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Joint effects of crude oil and heavy metals on the gill filament EROD activity of marbled rockfish *Sebastiscus marmoratus*

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ABSTRACT

The aim of this study was to characterize dose- and time-dependent responses of gill 7-ethoxyresorufin *O*-deethylase (EROD) activity from Juvenile marbled rockfish (*Sebastiscus marmoratus*) exposed to the water-accommodated fraction (WAF) of crude oil and heavy metal Cd(II) or Pb(II) alone or in mixture. Compared to the control group, gill filament EROD activity in *S. marmoratus* was significantly induced after exposure to the WAF from 80 to 320 μg/L for 5 days in dose response experiment and after exposure to 40 μg/L WAF for 6–10 days in time course experiment, respectively. In the other hand, gill filament EROD activity were not significantly affected compared to the control group or related WAF groups no matter in the dose response experiment or in the time course experiment of Cd(II), Pb(II) or its mixture with WAF. The results suggest the use of gill filament EROD activity as a biomarker of exposure to waterborne AhR agonists in marine ecosystems while simultaneously being exposed to environmental concentrations of Cd(II) or Pb(II).

1. Introduction

Crude oils are widely prevalent pollutants in the marine environment. They are mainly derived from natural petroleum seeps, marine oil spill, offshore oil production, marine transportation, air transportation, urban pollution and discharge, etc. Crude oils are either chronically or accidentally introduced into many marine areas around the world. They can cause strong-acute and chronic impact on marine ecosystems, including effects from physical damage and biological toxicity of their chemical compounds (Almeda et al., 2013; Alonso-Alvarez et al., 2007; Bellas et al., 2013; Engraff et al., 2011; Fleeger et al., 2003; González et al., 2009; Lee and Lin 2013; NRC, 2003; Wiese and Ryan 2003). Crude oils comprise hundreds of complex gaseous, liquid and solid organic compounds of which hydrocarbons are the most abundant (Kennish, 1992; Stehr et al., 2003; Stentiford et al., 2003; Vethaak et al., 1996). In particular, the presence of polycyclic aromatic hydrocarbons (PAHs) in crude oil is of concern, as they exhibit high chronic toxicity in the marine ecosystem (Achuba and Osakwe, 2003; Gonzalez-Doncel et al., 2008; Kerambrun et al., 2012). PAHs or their metabolites can cause physiological, biochemical and histological changes in fish (Katsumiti et al., 2009; Simonato et al., 2008).

Heavy metals are widespread and persistent in the marine environment and are generally present together with petroleum hydrocarbon in polluted areas (Lemaire-Gony et al., 1995; Muniz et al., 2004; Sprovieri et al., 2007). Numerous aquatic studies of metal–PAH co-toxicity have emerged in the past two decades. However, the question of whether joint toxicity is additive or non-additive has rarely been addressed, the reports describing the mechanisms of metal–PAH co-toxicity are scarce and the nature of co-toxic interactions remains virtually unknown (Fleeger et al., 2007; Gauthier et al., 2014; Gust, 2006; Naddafi et al., 2011; Wang et al., 2011). As such, the limited number of studies investigating the additivity of metal–PAH mixtures have had to avail to coarse endpoints (e.g., mortality) and ultimately have produced ambiguous results with limited mechanistic explanation. Therefore, more data are required on the co-toxic interaction between heavy metals and PAHs.

Induction of cytochrome P450 1A (CYP1A) enzyme activity in fish is routinely used as a biomarker of exposure to Ah-receptor agonists, mainly including PAHs (Goksøyr and Förlin, 1992; Jönsson, 2003; Payne et al., 1987). In fish, a frequently used method for analysis of CYP1A induction is measuring the increase of the hepatic 7-ethoxyresorufin *O*-deethylase (EROD) activity.

Fish gills are highly exposed to waterborne pollutants and an important organ of absorption of pollutants to the body. As in many other organs, CYP1A is also induced in fish gills following exposure to PAHs, and a handy, robust, relatively cheap and sensitive method to determine EROD activity in gill filaments has

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recently been described by Jönsson et al. (2002). After that, the gill EROD assay has been applied in a number of other species (Andersson et al., 2007; Chen et al., 2010; Jönsson et al., 2003; Jönsson et al., 2009; Mdegela et al., 2006; Nahrgang et al., 2010). However, to date, CYP1A activity in gills has received less attention than that in livers (Costa et al., 2011; Nogueira et al., 2011; Oliva et al., 2014) and only a few of papers about the activity in relation with heavy metals were reported (Jönsson et al., 2006; Oliva et al., 2014).

The aim of the present study was to examine the response of the gill filament EROD activity in marbled rockfish *Sebastiscus marmoratus* exposed to water borne crude oil and heavy metal (cadmium or lead) alone or in mixture, respectively. Furthermore, the combined effects of waterborne crude oil and the two heavy metals on gill filament EROD activity were investigated.

2. Materials and methods

2.1. Fish

Juvenile marbled rockfish (*S. marmoratus*) were obtained from Dongshan fish hatchery, Fujian Province, China. All animal procedures were conducted in accordance with the animal care and use guidelines of the China Council on Animal Care (Regulations for the Administration of Affairs Concerning Experimental Animals approved by Decree No. 2 of the State Science and Technology Commission on November 14, 1988). The mean weight and length of fish were 24.92 ± 0.47 g and 11.59 ± 0.07 cm (mean $\pm$ S.E), respectively. The fish were acclimatized in a 3-ton cement tank containing sand-filtrated seawater in the aquarium with a temperature of 20 ± 1 °C, salinity of 28 ± 1‰, pH 8.0 ± 0.1 and a natural daylight cycle and fed with commercial fish bait at 1% of body weight daily for 2 weeks prior to the laboratory exposure.

2.2. Chemicals and materials

The crude oil is from the terminal treatment plant of Weizhou offshore oil field, China. Cadmium chloride, lead (II) nitrate, 7-ethoxyresorufin, and sodium salt of resorufin were purchased from the Sigma-Aldrich, USA; 96-well Fluoronunc plates were from Corning, USA; and all the other chemicals from Sinopharm Chemical Reagent Company, China. All the chemicals used were of analytical grade.

2.3. Water-accommodated fraction (WAF) of crude oil and heavy metals

The crude oil was mixed with seawater (1:10 v/v) in a sealed container with minimum head space for 8 h at 18 °C. The vortex was adjusted to no more than one third of the height of the mixture from the oil–water interface (Singer et al., 2000). The mixture was allowed to settle for 16 h for the separation of water and oil phases and the water phase (namely water-accommodated fraction) was collected for the experiment. The crude oil WAF concentration was measured using fluorescence spectrophotometry following "The specification for marine monitoring - Part 4: Seawater analysis" (AQSIQ, 2007). The excitation wavelength was 310 nm, and the emission wavelength was 365 nm, and a 20–3 oil sample from the National Marine Environmental Monitoring Center was used as a standard. Concentrations of the 16 PAHs compounds were measured by gas chromatography-mass spectrometry (GC–MS) according to Zheng et al. (2000). $CdCl_2$ (Cd (II)) and $Pb(NO_3)_2$ (Pb(II)) were dissolved in ddH_2O. Concentrations of the WAF and heavy metals for exposure experiment were described in Table 1.

Table 1.
Concentrations of xenobiotics used in dose response experiment.

Pollutant type		Experimental concentrations (μg/L)					
WAF		20	40	80	160	320	–
Cd(II)		0.8	4	8	40	80	–
Pb(II)		0.8	4	8	40	80	–
Mixture of WAF and Cd (II)	WAF	40	40	40	80	80	80
	Cd(II)	4	8	40	4	8	40
Mixture of WAF and Pb (II)	WAF	40	40	40	80	80	80
	Pb(II)	4	8	40	4	8	40

2.4. Exposure experiments

Glass tanks (75 cm × 40 cm × 35 cm) were used and kept in the aquarium at 20 °C. Each tank contained 60 l sand-filtrated seawater with the given concentration of xenobiotics alone or in mixture.

For the dose response experiment, groups of *S. marmoratus* ($n=10$) were exposed to different concentrations of xenobiotics for 5 days (Table 1), and a healthy control (blank control) was maintained. The experimental concentrations were chosen with reference to the "Seawater quality standard of China GB 3097–1997" (SEPA, 1997) and the ambient concentrations in seawater (Dai et al., 2009; Gao et al., 2014; Han et al., 2010; Jitar et al., 2015; Liu et al., 2014; Wang et al., 2010).

For the time course experiment, groups of *S. marmoratus* ($n=10$) were exposed to 40 μg/L WAF, 4 μg/L Cd(II), 4 μg/L Pb(II) or the mixture of WAF and Cd(II) or Pb(II) for 2, 4, 6, 8 and 10 days, and a healthy control (blank control) was maintained in the same volume of the sea water.

During the exposure experiments, the water containing different concentrations of xenobiotics was renewed every other day. The fish were fed with commercial fish bait at 1% of body weight daily until the second day before sampling. No in vivo physiological effect or mortality was observed during the experiments.

2.5. Gill filament preparation and EROD activity determination

Sampling was from 08:00 to 11:00 a.m. in order to minimize diurnal variability. The fish were killed by a blow on the head, then weighed and measured. The gill arches were excised and placed in ice-cold HEPES-Cortland (HC) buffer (0.38 g of KCl, 7.74 g of NaCl, 0.23 g of $MgSO_4 \cdot 7H_2O$, 0.23 g of $CaCl_2 \cdot 2H_2O$, 0.41 g of $NaH_2PO_4 \cdot H_2O$, 1.43 g of HEPES, and 1 g of glucose per 1 L of ddH_2O; pH7.8). The method described by Jönsson et al. (2002) for rainbow trout, adopted for *S. marmoratus* by our laboratory (Chen et al., 2010), was used for preparation and determination of the gill filament EROD activity with a small modification. The gill filaments were cut off from the cartilage part of the gill arches. From each fish, about 3 mm tip pieces were carefully selected by comparison with a standard, and duplicate groups of 7 tip pieces were transferred to 1.5 mL centrifuge tube containing 1 mL HC buffer. The HC buffer was replaced with 0.5 mL reaction buffer, consisting of 1 μM 7-ethoxyresorufin and 10 μM dicumarol in HC buffer. The incubation took place under intermittent shaking for 10 min at 35 °C. Subsequently, the reaction buffer was renewed with 0.7 mL and incubation proceeded. After 10 and 30 min of incubation (as above), 0.2 mL samples were transferred from each tube to a 96-well Fluoronunc plate. The fluorescence was determined in a multi-well plate reader (Tecan, Swiss) using the wavelengths 544 (ex) and 590 nm (em). Aliquots of resorufin standard solutions (0–200 nM) were prepared with HC buffer from a 0.1 mM stock solution in methanol. EROD activity was calculated and expressed as femtomole of resorufin per mm filament and minute.