

# Relationship of Genetics and Cs-137 in Asian Green Mussel (*Perna viridis*) from Nuclear Activities in Asia-Pacific Region

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## ABSTRACT

This study focuses on the relationship of genetics and Cs-137 radiation doses in Asian green mussel (*Perna viridis*) collected from Chonburi province, Thailand. They might accumulate the radiocaesium from the nuclear power plants in the Asia-Pacific region including the Fukushima-Daiichi nuclear power plant via their routine or accidental releases. The radiation doses, estimated using ERICA Tool in the bivalves categorized into 3 different size classes including 4-6, 6-8, and 8-10 cm, were below 0.02  $\mu\text{Gy/h}$ . In parallel, Micronucleus test and Comet assay were used to investigate genetic responses in the mussels. They revealed minimum micronucleus frequency (MNF) and %Tail DNA varying from 1.80-2.90‰ and 1.36-1.70%, respectively. Our result indicates that neither particular accumulation of Cs-137 nor genetic responses among different size classes of the animals were observed. Furthermore, the radiation doses in the mussels were below the dose limit of 10  $\mu\text{Gy/h}$ . Therefore, no radiation effect caused by Cs-137 was found and it was also confirmed by minimal genetic damages. Data obtained can be used as site-specific data for radiological dose and impact assessment and as baseline data to establish the national radiation safety levels to protect Thai marine biota from any possible future nuclear accidents.

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## 1. INTRODUCTION

Presently, nuclear technology is frequently used in various aspects such as medical, agricultural and industrial applications. One of the notable utilizations is nuclear power widely known as low carbon emission energy. In Asia, 128 nuclear power reactors are on operation and 40 are under construction in order to generate electricity to meet the demand of rapidly growing population in the region (World Nuclear Association, 2017). In spite of so-called environmentally friendly energy, the risk of radioactive wastes consisting of several fission products released into the nature through either nuclear accident or routine operation still remains. Therefore, radioactivities in the environment would be elevated leading to an increase of radiation doses in both human and non-human biota. In those cases, not only the countries where nuclear power plants (NPPs) inhabited will be affected, but the seas nearby are also prone to be radioactively contaminated. As reported by Steinhäuser et al. (2014), over 80% of radionuclides

discharged from the Fukushima Daiichi nuclear accident were transported offshore and settled in the Pacific Ocean. Although several Fukushima-derived radionuclides were accidentally and deliberately released into the ocean, some of them including I-131 with a half-life of 8 days had decayed away from the environment few months after the accident. Cs-137, however, remains to be detected and shows high levels of contamination in the Pacific Ocean (MEXT, 2011; TEPCO, 2011; Battle et al., 2014). This artificial radionuclide could possibly be dispersed by the surface water circulation leading to possible radioactive contamination in several distant regions including the South China Sea and the Gulf of Thailand. In addition, migratory animals such as bird, fish, and turtle living in the Fukushima area and contaminated debris are expected to quickly spread radioactive material across the globe. It was reported that an elevated Cs-137 radioactivity was found in the Pacific Bluefin tuna migrating from Japan to California, USA after the accident in 2011 (Madigan et al., 2012). However, the radiocaesium

persisting in the ocean is not only from the Fukushima Daiichi nuclear accident, but also from the historical nuclear weapon tests and the routine releases from any other operating NPPs (Aarkrog et al., 1997; Hassona et al., 2008). Recently, Cs-137, a beta- and gamma-emitting radionuclide with a physical half-life of 30 years, plays a key role as a main contributor to radiation doses received by coastal and marine biota, excluding K-40 and Po-210 which are naturally occurring radioactive material. Accumulation of Cs-137 in such organisms belonging to different trophic levels is more likely to be high due to high water solubility and chemically similar behaviour to K (Peters and Newman, 1999; Leung and Shang, 2003). Gamma radiation from radiocaesium is able to penetrate into organisms causing DNA damages, cell abnormalities, increased mutation rates, and reduced individual and population fitness in those biotas with varying degrees of severity depending on exposure time and DNA repair mechanisms.

Prior to the Fukushima Daiichi nuclear accident, only one comprehensive marine environmental radioactivity measurement was conducted by Office of Atomic Energy for Peace (OAEP), Thailand in collaboration with the International Atomic Energy Agency (IAEA) during 1989-1991. Radioactivities from both natural and artificial radionuclides including Cs-137 in seawater, sediment, and marine animal collected from the Gulf of Thailand were reported (Mahapanyawong et al., 1992). There was no radiation dose in the studied marine biota estimated. Until recently, as the result of the recent nuclear accident, radiation dose and risk assessments became very important to reveal whether or not marine organisms are radiologically impacted. This is due to the fact that there are no safety levels in terms of concentration established for radiological impact assessments. ERICA Tool and the dose limit of 10  $\mu\text{Gy/h}$  (UNSCEAR, 2011) were used to estimate radiation dose rates and to assess radiological risks for marine organisms, respectively. No observable impact is found if they receive radiation doses below the dose limit. It has, nevertheless, been widely known that not only ERICA Tool but also other predictive computer software, RESRAD-Biota for example, contains all parameters derived from their own animal & plant species and environment. Hence, applying those

radiation dose and risk assessment software in other regions might not generate accurate results unless site-specific data such as radioactivity, concentration factor (CF), distribution coefficient (Kd), and dose-response relationship are used as the input in the software. Even few previous studies performed radiation dose and impact assessments using ERICA Tool and the radioactivity in the seawater and the marine animals, dose-response correlation was not investigated (Tumnoi, per.com.). Future thorough works are, therefore, needed to generate data necessary for the software.

Mussel, a group of bivalves, has worldwide distribution. Several species of them are commercially important. Furthermore, since the mollusc is sessile animal and filter feeder, it is widely used as a sentinel organism for aquatic pollution monitoring in particular radiation impact (AlAmri et al., 2012; Hagger et al., 2005; Jaeschke and Bradshaw, 2013; Jaeschke et al., 2015; Jaeschke et al., 2011, Jha et al., 2005; Jha et al., 2006). Asian green mussel *Perna viridis* (Family Mytilidae) is native in the Asia-Pacific region as well as Thailand which can be found in both the Gulf of Thailand and the Andaman Sea.

The present study aimed (1) to evaluate Cs-137 radiation dose rates in different shell lengths of Asian green mussel *Perna viridis* and (2) to investigate genetic responses in the mussel using micronucleus test and comet assay. Data obtained will enable the national regulatory body to be able to correctly assess radiation doses and radiological risks on local organisms. In addition, the information can be used as the national, regional, and international baseline data in case of future radioactive contaminations in the marine environment. More appropriate guidelines and regulations can be established based on the data obtained to protect local marine biota from radiological impacts caused by radioactive material from the NPPs and any possible emerging nuclear threats to the marine environment in the region.

## 2. METHODOLOGY

### 2.1 Sample Collection

A total of 153 kg of *Perna viridis* was collected from mussel rafts in the coast of Sriracha, Chonburi Province, Thailand in dry season (December 2015 and January 2016). The sample was then categorised into three different size classes

based on the total shell length: 4-6, 6-8, 8-10 cm (51 kg each). Forty litres of seawater were sampled at the mussel culture area.

## 2.2 Cs-137 Concentration Measurements and Dose & Risk Assessments

An individual mussel flesh was extracted from the shell of a total 50 kg mussel wet weight from each size class and was freeze-dried prior to thorough grinding. The prepared mussel samples were homogenized and 100 g were then taken for Cs-137 measurement using a High Purity Germanium (HPGe) detector with relative efficiency at 0.662 MeV Cs-137 of 0.76%, standard uncertainty of 8%, and counting time of 100,000 secs. Triplicate measurements were performed to provide sufficient data for subsequent statistical analysis.

Following the filtration, seawater samples were co-precipitated using Ammonium Phosphomolybdate (AMP) technique to extract Cs-137 from the seawater. Radiocaesium measurement was then carried out using HPGe with relative efficiency at 0.662 MeV Cs-137 of 2.5%, standard uncertainty of 7%, and counting time of 100,000 secs.

ERICA Tool was used in conjunction with the Cs-137 concentrations in the mussels and in the seawater analysed to estimate internal and external doses, respectively, and total doses received by the bivalves of interest. The external dose rates received by the mussels were only calculated from their surrounding seawater because they spend their entire life time in water column, not at the sediment. In addition, Cs-137 radiation emitted from sediment is absorbed by seawater before reaching to the mussels, therefore, the external dose rates from the sediment is negligible. The total doses were then compared with the dose limit of 10  $\mu\text{Gy/h}$  to reveal any possible radiation effects.

## 2.3 Haemolymph extraction, Total Haemocyte Count, and Cell Viability

At least 170  $\mu\text{l}$  of haemolymph from individual mussel was withdrawn from the posterior adductor muscle sinus using a 1-ml syringe with a 26G  $\frac{1}{2}$  needle. The sample was transferred to an Eppendorf tube and placed on ice. The total haemocyte count (THC) and the cell viability were examined before micronucleus test (MN) and comet assay to ensure that the haemocyte would be

sufficient to perform the assays. A 20  $\mu\text{l}$  aliquot of each Eppendorf tube was mixed at 1:1 with trypan blue and consequently loaded into a haemocytometer. THC and their viability were counted under a light microscope. Dead cells stained blue. The sample contained minimum  $1 \times 10^6$  cells/ml and 75% viability were used in the assays. The remaining samples of 2 qualified individuals were pooled so that the volume would be plenty for further analyses and to reduce inter-individual variation. Ten replicates are required for an individual mussel size class.

## 2.4 Micronucleus Test

A 50  $\mu\text{l}$  of each sample pool was fixed in a 50  $\mu\text{l}$  fixative (methanol:acetic acid at 3:1). The solution was spread over glass slide (2 slides/pool), allowed to dry at room temperature, and stained with Giemsa for 10 min. The slides were washed with distilled water, air dried, mounted with DPX, and scored under a light microscope. Thousand cells were counted per pool and reported as MN frequency (MNF).

## 2.5 Comet Assay

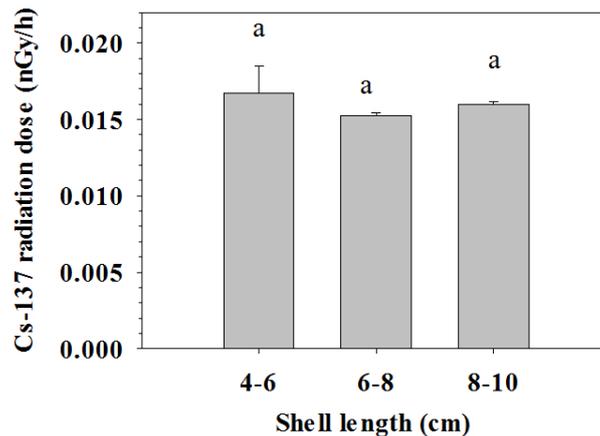
A 250  $\mu\text{l}$  of remaining haemolymph was suspended in a 250  $\mu\text{l}$  2% low melting point agarose (LMP; in PBS). An aliquot of 150  $\mu\text{l}$  of the solution was sandwiched between 1% LMP (in PBS) on microscopic slide and then placed to solidify at 4°C (3 slides/pool). At this point onwards, all steps were carried out in the dark at 4°C. For cellular protein removal and DNA unwinding, the slides were immersed in lysis solution for 60 min and electrophoresis buffer for 30 min, respectively. Electrophoresis for DNA migration was run at 300 mA, 20 V for 30 min. Then the slides were neutralised using neutralisation buffer for 10 min prior to dehydration with methanol for 15 min. After rinsing with distilled water, they are stained with ethidium bromide for 15 min and washed again with distilled water. The images of cell nuclei were taken using a camera attached to a fluorescence microscope. For each pool, 100 nuclei were randomly selected and graded for DNA damage (%Tail DNA) using LUCIA comet assay. In addition, positive control was performed using 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  as reference genotoxicant.

### 3. RESULT AND DISCUSSION

#### 3.1 Cs-137 radiation dose

The total radiation doses of Cs-137 in different size classes of *Perna viridis* from Sriracha coast were assessed from external and internal doses the organism exposed. The highest dose rate ( $0.0167 \pm 0.0018$  nGy/h) was found in the smallest shell length (4-6 cm) while the lowest

( $0.0152 \pm 0.0002$  nGy/h) was recorded in the middle size class of 6-8 cm (Figure 1). However, there were no significant differences in the dose rates among three groups categorized ( $p > 0.05$ ). The results obtained also revealed that all mussels of interest received radiation doses from Cs-137 less than  $10 \mu\text{Gy/h}$ .



**Figure 1.** Cs-137 Radiation doses in green mussel *Perna viridis* with different size classes

The radiation doses of Cs-137 obtained in three different shell ranges of the bivalve in the current study are closely matched with the results ranging between 0.0063 and 0.0144 nGy/h in the same species sampled from three areas in the Gulf of Thailand and Andaman Sea (Sookjuntra et al., 2016). This is probably the consequence of similar technique applied for assessment including ERICA Tool and the site-specific radioactivity data. Our findings are, however, much lower than the averaged Cs-137 dose rates received by sea mussel ( $0.005 \pm 0.002 \mu\text{Gy/h}$ ), blood cockle ( $0.003 \pm 0.001 \mu\text{Gy/h}$ ), shrimp ( $0.010 \pm 0.005 \mu\text{Gy/h}$ ), and squid ( $0.019 \pm 0.009 \mu\text{Gy/h}$ ) from the Thai sea (Tumnoi, 2012). It has to be noted here that high dose rates reported in Tumnoi (2012) was due to few assumptions. Firstly, gross beta concentrations which normally contain several radionuclides including Cs-137 were used as the Cs-137 levels in the seawater samples for external dose estimation. Secondly, concentration factors (CFs) for the marine animals of interest were applied for internal dose calculation due to the fact that during the seawater collection none of the marine animals were sampled. Elevated Cs-137 dose rates were obtained as the results of those assumptions leading to some

concerns of possible radioactive contamination caused by the Fukushima Daiichi nuclear accident in our marine organisms. This will also clearly demonstrate that if inappropriate data or non-site specific data was applied, results might not be accurate, reliable, and suitable for being used for radiation protection purposes, unless there is no other mean of doing so. However, the results from this work are consistent with those reported in several marine organisms collected from a number of countries in the Asia-Pacific region (IAEA, 2016).

In addition, the maximum and the minimum Cs-137 dose rates were found in the mussels with the smallest (4-6 cm) and the middle size class (6-8 cm), respectively while the mollusc with the largest shell length (8-10 cm) containing intermediate levels of radiation dose indicated no relationship between Cs-137 dose rates and the mussel sizes. The current observation is believed to be the first attempt to examine the relationship between radiation doses and body sizes. Due to the lack of that information, it is difficult to compare our finding with the results from previous studies. A majority of the previous works mainly focused on radioactivity and size relationship (Alam et al.,

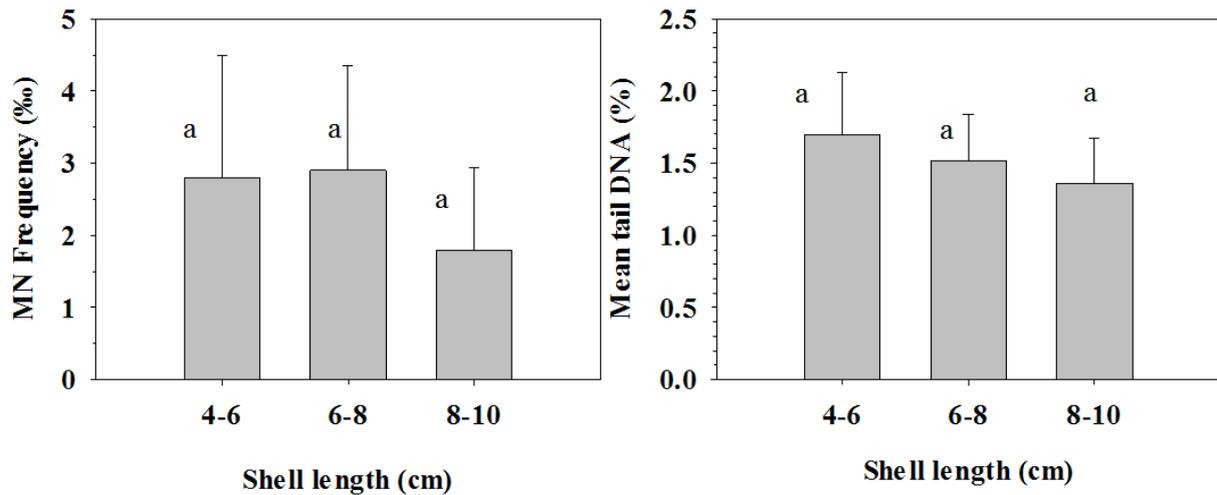
2000; Bustamante et al., 2002; Carvalho et al., 2010; Elliott et al., 1992; Khan et al., 2014; Matsumoto et al., 2015; Smith et al., 2002; Sundbom et al., 2003; Tsuboi et al., 2015; Uğur et al., 2011). However, a comparison between our results and their results should still be feasible because our dose rates were estimated based on radioactivity data in seawater and in mussel representing internal and external radiation doses, respectively. The recent finding is in agreement with the research conducted by Alam et al. (2000). Two species of mussels including *P. viridis* and estuarine mussel *Modiolus striatulus* were collected from the southern coast of Bangladesh to measure Cs-137 activity and to reveal the effect of mussel sizes on the radiocaesium accumulation in their body. The size effect from this investigation was not observed since those activities were below the detection limit (Alam et al., 2000). Possible causes for both findings could be (1) very low Cs-137 concentration in the samples and (2) minimal bioaccumulation of radiocaesium in the mussel due to its trophic position. Both negative and positive correlations between radionuclide concentration and size were nevertheless reported. The former found in mussels shows that smaller mussels appeared to concentrate higher radioactivity of natural radionuclides including Po-210, Pb-210, Ra-226, U-238, and K-40 (Alam et al., 2000; Bustamante et al., 2002; Carvalho et al., 2010; Khan et al., 2014; Uğur et al., 2011) while the latter was observed in fish where larger fish contained higher Cs-137 concentration in their body (Elliott et al., 1992; Matsumoto et al., 2015; Smith et al., 2002; Sundbom et al., 2003; Tsuboi et al., 2015). Some authors indicated that Cs-137 variation could be affected by fish trophic level (Sundbom and Meili, 2005; Sundbom et al., 2003). In fact, on the basis of biomagnification, high trophic level is able to accumulate more Cs-137 through its prey than lower trophic level. Asian green mussel is herbivorous, feeding on phytoplankton, small zooplankton, and organic matter, which is defined as low trophic level (Poutiers, 1998).

When compared with 10  $\mu\text{Gy/h}$  dose limit, the results gained from the present investigation were far below the safety level. This implies that any radiation hazard cannot be observed in the marine species studied (Andersson et al., 2009; Beresford et al., 2007; Garnier-Laplace et al., 2008; UNSCEAR, 2011). It is also in agreement with the previous finding where no reproductive effect on the biotas exposed to the dose rate lower than 200  $\mu\text{Gy/h}$  would take place (Coppelstone et al., 2008; Garnier-Laplace et al., 2008). Radiation exposure to a small proportion of the individuals in aquatic populations of organisms at the maximum dose rate of 400  $\mu\text{Gy/h}$  would be unlikely to have any significant impact at the population level (UNSCEAR, 2011). ICRP recommended the globally accepted principle that additional exposure to ionising radiations should be kept to as low as reasonably achievable (Khan et al., 2014).

### 3.2 Genetic responses

DNA damage evaluated using MN test indicated that the middle shell size of 6-8 cm and the large group of 8-10 cm revealed maximum and minimum MNF of  $2.90 \pm 1.45\%$  and  $1.80 \pm 1.14\%$ , respectively (Figure 2). No statistic differences in MNF were reported among the various size classes ( $p > 0.05$ ). %Tail DNA representing single strand break in the haemocyte showed high damage ( $1.70 \pm 0.43\%$ ) in the small size class (4-6 cm) and continuously decreased to the lowest value ( $1.36 \pm 0.31\%$ ) in the largest shell range of 8-10 cm (Figure 2). Although %Tail DNA seemed to have negative relationship to the bivalve size, it was not significantly different ( $p > 0.05$ ). Furthermore, %Tail DNA examined from positive control was  $63.26 \pm 1.11\%$  which had statistical differences from the results of all shell lengths ( $p < 0.05$ ).

According to the correlation among Cs-137, MNF, and %Tail DNA (Table 1), neither Cs-137 dose and MNF nor Cs-137 dose and %Tail DNA showed significant relationship ( $p > 0.05$ ). In contrast, there was a strong positive association between MNF and %Tail DNA ( $p < 0.01$ ).



**Figure 2.** Micronucleus frequency and %Tail DNA in green mussel *Perna viridis* with different size classes

**Table 1.** Estimative of Pearson's product-moment correlation coefficients among Cs-137 dose, MNF and %Tail DNA

Variables	r	p-Value
Cs-137 dose and MNF	-0.032	0.867
Cs-137 dose and %Tail DNA	0.198	0.295
MNF and %Tail DNA	0.741	0.000

MNF and %Tail DNA observed were in the similar range of mussels collected from clean or reference sites (Table 12). Additionally, the %Tail DNA can be categorised as minimal DNA damage because less than 10% was detected according to the criteria. While low damage is the DNA strand breaks of 10-25% (Mitchelmore et al., 1998). Minimal DNA damage data obtained in conjunction with low Cs-137 dose rates in the mussel can therefore be used to indicate no radiation impact taking place in our mussels. Besides, radiocaesium dose rate showed no significant relationship to

genetics. This is probably in consequence of very low radiation dose rates observed. Godoy et al. (2008) reported that no detrimental effect of Po-210 dose rate on MNF and DNA break in *Perna perna* was found as a result of the low dose rate of 0.02 mGy/d which is far below the suggested potential dose limit of 10 mGy/d. Nevertheless, a strong positive relationship between MNF and %Tail DNA reflects that these two techniques are reliable and can be used as powerful tools for future genetic impact assessment.

**Table 1.** Literature data related to MNF and %Tail DNA in mussels from clean sites

Mussel	Location	MNF (%)	%Tail DNA	Reference
<i>Perna viridis</i>	Hong Kong	<1	<25	Siu et al. (2004)
<i>P. viridis</i>	Singapore	<2	-	Liu et al. (2014)
<i>P. viridis</i>	Chonburi, Thailand	-	2.69	Sripromtong and Vejaratpimol (2009)
<i>Mytilus edulis</i>	Cornwall, UK	2-5	<10	Jha et al. (2005)
<i>M. galloprovincialis</i>	Necujum, Croatia	<4	<2	Klobučar et al. (2008)

#### 4. CONCLUSIONS

The Cs-137 radiation dose investigated for each size range of Asian green mussel *Perna viridis* were between 0.0152 and 0.0167 nGy/h. The dose rates that did not exceed the guideline level of 10 µGy/h indicate that no all biotas have been radioactively affected. This can be confirmed by the genetic results including MNF and %Tail DNA. They showed very low values of 1.80-2.90‰ and 1.36–1.70%, respectively, indicating no or very low damages. Both parameters are in the similar range reported from unpolluted sites. In addition, no effect of size class was observed on Cs-137 dose rate, MNF, and %Tail DNA which might be because of minimal Cs-137 concentration in the study area. However, more investigations need to be carried out in the future, especially, in the controlled laboratory where experimental organisms are exposed to varying degrees of radiation doses. This will generate more useful data for improving the national capability in radiation dose and radiological risk assessment. Data obtained will enable the national regulatory body and relevant organizations to establish the national guideline level and the national environmental management plan & strategy to protect our coastal and marine organisms from any future radiation impacts.

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