Marine Pollution Bulletin 57 (2008) 524–528

Contents lists available at ScienceDirect

Marine Pollution Bulletin

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

Effects of heavy oil in the developing spotted halibut, *Verasper variegatus*

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ARTICLE INFO

Keywords:
Heavy oil
Spotted halibut
Nervous system
Development

ABSTRACT

It is well known that heavy oil (HO) on the sea surface causes serious problems in the aquatic environment. In particular, some species of teleosts which develop on the sea surface are thought to be affected by the HO which flows out from tankers or coastal industry. However, the toxicological effects of HO are not fully understood. We performed exposure experiments using the Pleuronectiformean fish, spotted halibut (*Verasper variegatus*), which is an important fishery resource in Japan. In course of the development, HO-exposed embryos showed remarkable delay in developmental processes including somite formation. We further observed abnormal development of the head morphology. Notably, treated embryos had relatively small eyes and craniofacial structures. These findings strongly suggest that HO seriously affects the cell proliferation and differentiation of the embryo. In addition, HO-exposed embryos showed abnormal neuronal development. We also performed the exposure in the larval stage. Treatment of post-hatching larvae with HO resulted in significantly greater mortality compared with controls. Through these observations, we finally conclude that HO is strongly toxic to halibut in their early life stages.

1. Introduction

Oil spills associated with marine transport such as tanker accidents and offshore production facilities are the main route of bulk crude oil release into the open sea (Ramachandran et al., 2006). Approximately 5 million tons of crude oil from a variety of sources enters the marine environment each year (Neff, 1990; Kennedy and Farrell, 2005). Crude oil consists of different components, including various hydrocarbons, nitrogen–oxygen compounds and heavy metals. Their solubility depends on their octanol–water partition coefficients (K_{ow}) (Baršienė et al., 2006; Wake, 2005; Ramachandran et al., 2006). Among these components, polycyclic aromatic hydrocarbons (PAHs) rank as relatively soluble, and are more soluble than alkanes having an equal number of carbon atoms (McAuliffe, 1987; Ramachandran et al., 2006). PAHs are known to produce myriad lethal and sublethal effects in a wide range of marine biota (Kennedy and Farrell, 2005).

The acute toxicity of oil and its components have been well documented for several teleost species (Anderson et al., 1974; Rice et al., 1987), and have been shown to cause morphological and histopathological changes and genetic damage in larval and juvenile fish (Brown et al., 1996; Carls, 1987; Carls et al., 1999; Heintz et al., 1999; Hose et al., 1996; Kocan et al., 1996; McGurk and Brown, 1996; Norcross et al., 1996). Exposure of adult fish to parts per million (ppm) concentrations of crude oil has been reported to elevate adrenaline, noradrenaline and cortisol concentrations in plasma (Thomas and Rice, 1987; Alkindi et al., 1996). Thus, oil pollution can be serious stressor for fish, and the oil also affect to fish hematological and immune system (Alkindi et al., 1996). The effect of crude oil on the developing fish has been studied and known that fish eggs and larvae are sensitive to oil spil (Carls et al., 1999; Couillard, 2002; Colavecchia et al., 2004; Heintz et al., 1999; Pollino and Holdway, 2002; Kazlauskiene et al., 2004). However, detailed morphological defects, especially in the developing nervous system, have not been fully understood. In this study, we studied the effect of HO in developing spotted halibut, which is one of the most important cultured flatfish species in Japan. Furthermore, to clarify the effect of HO, we further studied nervous system defects using immunohistochemistry.

2. Materials and methods

2.1. Spotted halibut (Verasper variegatus) embryos

The eggs were artificially fertilized in Ehime Prefecture Fish Experiment Station. Eggs were collected within 1 h after spawning, placed in an aerated seawater tank, and cultured in the sea water at 12 °C. Floating eggs were then collected and used for experiments.

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0025-326X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marpolbul.2008.02.043

2.2. Heavy oil (HO) exposure

Heavy oil (HO; Bunker C) was obtained from an oil company and used for exposure experiments. Experimental treatments comprised 8 L of seawater in 13 L tanks supplied with filtered seawater with continuous aeration at 12 °C. HO was added at graded concentrations (1, 9, 50 mg/L) in each tanks and no exposure was added in a control tank. To study the toxic effect in early developmental stages, embryos at 20 h post-fertilization (hpf) were exposed. Twenty embryos were used for individual exposures, and two independent experiments were performed. To study the effect in the post-hatching larva, larvae at 5 days post-hatching (dph) were exposed in the same condition of the early-stage embryos, and three independent experiments were performed.

2.3. Whole-mount immunostaining

For whole-mount immunostaining, embryos were prepared as described previously (Kuratani and Eichele, 1993) with minor modifications. After fixation with PFA/PBS at 4 °C for 1 day, embryos were dehydrated in a graded methanol series (50%, 70%, 90%, 100%), and stored at −20 °C. The samples to be stained were treated with H_2O_2/methanol (10:1) overnight. After washing in TBST/DMSO (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 5% dimethyl sulfoxide), the samples were sequentially blocked with 5% skim milk in TBST/DMSO (TSTM).

For whole-mount immunostaining of the cranial nerves, embryos were incubated in a monoclonal antibody raised against acetylated tubulin (T-6793, Sigma, St. Louis, MO; diluted 1/1000 in TSTM containing 0.01% sodium azide) for 2–4 days at room temperature. After washing with TBST/DMSO, embryos were incubated in the second antibody (horseradish peroxidase [HRP]-conjugated goat anti-mouse IgG [ZYMED Lab. Inc., San Francisco, CA] diluted 1/200 in TSTM). After a final wash in TBST/DMSO, the embryos were pre-incubated with the peroxidase substrate 3,3′-diaminobenzidine (DAB, 100 mg/mL) in TBST for 30 min and allowed to react in TBST with the same concentration of DAB with 0.01% H_2O_2 for 20 min at room temperature, then the reaction was stopped by TBST.

Table 1
Somite numbers in control and HO-exposed embryos

	Control	1 mg/L HO	9 mg/L HO	50 mg/L HO
Number of somites	10–13 (n = 5)	10–11 (n = 3)	8–9 (n = 4)	6–8 (n = 7)
Average number	11.4	10.7	8.5	6.7

3. Results and discussion

3.1. Early development of the spotted halibut

The artificial fertilization was performed twice, and no significant temporal differences was observed. At 20 hpf, immediately after HO treatment, cell proliferation occurred in the animal pole

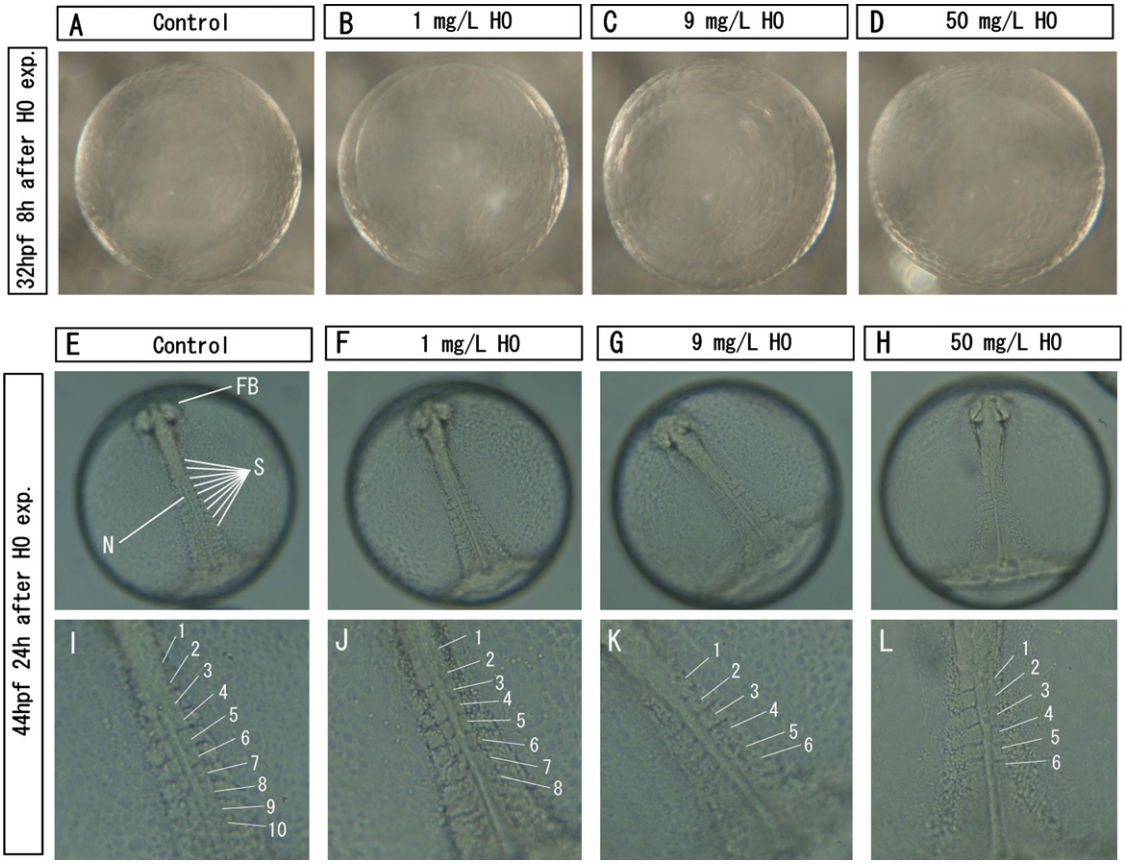

Fig. 1. Early development of the spotted halibut. (A) Control embryo at 32 hpf. In this stage, epiboly movement was almost complete. (B–D) Embryos at 32 hpf after 8 h of HO exposure in the concentrations of 1 mg/L (B), 9 mg/L (C) and 50 mg/L (D). No differences were observed in the HO-exposed embryos. (E) Control embryo in 44 hpf embryogenesis had begun. The forebrain vesicle (FB) had already appeared in the anterior end, and the somites (S) were clearly seen on both sides of the notochord (N). (F–H) Embryos at 44 hpf after 24 h of HO exposure in the concentrations of 1 mg/L (F), 9 mg/L (G) and 50 mg/L (H). (I–L) Higher magnification view of the embryos corresponding to (E–H). (I) Control embryo. (J–L). Embryos after 24 h of HO exposure at the concentrations of 1 mg/L (J), 9 mg/L (K) and 50 mg/L (L). The number of somites is shown in the right side of the embryos. The somite number was reduced and six somites could be identified in the highest-dose treatment.