TOXICITY OF DIFENOCONAZOLE PESTICIDE ON FRESHWATER SHRIMP, *Palaemonetes paludosus*

Harlina Ahmad*, Muhammad Radzi Rahman and Shahizatul Fatihah Jamal Abdul Nasir

School of Industrial Technology, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia.
**Email: Harlinaa@usm.my*

ABSTRACT

Laboratories study on the toxicity of the difenoconazole compound in the triazole fungicide group was conducted on freshwater shrimp, Palaemonetes Paludosus species. These freshwater shrimps were exposed to five different concentrations of the difenoconazole compound. The concentrations of difenoconazole were 0.33, 0.65, 1.30, 3.25 and 6.50 mg/L respectively. The estimated LC50 of P. Paludosus was 2.987mg/L. This study shows that difenoconazole in the fungicide group has potential toxicity onto non-targeted organism particularly freshwater shrimp, Palaemonetes Paludosus.

Keywords: Difenoconazole, acute toxicity test, *Palaemonetes Paludosus*, lethal concentration 50 (LC_{50}).

INTRODUCTION

After World War II, pesticide was introduced nationwide to kill or control unwanted organisms including insects, weeds, fungi and nematodes. During the early generation of pesticides, the rate of toxicity was too high and dangerous. Nowadays, pesticide usage is more focusing on the negative effects to the environment and human health [1]. Pesticides are also potentially toxic to aquatic life and usage of pesticides will affect the water quality [2]. Pesticides can be grouped into different types, including pesticides formulations which consist of more than eight hundred active ingredients [3]. The use of pesticides at paddy fields is reported to be more than 90% [4]. The use of pesticides in the agricultural sector in Malaysia has been increasing over the years. Few of the aged farmers still maintains the use of pheromone and biological pest control methods for certain pests [5]. These pesticides can be classified into various and different types of chemicals with over eight hundred active ingredients in over tens of thousands of formulations [4]. They can roughly be divided based on the organisms intended to kill such as fungicides, insecticides, herbicides, molluscicides, nematocides, rodenticides and others [6].

For the paddy plantation, fungicide is one of the crucial groups of pesticide that are being used by the farmers. Controlling diseases caused by fungi, fungicides are used at paddy field [7]. A number of fungicides produced to control diseases caused by fungi [8]. Various fungicide products in the market recommend the time of use is in between reproductive and harvesting stage. Difenoconazole is a fungicide used in many countries as well as Malaysia due to the ability of this compound to control fungal disease. IUPAC name of difenoconazole is cis, trans-3-chloro-4-[4-methyl-2-(1H-1, 2,4-triazol-1-yimethyl)-1,3-dioxolan-2-y] pheny-4-chlorophenylether) and it has a good ability to interfere with the mycelia growth and inhibit the spore germination of pathogens due to the systemic sterol demethylation (DMI) that will ultimately result in inhibiting fungal growth [9]. Difenoconazole is relatively a persistent fungicide and degrades just 15% after two months and may sustain in water over long distance [10]. Basically, pesticides will be applied directly to the surface water of the paddy field. After the application or during the application of pesticides, these substances may accidentally transport via spray drift or runoff into nearby surface water [11]. The water flow from paddy field will carry along pesticides residues used from paddy fields [12]. Higher amount of rainfall after using of pesticides will increase the runoff rate of pesticides into water systems [13].

Difenoconazole fungicide is potentially toxic to the aquatic organism, therefore is one of the factors that contributes to the decreasing number of aquatic species in the river. In addition, difenoconazole may also be a threat to human by causing eye irritation. Even though difenoconazole aims to kill the fungi at the rice plant but the impact of the chemical towards non-targeted organisms is a main concern. Year by year, the decreasing number of shrimp production has been recorded [14]. The river or irrigation channel that is contaminated with pesticide residues can destroy freshwater shrimp population. The pesticide can easily penetrate other organisms or substrate like human, soil, meat and animal and can be transferred or migrated further than predicted due to the persistency of the pesticide [15]. So, it is important to evaluate the potential toxicology effect of pesticides on freshwater shrimp. There are many test methods to determine the toxicity of pesticides, chemicals and the effluent to aquatic organisms [16,17,18]. However, the standard method for the toxicity of freshwater shrimp is still unavailable. The aims of this study are as follows: determine the relationship between dosage of difenoconazole and time exposure and lastly to determine the lethal concentration (LC_{50}) of difenoconazole on freshwater shrimp, *P. paludosus.* Simple dilution was used to find five different concentrations of difenoconazole that were used for the toxicity study. Statistical analysis was conducted using One-Way ANOVA to analyze data obtained from this study.

METHODOLOGY

Shrimp collection and maintenance of culture

P. paludosus were purchased from the local fish shop and transferred to the laboratory. *P. paludosus* were transferred to a big aquarium tank containing dechlorinated tap water. Tap water was allowed to stand for at least 1 week to de-chlorinate the tap water. Water temperature was maintained in the range of 26-27^0 C.

Test chemical

Difenoconazole was purchased from an agricultural store at Permatang Pauh, Penang, Malaysia. The composition of the formulations of the active ingredients are 23% w/w; 250 g/l EC with trade name ARIMO 23EC from Advansia Sdn Bhd. All working stock solutions were made immediately prior to use.

Toxicity test

Five different concentrations and a control were used in the toxicity test. 1L beaker was used for this toxicity test. 20 shrimp were used for each concentration and in the control. Each experiment was repeated three times. Conductivity, pH, and dissolved oxygen were measured every day until the test ended. The beaker was covered with a plastic film to reduce volatilization. The beaker was aerated to supply enough oxygen. Shrimps were not fed during the experiment. The number of dead shrimps was counted and removed every 12 hours for the end test of 96 hours. The criterion for death was the absence of movement when shrimps were gently probed with the glass rod. The mortality of the shrimps was recorded after 24 hours and 96-hour exposure.

RESULTS AND DISCUSSION

The relationship between the concentration of Difenoconazole used and time exposure

Table 1 shows the result of the number of freshwater shrimp *P. paludosus* that had died after being exposed for 96 hours to different concentrations of difenoconazole pesticides. The mortality of the *P. paludosus* was recorded after 24h, 48h, 72h and 96h exposure. The toxicity test had five different test concentrations and a control, with three replicates per treatment.

Table 1. Variable of Difenoconazole fungicide concentrations and means Mortality of *P. paludosus* (N=20)

Concentration of Difenoconazole (mg/L)	**Exposure duration**				**Mean Mortality of shrimp died**
	24 hour	48 hour	72 hour	96 hour	Total
Control	X	X	X	X	X
0.33	X	X	X	X	X
0.65	X	X	X	X	X
1.30	X	X	X	X	X
3.25	X	8	2	3	13
6.50	X	18	1	X	19
X = No mortality					

Figure 1 shows the relationships between the concentrations of difenoconazole fungicide used with time exposure for the mortality of *P. paludosus*. For the first 24-hour exposure, no mortality of shrimp was recorded for all concentrations including control. At this duration exposure, for concentration 0.33 mg/L, 0.65 mg/L and 1.3 mg/L the shrimps were observed to be in stable condition. For the concentration of 3.25 mg/L and 6.5 mg/L, the moving ability of shrimps was getting lesser. For the 48-hour exposure, mortality of the shrimp was observed at concentration 3.25 mg/L and 6.5 mg/L. At concentration 3.25 mg/L, the number of the dead shrimps was eight which is equivalent to 40% mortality. For concentration 6.5 mg/L, almost all shrimps had died which means 90% mortality was recorded. Mortality rate for 72 hrs exposure for 3.25 mg/L and 6.5 mg/L concentration is 10% and 5% respectively. For the first three concentrations, which are 0.33 mg/L, 0.65 mg/L and 1.3 mg/L, 0% mortality recorded. The shrimps could still adapt to these concentrations. For the last duration, which is 96 hours' exposure, the mortality for the first three concentrations were still 0% which means the probability for concentration 0.325 mg/L, 0.65 mg/L and 0.65 mg/L causing death was low. For concentration 3.25 mg/L, the mortality recorded was 15% and for concentration 6.5 mg/L is 0% mortality. *P. paludosus* shows sensitivity towards difenoconazole where the effects can be observed within n 42 hours of exposure. The test wi*th P. paludosus* showed good control performance, and reproducibility of difenoconazole toxicity was good. This proves that this freshwater shrimp is suitable as standard toxicity test organism.

LC50 of *Palaemonetes Paludosus*.

The mortality rate was interpreted by using one-way ANOVA test and probit analysis. The probit analysis was used to determine the lethal concentration (LC) for certain percent of the population left. After 96 hours' exposure, LC_{50} value 2.987 mg/L. LC_{50} is the concentration of difenoconazole that kills 50 % of *P. paludosus*. The value for LC_{75} is 3.903mg/L with 95% confidence limit. There have been less published studies on toxicity study of difenoconazole onto *P. paludosus* but according to The European Food Safety Authority, difenoconazole had a higher rate of toxicity to aquatic organisms based on its high toxicity to Daphnia Magna [19]. Besides, difenoconazole has also a negative effect on Zebrafish during adult and early life stages [20].

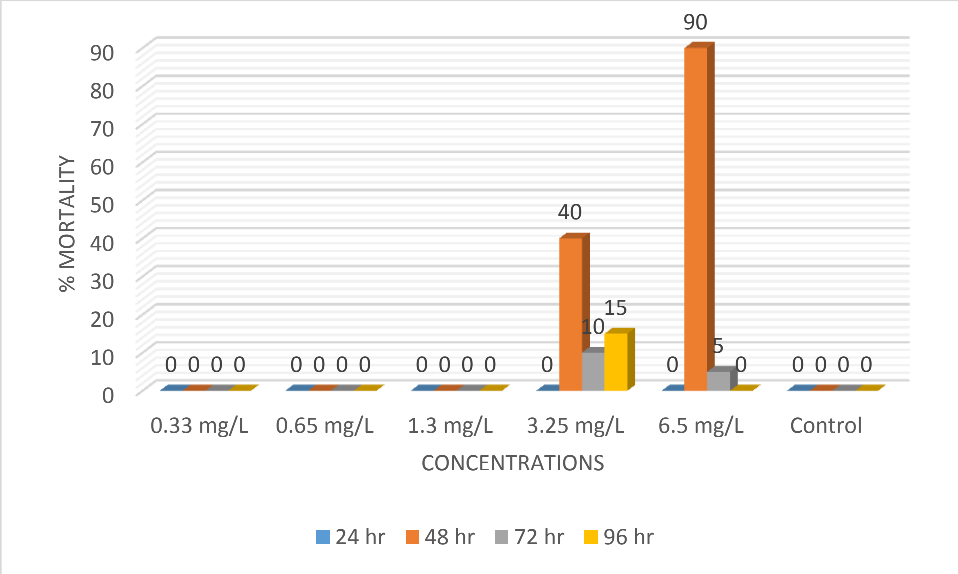

Figure 1. Percentage of mortality against exposure duration, hr of *P. paludosus* at five difference concentrations of difenoconazole fungicide.

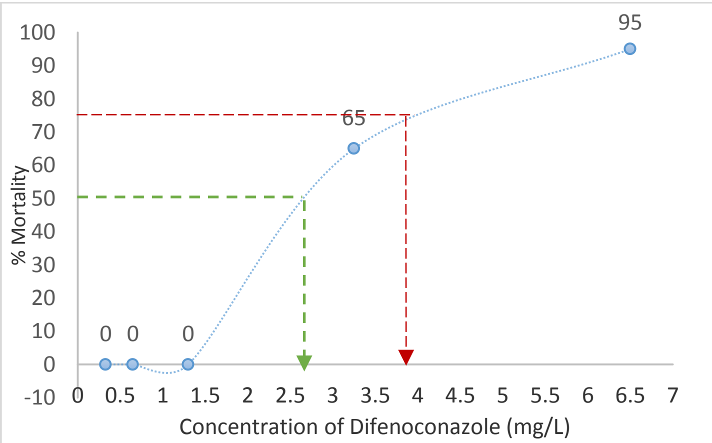

Figure 2. LC_{50} Difenoconazole onto *P. paludosus*

DISCUSSION

Sensitivity of Difenoconazole towards aquatic crustacean

There is no research conducted on acute toxicity test of difenoconazole towards *P. paludosus*. However, the toxicity of difenoconazole on crustaceans such as *Daphnia magna* was widely reported. Geometric mean of LC_{50} between 430, 770, 830 940, and 1100 µg/L on parameter mortality/immobilisation for *D. Magna* was 778 µg/L [21]. *Palaemonias alabama* sp. in Alabama state in USA was endangered by difenoconazole but was not categorized in critical habitat [22]. The LC_{50} after 96 hours' exposure for *Daphnia magna* and mysid shrimp is 0.77 mg/l and 0.15 mg/l respectively [23]. The presence of difenoconazole did not persist in a length of time that may be harmful to the environment [24]. Aquatic organisms at most of the agriculture area have the probability to be exposed to a very low concentration of pesticides caused by runoff and spray drift of pesticides [25].

Freshwater shrimp as acute toxicity test organism.

In Malaysia, the acute toxicity data on freshwater shrimp are limited. Toxicity test on fish and *Daphnia magna* were likely to be found. This may be due to the abundance of toxicity data on these species that is usually conducted in temperate environment. *Daphnia* is largely absent from the tropics and commonly inhabit the temperate region . Based on the standard acute toxicity test, all species involved is commonly found in temperate region [26]. This creates a bias towards the tropical ecosystem and may not represent the exact eco-toxicological risk. There are some differences between the organisms that live in temperate and tropical climates such as the amount of light exposure, the light intensity and climate changes [27]. The pesticides that showed high sensitivity towards freshwater shrimp were GABA-gated chloride channel antagonist and sodium channel modulator insecticides while for acetylcholinesterase inhibiting insecticides and fungicides, a moderate and low sensitivity respectively was shown [28]. Previous study indicated that there is inadequate information regarding the toxicity of difenoconazole on freshwater shrimp. There are several studies on the toxicity of other pesticides on freshwater shrimp. The acute toxicity test using *C. laevis* may be a suitable alternative for determining the potential risk of chemicals under tropical conditions after *C. laevis* showed similar sensitivity for diazinon and lambda cyhalothrin, much greater sensitivity for endosulfan and paraquat and much lower sensitivity for carbofuran [29]. Carbaryl, chlorpyrifos, cypermethrin, dimethoate, diuron and fenarimol to freshwater shrimp (*Paratya australiensis*) and after 96 hrs exposures, the pesticides that were most toxic to the shrimp was cypermethrin [25]. It can be observed that the most sensitive pesticide towards various freshwater shrimps came from the insecticide group followed by fungicide and herbicide.

Table 2. 96 h LC_{50} values for different pesticides during acute exposures towards different freshwater shrimps

Pesticide	Class of pesticide	Type of freshwater shrimp	96 hrs LC_{50}	Sources
Temephos s-Methoprene Pyriproxyfen	Insecticide Insecticide Insecticide	*Leander tenuicornis* sp.	0.0112 mg/L 14.32 mg/L 0.098 mg/L	[30]
Malathion	Insecticide	*Palaemonetes pugio* sp.	0.03819 mg/L	[31]
Carbofuran Diazinon Endosulfan Lambda cyhalothrin Profenofos 2,4-D-dimethylamine Paraquat	Insecticide Insecticide Insecticide Insecticide Insecticide Herbicide Herbicide	*Caridina laevis* sp.	0.95 mg/L 1.32 μg/L 1.02 μg/L 0.33 μg/L 1.42 μg/L 872 mg/L 35.8 μg/L	[29]
Cypermethrin Carbaryl Chlorpyrifos Dimethoate Diuron Fenarimol	Insecticide Insecticide Insecticide Insecticide Herbicide Fungicide	*Paratya australiensis* sp.	0.019 μg/L 12 μg/L 0.063 μg/L 0.8 mg/L 8.8 mg/L 3.4 mg/L	[25]
Glyphosate	Herbicide	*Caridina nilotica* P. Roux sp.	60.96 mg/L	[32]

CONCLUSIONS

P. paludosus is a suitable test organism because this shrimp can act as a representative of the freshwater aquatic organism that is available at the paddy field area. *P. paludosus* showed good control performance because 0% mortality recorded for control test even the shrimps were not fed along 96 hours' exposure. Results for this study showed that LC_{50} for the *P. paludosus* is estimated to be at 2.987 mg/L. Besides, this study also shows that difenoconazole can kill non-target organisms. Therefore, it is important for present researchers to carry out more studies about the negative effects of difenoconazole not only to the aquatic organisms but also to humans and the environment.

ACKNOWLEDGEMENT

The authors acknowledge the Malaysian Ministry of Education and Universiti Sains Malaysia (USM) for awarding the Fundamental Research Grant Scheme (FRGS) to conduct this research (Grant no: 203/PTEKIND/6711466).

REFERERENCES

[1] Chambers H.W., Boone J.S., Carr R.L., & Chanbers J.E. (2001). Chemistry of organophosphorus insecticides. In: Robert IK, editor. Handbook of Pesticide Toxicology. secondth ed. . p. 913–7. California: Academic.

[2] Helfrich, L.A., Weigmann, D.L., Hipkins, P., & Stinson, E.R. (2009). Pesticides and aquatic animals: a guide to reducing impacts on aquatic systems.

[3] Hernández, A. F., Parrón, T., Tsatsakis, A. M., Requena, M., Alarcón, R., & López-Guarnido, O. (2013). Toxic effects of pesticide mixtures at a molecular level: their relevance to human health. *Toxicology*, 307, 136-145.

[4] Abdullah, A. R., Bajet, C. M., Matin, M. A., Nhan, D.D. & Sulaiman, A. H. (1997). Ecotoxiology of pesticides in the tropical paddy field ecosystem. *Environmental Toxicology and Chemistry*, 16, 59-70.

[5] Idayu, M., Anis, N., Akmal, S., & Esa, N. (2014). A Survey on Use , Hazards and Potential Risks of Rice Farming Pesticides in Permatang Keriang , Pulau Pinang (Malaysia), *International Journal of Scientific and Research Publications*, 4(10), 1–11.

[6] Aktar, W., Sengupta, D., & Chowdhury, A. (2009). Impact of pesticides use in agriculture: their benefits and hazards. *Interdisciplinary toxicology*, 2(1), 1-12.
[7] Kumar, M. P., Gowda, D. S., Moudgal, R., Kumar, N. K., Gowda, K. P., & Vishwanath, K. (2013). Impact on fungicides on rice production in India. Fungicides-showcases of integrated plant disease management from around the world. ISBN, 978-953.
[8] Khalidah A. L., Nor Kartini, A. B. & Nurzawani, M. I. 2010. Preliminary study of difenoconazole residure in rice paddy watersheds. *Malaysian Journal of Science*, 29.
[9] Reuveni*, M., & Sheglov, D. (2002). Effects of azoxystrobin,difenoconazole, polyoxin B(polar) and trifloxystrobin on germination and growth of Alternaria alternata and decay in red delicious apple fruit. *Crop Protection*. 21, 951-955.
[10] Rodriguez-Cabo, T., Rodriguez, I., Ramil, M., Cela, R., (2013). Assesssment of silicone as support to investigate the transformation routes of organic chemicals under environmental conditions and UV exposure. Application to selected fungicides. *Analytical and Bioanalytical Chemistry*. 405, 4187-4198.
[11] Schulz, R., 2004. Field studies on exposure, effects, and risk mitigation of aquatic nonpoint-source insecticide pollution: a review. *Journal of Environmental Quality*. 33, 419–448.
[12] Inao K., Ishi Y., Kobara Y., Kitamura Y. (2003). Landscape scale simulation of pesticide behavior in river basin due to runoff from Paddy Fields using Pesticide Paddy Field Model (PADDY), *Journal of Pesticide Science*. 28.24 – 32.
[13] Parveen, S., kohguchi, T., Shimazawa, H., Nakagoshi, N., (2005). Measuring of some selected herbicides in paddy surface water in the Saijo Basin, Western Japan. *Agronomy for Sustainable Development*. 25, 55 – 61.
[14] Reyes, G. G., Villagrana L, C., & Alvarez, G. L. (1999). Environmental conditions and pesticide pollution of two coastal ecosystems in the Gulf of California, Mexico. *Ecotoxicology and Environmental Safety*, *44*(3), 280–286. http://doi.org/10.1006/eesa.1999.1836
[15] Mohd Fuad, M.J., Junaidi, A.B., Habibah, A., Hamzah, J., Toriman, M.E., Lyndon, N., Er, A.C., Selvadurai, S. and Azima, A.M. (2012). The Impact of Pesticides On Paddy Farmers and Ecosystem. *Advances in Natural and Applied Sciences*, 6(1): 65-70.
[16] OECD, 1984. OECD Guidelines for Testing Chemicals. Organization for Economic Cooperation and Development, Paris.
[17] ISO, 1996. Water Quality: Determination of the Inhibition of the Mobility of Daphnia magna Strauss (Cladocera, Crustacea)- Acute Toxicity Test. ISO 6341. International Organization for Standardization,Geneva.
[18] Lewis, P.A., Klemm, J., Lazorchak, J.M., Norberg-King, T.J., Peltier, W.H., Heber, M.A., (1994). Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. US Environmental Protection Agency EPA/600/4-9/002, Cincinnatti, OH.
[19] European Food Safety Authority (EFSA), Parma,Italy (2011), https://www.efsa.europa.eu/en/efsajournal/pub/1967 , [Accessed November 25, 2016].
[20] Mu. X, S. Pang, X. Sun, J. Gao, J. Chen, X. Chen, X. Li, C. Wang. (2013). Evaluation of acute and developmental effects of difenoconazole via multiple stage zebrafish assays. *Environmental Pollution*. 175, 147–157.
[21] Mensink, B. J. W. G. (2008). Environmental risk limits for difenoconazole. Retrieved from http://library.wur.nl/ebooks/hydrotheek/1890379.pdf
[22] Andrew, N., Ullagoddi, A. and Khan, F. (2011). Eclogical risk assessment for the proposed new uses of difenoconazole as foliar sprayfor carrot, chickpea, soybean, stone fruit (group 12), and strawberry and a seed treatment for oat and rye [Memorandom]. Washington, DC: United States Environmental Protection Agency.
[23] Arysta. (2010). Difenoconazole [Material Safety Data Sheet]. Retrieved from https://arystalifescience.co.za/files/Defender-MSDS.pdf
[24] Ab Latiff, K., Abu Bakar, N. K., & Md Isa, N. (2010). Preliminary study of difenoconazole residues in rice paddy watersheds. *Malaysian Journal of Science*, *29*(1), 73–79.
[25] Kumar, A., Correll, R., Grocke, S., & Bajet, C. (2010). Toxicity of selected pesticides to freshwater shrimp, Paratya australiensis (Decapoda: Atyidae): Use of time series acute toxicity data to predict chronic lethality. *Ecotoxicology and Environmental Safety*, *73*(3), 360–369. http://doi.org/10.1016/j.ecoenv.2009.09.001
[26] Dumont, H. J. (1994). On the diversity of the Cladocera in the tropics, *Hydrobiologia, 272: 27-38.*
[27]Segers, H., & Martens, K. (2005). *Aquatic biodiversity II* (1st ed., pp. 315-329). Springer.
[28] Daam, M. A., & Rico, A. (2016). Freshwater shrimps as sensitive test species for the risk assessment of pesticides in the tropics. *Environmental Science and Pollution Research*, 1–9. http://doi.org/10.1007/s11356-016-7451-1
[29] Sucahyo, D., van Straalen, N. M., Krave, A., & van Gestel, C. A. (2008). Acute toxicity of Pesticides to the tropical freshwater shrimp Caridina laevis. *Ecotoxicology and Environmental Safety*, 69(3), 421-427. doi: 10.1016/j.ecoenv.2007.06.003
[30] Brown, M. D., Thomas, D., Watson, K., Greenwood, J. G., & Kay, B. H. (1996). Acute toxicity of selected pesticides to the estuarine shrimp Leander tenuicornis (Decapoda:Palaemonidae). *Journal of the American Mosquito Control Association*, 12(4), 721–724. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9046481
[31] Key, P. B., Fulton, M. H., Scott, G. I., Layman, S. L., & Wirth, E. F. (1998). Lethal and sublethal effects of malathion on three life stages of the grass shrimp, Palaemonetes pugio. *Aquatic Toxicology, 40*, 311–322. http://doi.org/10.1016/S0166-445X(97)00068-4
[32] Deepananda, K. H. M. A., Gajamange, D., de Silva, W. A. J. P., & Wegiriya, H. C. E. (2011). Acute toxicity of a glyphosate herbicide, Roundup??, to two freshwater crustaceans. *Journal of the National Science Foundation of Sri Lanka*, *39*(2), 169–173. http://doi.org/10.4038/jnsfsr.v39i2.3178