VOLUME 9 ISSUE 1 2023

ISSN 2454 – 3055

INTERNATIONAL JOURNAL OF ZOOLOGICAL INVESTIGATIONS

Forum for Biological and Environmental Sciences

Published by Saran Publications, India

International Journal of Zoological Investigations Vol. 9, No. 1, 1-7 (2023)

International Journal of Zoological Investigations

Contents available at Journals Home Page: www.ijzi.net

Editor-in-Chief: Prof. Ajai Kumar Srivastav

Published by: Saran Publications, Gorakhpur, India

ISSN: 2454-3055

Fluorene is Highly Toxic to Zoea Larvae of the Red-Clawed Crab *Chiromantes haematocheir*

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Received: 14th December, 2022; **Accepted:** 1st January, 2023; **Published online:** 6th January, 2023

https://doi.org/10.33745/ijzi.2023.v09i01.001

Abstract: In order to examine the toxicity of polycyclic aromatic hydrocarbons (PAHs) on marine crustaceans, we investigated the toxic influence of sixteen typical PAHs [as determined by the United States Environmental Protection Agency (US EPA)] on the zoea larvae (stage 1) of the red-clawed crab *Chiromantes haematocheir*. The 12 and 24 h survival rates for 16 PAHs (each 10^{-5} M) showed that fluorene was the most toxic, with survival rates of 78.3% and 6.7% for 12 and 24 h exposures, respectively. Fluorene was more toxic to zoea larvae than benzo[*a*]pyrene, which is known to be a toxic substance, with survival rates of 98.3% and 90% for 12 and 24 h exposures to benzo[*a*]pyrene (10^{-5} M), respectively. The 24 h exposure to acenaphthene and pyrene resulted in a survival rate of 98.3%. In the other 12 PAHs except for fluorene, benzo[*a*]pyrene, acenaphthene, and pyrene, however, survival was unaffected, at least in the present conditions. EC_{50} for 24 and 48 h after fluorene exposure were 8.96×10^{-6} M and 7.05×10^{-6} M, respectively. Until now, toxic evaluations have been focused on only a few PAHs that are well known to be highly toxic, such as benzo[*a*]pyrene. In the present study, for the first time, fluorene was found to be the most toxic PAH on zoea larvae of the red-clawed crab among 16 PAHs as decided upon by the US EPA. In the future, the toxicity of this compound to marine animals, including crabs, will be studied in detail.

Keywords: Polycyclic aromatic hydrocarbons, Fluorene, Zoea larva, Red-clawed crab, Toxicity

Citation: Kawamura Ryoya, Rafiuddin Ahya Muhammad, Toyota Kenji, Honda Masato, Amornsakun Thumronk, Tabuchi Yoshiaki, Hattori Atsuhiko, Harumi Tatsuo, Hirayama Jun, Urata Makoto, Matsumoto Kyoko, Nishimoto Sogo, Srivastav Ajai K., Suzuki Nobuo and Matsubara Hajime: Fluorene is highly toxic to zoea larvae of the red-clawed crab *Chiromantes haematocheir.* Intern. J. Zool. Invest. 9(1): 1-7, 2023.

https://doi.org/10.33745/ijzi.2023.v09i01.001

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds derived from fossil fuels and incomplete combustion; they are widely distributed in the air, water, and soil (Honda and Suzuki, 2020). In aquatic environments, oil spills were well correlated with the major shipping routes (Brekke and Solberg, 2005) and directly cause PAH pollution that affects marine organisms (Peterson, 2003). Furthermore, accidental oil spills from the Deepwater Horizon and sunken oil tanker ships such as the Exxon Valdez and the Nakhodka caused PAH pollution in the sea area and seriously impact the marine environment (Bue *et al*., 1998; Heintz *et al*., 2000; Hayakawa *et al*., 2006; de Soysa *et al*., 2012). Therefore, there is a need to conduct studies to assess the toxicity of PAHs to marine organisms.

In the present study, we focused on red-clawed crabs as a marine organism. The red-clawed crab *Chiromantes haematocheir* (De Haan, 1833) (Fig. 1A) is a crustacean belonging to the decapod family Crassulaceae that inhabits coasts and forests from the mainland to the southwest Islands of Japan (Nakayama and Yanai, 2020). Adults of the red-clawed crab live in forests, but they have a unique life history in which they temporarily live in the sea during the growth process from the zoea and megalopa stages until they become juvenile crabs (Saigusa and Hidaka, 1978; Murayama *et al*., 2019). Notably, a single release of one female can yield approximately 20,000–30,000 zoea larvae. Therefore, it is possible to conduct experiments using these zoea larvae under various conditions at one time.

On the other hand, toxic evaluations have been focused on only a few PAHs that are well known to be highly toxic, such as benzo[*a*]pyrene (Bihari and Fafandel, 2004; Wen and Pan, 2016; Yu *et al*., 2018). It is likely that several PAHs are more toxic to aquatic animals than benzo[*a*]pyrene. For the purpose of studying the toxicity of PAHs on marine crustaceans, we examined the toxic influence of 16 typical PAHs [as determined by the United States Environmental Protection Agency (US EPA)] on the zoea larvae of the red-clawed crab *C. haematocheir*.

Materials and Methods

Animals:

We collected female red-clawed crabs *C. haematocheir* that laid eggs on the coast of Tsukumo Bay, Noto Peninsula, Japan, from August to September, 2022. Crabs with eggs were placed in a bucket of natural seawater in the evening and left overnight. The next morning, zoea larvae (stage 1) (Fig. 1B) released into the bucket were collected and used in the toxicological experiment.

Survival rate (%) of zoea larvae at 12 and 24 hours after each exposure to 16 typical PAHs:

We used 16 typical PAHs: naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, chrysene, benz[*a*]anthracene, benzo[*b*] fluoranthene, and dibenzo[*a,h*]anthracene (Tokyo

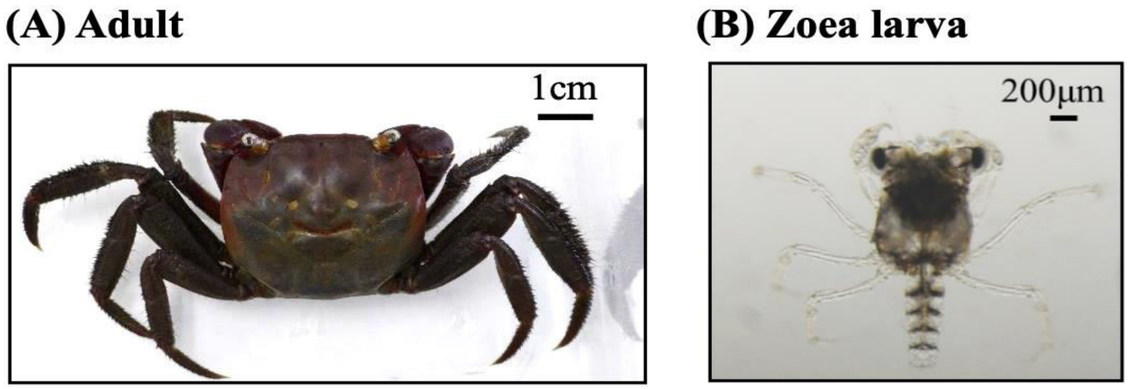


Fig. 1: Red-clawed crab *Chiromantes haematocheir* (A: adult; B: zoea larva, stage 1) collected around Tsukumo Bay, Noto Peninsula, Japan.

Chemical Industry Co., Tokyo, Japan); benzo[*k*]fluoranthene and indeno[*1,2,3-c,d*]pyrene (Toronto Research Chemicals, Inc., Toronto, ON, Canada); benzo[*a*]pyrene (Sigma-Aldrich Co., St. Louis, MO, USA); and benzo[*g,h,i*]perylene (Fluorochem Ltd., Derbyshire, UK). These PAHs were first diluted with dimethyl sulfoxide (DMSO) at a concentration of 10^{-2} M and then with seawater at a concentration of 10^{-5} M. The toxicity of each PAH on the survival rate (%) of zoea larvae was evaluated. Twenty zoea larvae were placed into each well of a 6-well plate to determine their survival 12 and 24 h after exposure. Twenty zoea larvae were used for each PAH treatment. These zoea were incubated at 25°C and under light and dark cycles (12 h:12 h).

Survival rate of zoea larvae for the most toxic substance:

The survival rates (%) of zoea larvae after exposure for 12, 24, 36, and 48 h to a range of concentrations from 10^{-8} to 10^{-5} M were examined using the most toxic PAH for zoea larvae among 16 PAHs. Twenty zoea larvae were also used in each concentration and incubated in the same conditions as described above. The experiment was conducted twice to confirm reproducibility.

Statistical analysis:

A survival analysis was performed to compare the survival curves among the tested concentrations of PAH for each experiment with a Kaplan–Meier method and a log-rank test stratified by the brood identity using Statcel 4 (Yanai, 2015).

Results

Survival rate (%) of zoea larvae at 12 and 24 hours after each of 16 typical PAH exposures:

The results are indicated in Table 1. Fluorene (Fig. 2) was the most toxic PAH among the 16 PAHs as decided upon by the US EPA. The survival rates 12 and 24 h after exposure were 78.3% and 6.7%, respectively. In the case of benzo[*a*]pyrene, survival rates were 98.3% and 90%, respectively. The 24 h exposure to acenaphthene and pyrene resulted in a survival rate of 98.3%. In the other 12 PAHs except for fluorene, benzo[*a*]pyrene, acenaphthene, and pyrene, however, survival was unaffected, at least in the present conditions.

Survival curve and EC_{50}s for fluorene:

The survival curve of zoea larvae after 12, 24, 36, and 48 h exposures are indicated in Figure 3. The results showed a decrease in the survival of zoea larvae only at concentrations of 10^{-5} and 10^{-4} M. Survival curves were very consistent between experiment 1 (Fig 3A) and experiment 2 (Fig. 3B). Significant differences in larval survival curves were detected by a log-rank test among the tested concentrations for both experiments (experiment 1, $\chi^2 = 92.7$; experiment 2, $\chi^2 = 115.8$; df = 5 and $p < 0.001$).

On the basis of above experiments, we calculated the half maximal (50%) effective

Table 1: Survival rate (%) of zoea larvae at 12 and 24 h after each PAH exposure

	12 h	24 h
Control	100	100
Naphthalene	100	100
Acenaphthylene	100	100
Acenaphthene	100	98.3
Fluorene	78.3	6.7
Anthracene	100	100
Phenanthrene	100	100
Fluoranthene	100	100
Pyrene	100	98.3
Chrysene	100	100
Benz[*a*]anthracene	100	100
Benzo[*b*] fluoranthene	100	100
Benzo[*k*]fluoranthene	100	100
Benzo[*a*]pyrene	98.3	90
Dibenzo[*a,h*]anthracene	100	100
Indeno[*1,2,3-c,d*]pyrene	100	100
Benzo[*g,h,i*]perylene	100	100

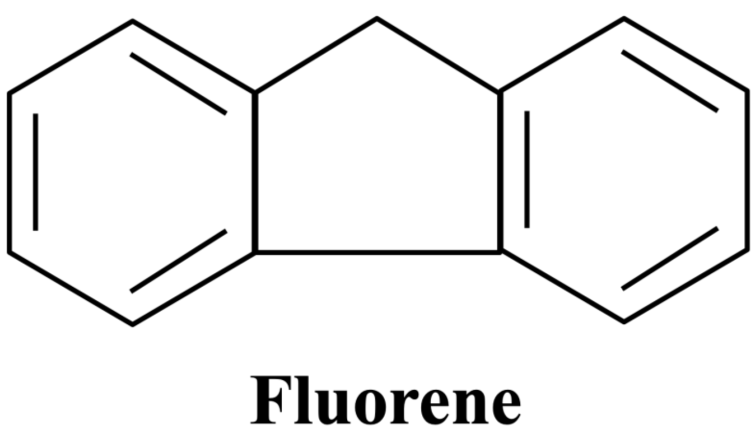


Fig. 2: Chemical structure of fluorene. Formula: $(C_6H_4)_2CH_2$; Molecular weight: 166.22.

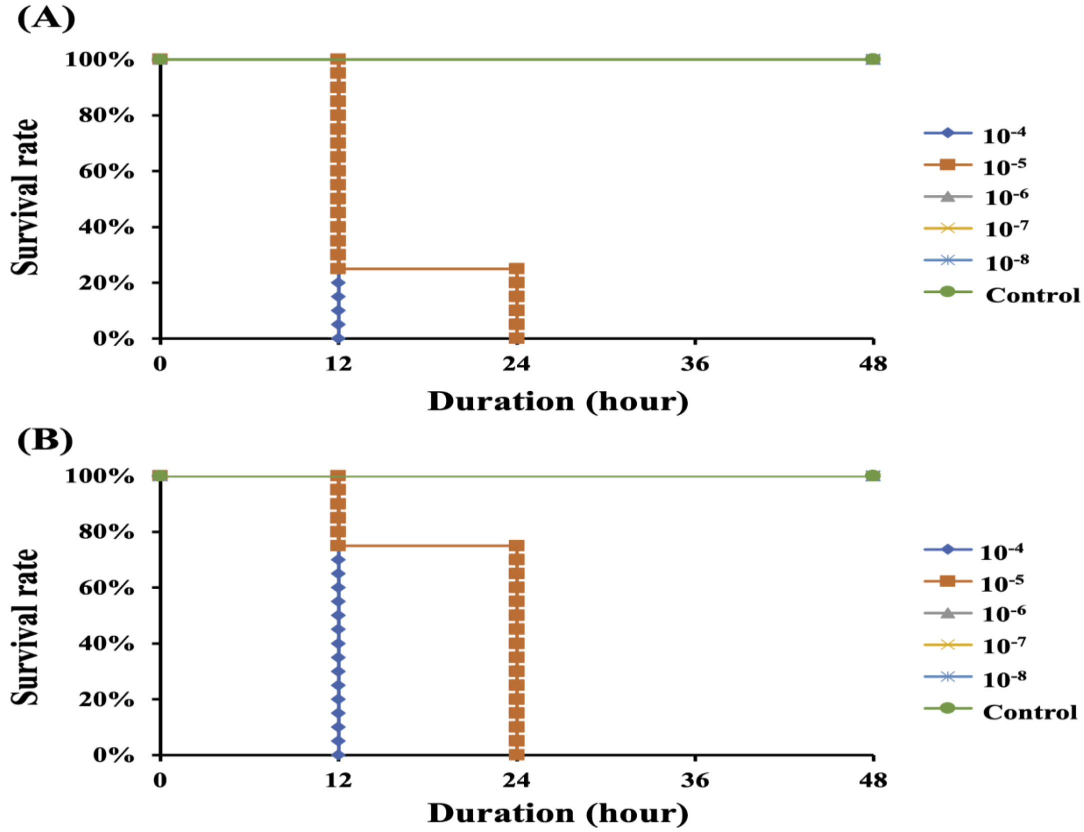
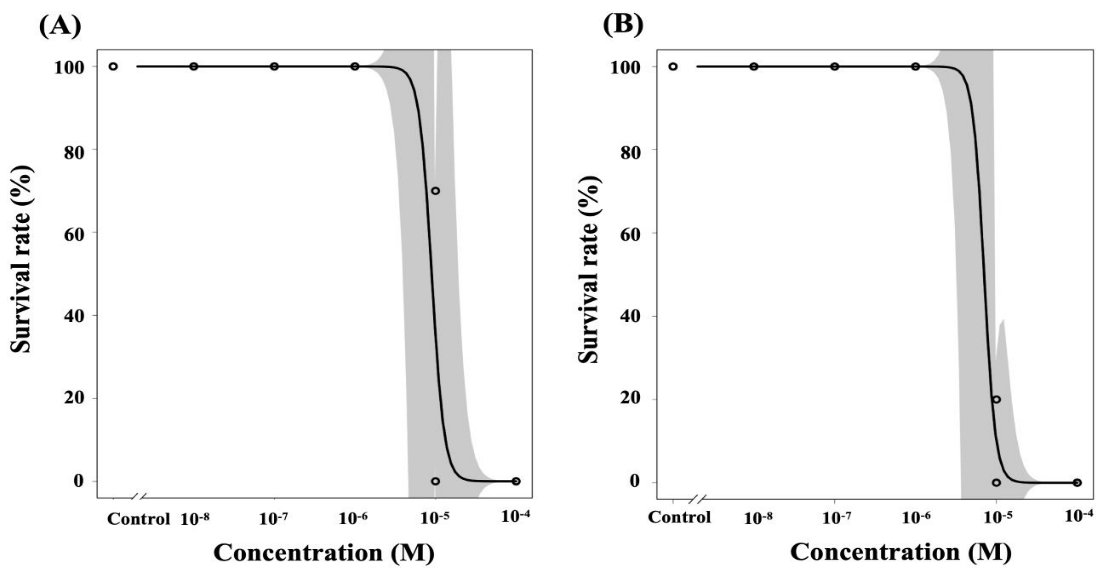


Fig. 3: Changes in the survival rate of zoea larvae (stage 1) for 48 hours after treatment with fluorene at five different concentrations.



(C)

Time point	EC_{50} (M)	Lower (95% CI)	Upper (95% CI)
24 h	8.96×10^{-6}	8.08×10^{-7}	1.87×10^{-5}
48 h	7.05×10^{-6}	-1.72×10^{-5}	3.13×10^{-5}

Fig. 4: Regression curve of the survival rate at 24 hours (A) and 48 hours (B) in response to fluorene treatments on zoea larvae (stage 1). Gray shading indicates 95% confidence intervals (CI). EC_{50} and 95% CI values are shown in (C).

concentration (EC_{50}) for fluorene. The EC_{50} for fluorene at 24 and 48 h were 8.96 x 10^{-6} M and 7.05 x 10^{-6} M, respectively (Fig. 4).

Discussion

In crustaceans as well as other animals, toxic evaluations have been focused on only a few PAHs that are well known to be highly toxic, such as benzo[*a*]pyrene. The toxicity of benzo[*a*]pyrene was reported in the spiny crab *Maja crispata* (Bihari and Fafandel, 2004), the swimming crab *Portunus trituberculatus* (Wen and Pan, 2016), and the Chinese mitten crab *Eriocheir sinensis* (Yu *et al*., 2018). However, fluorene was more toxic to zoea larvae than benzo[*a*]pyrene, which is known to be a toxic substance. Survival rates for 12 and 24 h exposures to benzo[*a*]pyrene (10^{-5} M) were 98.3% and 90%, respectively, while fluorene had survival rates of 78.3% and 6.7% for 12 and 24 h exposures, respectively. In the present study, for the first time, fluorene was found to be the most toxic PAH (EC_{50}: 8.96 x 10^{-6} M at 24 h, 7.05 x 10^{-6} M at 48 h) for zoea larvae of the red-clawed crab *C. haematocheir* among 16 PAHs as decided upon by the US EPA.

Red-clawed crabs are released daily from July to September in Tsukumo Bay, Ishikawa Prefecture, Japan (Murayama *et al*., 2019). Therefore, it is very easy to obtain zoea larvae of red-clawed crabs in the summer season. Furthermore, toxic effects were observed in zoea larvae after 12 h of exposure to fluorene and benzo[*a*]pyrene. Therefore, the bioassay using zoea larvae of the red-clawed crab was an excellent assay and can be used to comprehensively examine the effects of other environmental contaminants in addition to PAHs. For example, we are focusing on styrene trimers, which are compounds derived from plastics (Saido *et al*., 2014; Kwon *et al*., 2017, 2018). Large quantities of plastic-derived styrene trimers (STs) (0.35 to 6.97 μg/l) (mean: 3.26 μg/l) were detected along the coast of Japan (Amamiya *et al*., 2020). Similar concentrations of a ST were detected in other oceans of the world (Kwon *et al*., 2017, 2018). Recently, we reported that STs had a toxicity for nibbler fish *Girella punctata* (marine teleost) (Kawago *et al*., 2021). This environmental pollutant derived from plastics has potent toxicity for zoea larvae of the red-clawed crab.

In grass shrimp *Palaemonetes pugio*, the toxicity of fluorene has been reported at 48 h of exposure (Unger *et al*., 2008). The Lethal Concentration 50 (95% fiducial limits) of naphthalene and fluorene for *P. pugio* were 2,111 mg/l (2,057–2,161 mg/l) and 616 mg/l (593–637 mg/l), respectively. In the shrimp as well as the crab, fluorene was more toxic than naphthalene. Few studies have examined the toxicity of fluorene in marine animals. Fluorene may be highly toxic to marine animals and influence larval growth. In the future, we will examine the toxicity of this compound to marine animals, including crabs, in detail.

Conclusion

We investigated the toxic influence of 16 typical PAHs [as determined by the US EPA] on the zoea larvae of the red-clawed crab *C. haematocheir*. Fluorene was more toxic to zoea larvae than benzo[*a*]pyrene, which is known to be a toxic substance. The survival rates at 12 and 24 h after exposure to fluorene were 78.3% and 6.7%, respectively. The EC_{50} for fluorene at 24 and 48 h were 8.96 x 10^{-6} M and 7.05 x 10^{-6} M, respectively. In the future, we are planning to examine the toxic influence of this compound on the larval growth of marine animals, including crabs, in detail.

Acknowledgements

This study was supported in part by grants to N.S. (Grant-in-Aid for Scientific Research [C] No. 20K06718 by JSPS) and to H.M. (Grant-in-Aid for Scientific Research [C] No. 21K05725 by JSPS). This work was partly supported by the cooperative research program of the Institute of Nature and Environmental Technology, Kanazawa University, Accept Nos. 22009, 22010, 22015, 22017, 22021, 22044, and 22051.

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