dioxin-like compounds and other planar aromatic hydrocarbons such as PCBs and PAHs (Santana et al., 2018). Similarly, in vitro EROD responses in cell culture bioassays (such as a rainbow trout liver cell line (RTL-W1) and rat hepatoma cell line H4IIE) showed EROD induction by PCBs and PAHs (Willett et al., 1997; Billiard et al., 2004).

In contrast, our results clearly demonstrate that in vitro EROD activity was inhibited by PAHs and PCBs. Similarly, Previous studies indicate that PAHs, such as benzo[*a*]pyrene, benz[*a*]anthracene, benzo[*b*]fluoranthene, 5-methylchrysene, dibenz[*a*,*c*]anthracene, dibenz[*a*,*h*]anthracene, dibenz[*a*,*j*]acridine, 3-methylcholanthrene (Goddard et al., 1987; Shimada and Guengerich, 2006), and polychlorinated biphenyl compounds (Hahn et al., 1993) could also inhibit EROD activity either in vivo or in vitro.

Binding to the arylhydrocarbon-receptor (AhR) followed by gene expression of EROD-mediating enzymes (CYP) represents the classical mechanism of EROD activity response, especially EROD induction observed either in vivo or cell-based in vitro assays. However, in our study the liver homogenates model was different from the whole fish model or the intact cell model. Therefore, the AhR-related mechanism might not be suitable here. We speculate that the change of EROD activity was possibly due to a direct chemical reaction between the CYP enzyme in liver homogenates and the tested chemicals, not by the biological process of AhR. Similarly, some studies have also indicated that the EROD response may not be mediated by AhR. For instance, the mechanism of EROD inhibition by organotins is probably not related to the AhR or CYP1A specifically, but rather through interference with the reductase components of the microsomal monooxygenase system (Fent and Bucheli, 1994). In addition, the fungicide clotrimazole was found to inhibit EROD activity in gizzard shad (*Dorosoma cepedianum*) by binding to the heme group of CYP1A (Levine et al., 1997). In our study, chemicals may directly interact with EROD enzymes and then change CYP enzyme structure, finally causing EROD activity induction (e.g., DCF and IBF) or inhibition (e.g., PAHs and PCBs). Further studies are necessary to elucidate the corresponding mechanism.

In addition, it is worth to note that the LOEC of PAHs decreased as the number of rings increased in the present study. Accordingly, the in vitro

Table 1
The lowest observed effect concentration (LOEC) and inhibition rates of EROD activity following PAHs exposure.

Number of benzene rings	LOEC (mg/L)	Inhibition rates (for LOEC)	Inhibition rates (for 10 mg/L)
2 (NaP)	100	17.69% (Nap)	2.31% (Nap)
3 (Ace, Flu, Ant, Phe)	10	16.44% (Phe)	16.44% (Phe)
4 (Pyr, FluA, Chr)	1	37.50% (Chr)	54.17% (Chr)
5 (BkF, BaP, BbF)	0.1	43.51% (BbF)	90.07% (BaP)
6 (InP, BghiP)	0.01	16.98% (InP)	86.16% (InP)

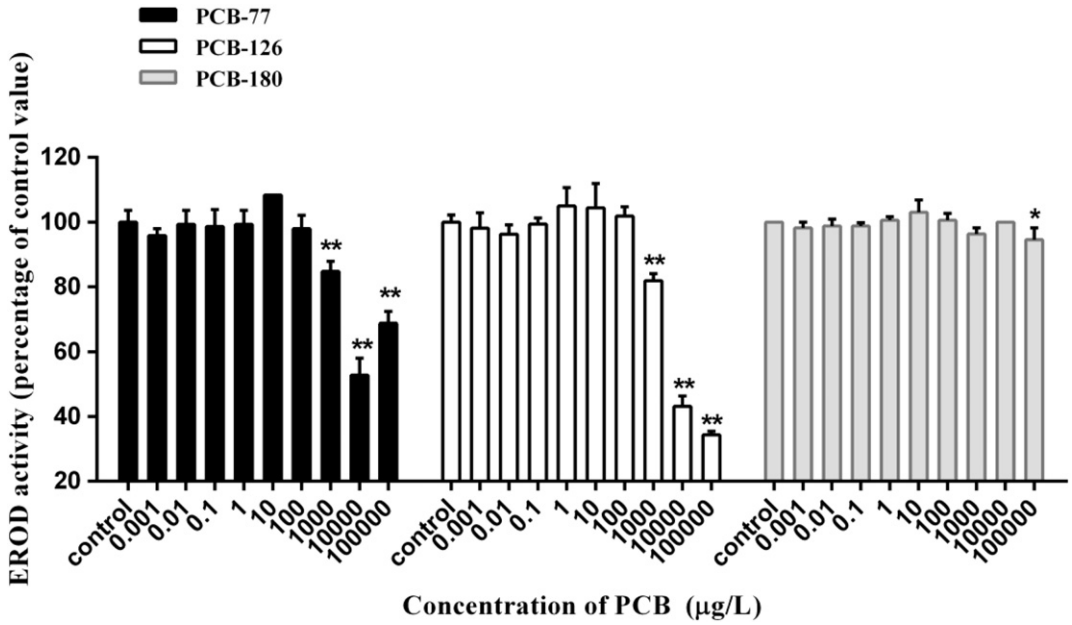

Fig. 3. Effects of 3 PCBs (PCB-77, PCB-126 and PCB-180) on EROD activity in the liver of *Mossambica tilapia* in vitro. Symbols (*) and (**) represent statistical significance relative to solvent control (DMSO): $^{*}P < 0.05$ and $^{**}P < 0.01$.

methods distinguished the impact of PAHs with different numbers of rings on EROD responses, showing that in vitro EROD-based bioassay seems to be a prospective method for toxicity screening of multiple contaminants.

In this study, the response of EROD activity to different PhACs was dissimilar. Similarly, previous studies indicated that clotrimazole and dexamethasone showed different potency to EROD activity. Clotrimazole identified as a potent inhibitor while dexamethasone did not affect EROD activity (Burkina et al., 2013). Burkina et al. (2015) reported that pharmaceuticals can cause decreases in CYP450 activity via either reversible or mechanism-based inhibition (non-receptor mediated mechanisms). Overall, the mechanism affecting EROD activity after exposure to PhACs remains unclear, and requires to be further studied.

5. Conclusions

An in vitro EROD assay based on hepatic homogenates was used to evaluate the toxicological effect of nine PhACs, 13 PAHs, and three PCBs. PAHs and PCBs decreased EROD activity in a dose-dependent manner. The inhibitory effect of PAHs increased with the number of benzene rings. PhACs had induction and inhibitory effects on EROD activity. Our hepatic homogenates-based in vitro EROD assay may be an effective supplement to in vivo or cell-based in vitro assays for the evaluation of toxicants, and risk assessment of chemicals.

Statement of novelty

In the present study, we demonstrated the utility of the heptatic homogenates-based in vitro approach for screening toxic effects of multiple kinds of pollutants, including nine PhACs, 13 PAHs, and three PCBs. The dose-response relationship between tested pollutants and EROD activity was summarized, providing data support that the liver homogenates-based in vitro bioassay could be an effective complementary method for toxicological screening of different contaminants.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Scientific Research Foundation of Third Institute of Oceanography, Ministry of Natural Resources, No. 2016044 and No. HE01-190702(6), and the foundation of Xiamen Haiyang Yanjiu Kaifa Yuan, No. 2018010.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.135775.

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