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A Trend of Zinc Uptake into *Tachypleus Gigas* Tissues After a Month of Exposure

Siti Noor Syuhada Muhammad Amin[a,*], Azman Bin Azid[b], Siti Maisarah Aziz[a], Salmiah Jamal Binti Mat Rosid[a], Azma Abdul Malek[a], Nurul Asma Hasliza Zulkifly[b], Nurulhuda Mohammad Yusoff[a]

[a] UniSZA Science and Medicine Foundation Centre, Universiti Sultan Zainal Abidin, Gong Badak Campus, 21300 Kuala Nerus, Terengganu.
[b] Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, Besut Campus, 22200 Besut, Terengganu.

***Corresponding author: syuhadaamin@unisza.edu.my**

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ABSTRACT

Tachypleus gigas is one of the species of horseshoe crabs found in Peninsular Malaysia. Metals in the surrounding water can accumulate into horseshoe crab tissues. This study was conducted to determine zinc (Zn) uptake into horseshoe crab tissues after continuous exposure to the metals. *T. gigas* that were sampled from Gelang Patah, Johor and Cherating, Pahang were reared in the control tank and Zn treatment tank (20 mg/L) with aeration supply for a month. Twelve horseshoe crabs from Zn treatment tank and 6 horseshoe crabs from control tank were dissected for tissues namely operculum, gills, chelicerae, leg, digestive tract, hepatopancreas and carapace in different intervals (Day 0, Day 10, Day 20 and Day 30). These tissues were freeze dried and digested with 65% nitric acid on a hotplate at 200°C. Zn were measured using Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES). Different concentrations of Zn were measured in different tissues. The tissues of *T. gigas* showed increased concentrations of Zn by following exposure in 30 days. The increasing pattern of Zn uptake into the tissues from day 0- 30 are clear. The gills showed highest concentrations of metals accumulation with the value of 6398.60 ± 909.51 µg/g (dw). Thus, uptake of Zn into *T. gigas* was confirmed, therefore it has the potential to become the suitable bio-indicator (especially their gills) to detect the metal pollution in the surrounding water.

Keywords: *Tachypleus gigas*, Zinc, Uptake, Exposure, Concentration

INTRODUCTION

Horseshoe crabs are the oldest creatures in the world, which live in this world since 450 years ago (Huang, 1997). They belong to the kingdom Animalia and phylum Arthropoda. This group also includes lobsters, crabs, insects, spiders and scorpions. They are crab-like animals, with a hard shell and claws and more closely related to scorpions and spiders from the subphylum Chelicerata due to their similar number of walking legs and mouthpart. They belong to the class Merostomata, order Xiphosura and family Limulidae.

To date, there are only four species worldwide which belongs to three genera of limulids, *Carcinoscorpius, Tachypleus* and *Limulus*. *Limulus* is restricted to eastern North America, ranging to Nova Scotia to the Yucatan region of Mexico while *Tachypleus* and *Carcinoscorpius* exist in Southern Asia (Mohan et al., 1984; Shuster, 1985). Malaysia has three species, which are *C. rotundicauda, T. gigas* and *T. tridentatus*.

Horseshoe crabs are consumed by local people in some areas such as the Gulf of Thailand, some eats the cooked eggs (Kungsuwan et al., 1987) and biomedically, scientists have applied horseshoe crab blood as a detector for bacterial endotoxins in drugs and intravenous devices (Gerhart, 2007).

Literatures have shown that metals can accumulate into horseshoe crab tissues. The metals do not decay and the tendency to accumulate into the organism is high. As metals can be found in the aquatic ecosystem, horseshoe crabs can be the suitable indicator to detect the metals in any particular area. The fact that they are consumed as delicacies, the evidence on the uptake of metals into their tissues could be a useful information to consumers.

To our knowledge, there is lack of laboratory studies on metals exposure towards the horseshoe crabs in Malaysia. Therefore, this study was conducted to determine the uptake zinc (Zn) into horseshoe crab tissues of one of the three species available in Malaysia, *T. gigas*. The study is carried out to predict the pattern of metals concentrations in different tissues of the animal after continuously exposed to the metals as well as to determine the potential of horseshoe crabs as metal indicator.

MATERIALS AND METHODS

Rearing samples

Eighteen horseshoe crabs, *Tachypleus gigas* were sampled from their habitats. The horseshoe crabs were sampled from Gelang Patah, Johor and Cherating, Pahang to run this project. Out of eighteen horseshoe crabs, seven were females. They were kept in the tank of clean seawater for acclimatization period in 1 week. Six horseshoe crabs were placed in the control tank and 12 horseshoe crabs were placed in the zinc (Zn) treatment tank with the aeration system. 200 L of clean seawater were filled in the tank with 20 mg/L of metal concentration was supplied. After Day 0, Day 10, Day 20 and Day 30, the horseshoe crabs from each tank were sacrificed by cold shock by placing them in the -20^{o}C freezer. The horseshoe crabs that were sacrificed on Day 0, also act as the control (without metal exposure). The weight and the width of the horseshoe crabs were measured as the allometric data.

Horseshoe crab dissection

Several different tissue samples were collected from each individual after the dissection, namely the genital operculum, book gills (gill 1-5), chelicerae, walking leg, digestive tract, hepatopancreas and carapace. The tissues were collected in labelled glass vials and the net weight was recorded in gram. For freeze drying process, the samples were taken out from freezer. Immediately, the mouth of the glass vials was sealed with parafilm. A few tiny holes were made on it to allow faster drying. The vials then were transferred to a freeze dryer (Labconco Freeze Dry System/ Freezone 6.0) and were allowed to dry for 24 hours or longer for samples which are harder to dissolve. After the freeze-drying period the tissues were reweighted to obtain the dry weight. The wet and dry weight was recorded.

Tissue digestion

All the samples were digested in concentrated nitric acid (65%) by using Hotplate Digestion. The samples were weighted around 100-300mg of the dried tissues and were placed in 50 ml conical flasks in order to undergo the digestion process of samples. 10 ml of 65% nitric acid were added into the conical flask by using a 10 ml pipette. These mixtures were heated on a hotplate at 200°C until it is completely dissolve. The remaining solutions were made up 10 ml by adding distilled water. These solutions were used to determine the metal concentrations in the tissues of horseshoe crabs.

Metal measurements

Zinc were measured by using a standard method of Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES). ICP-OES is a type of atomic spectrometry that is sensitive and capable of the determination of metals and metalloids at the ultratrace level on μl or μg samples. Combustion flames provide a remarkably simple means for converting inorganic analytes in solution into free atoms. It introduces an aerosol of the solution into an appropriate flame. The fraction or all of the metallic ions in the aerosol droplets are converted into free atoms. Once the free atoms formed, they were detected and determined quantitatively at the trace level of spectrometer.

Statistical analysis

The differences in the metal concentration between different tissues were determined by using One Way ANOVA. The differences in the metal concentrations between different tissues were considered as significant at $P < 0.05$.

RESULTS AND DISCUSSION

Allometric data of *Tachypleus gigas*

Table 1 shows the average weight and width of *T. gigas* in the control tank and Zn treatment tank.

Table 1. The average weight and width of *T. gigas*

			(mean ± SE)	
Treatment	N	Day	Average wet weight (g)	Average width (cm)
Control	6	0	301.53 ± 11.24	16.21 ± 0.19
Zinc	4	0	276.05 ± 20.32	15.90 ± 0.28
	3	10	267.21 ± 17.61	16.81 ± 0.25
	3	20	272.86 ± 17.87	15.95 ± 0.45
	2	30	470.14 ± 184.09	19.16 ± 2.33

Zinc concentrations in horseshoe crab tissues without exposure

Table 2. Zn concentrations in gills (gills 1-5) among horseshoe crab tissues without exposure

	Zinc concentrations (μg/g; mean ± SE)	
Tissues	Control	Zinc treatment (Day 0)
Gill 1	81.89 ± 14.66	62.19 ± 16.04
Gill 2	118.24 ± 21.36	67.38 ± 17.14
Gill 3	89.26 ± 10.21	65.33 ± 7.55
Gill 4	96.18 ± 10.88	66.36 ± 9.35
Gill 5	90.51 ± 13.96	55.11 ± 10.31
P * value	**0.490**	**0.959**

Table 3. Zn concentrations among the other tissues in horseshoe crab without exposure

	Zinc concentrations (μg/g; mean ± SE)	
Tissues	Control	Zinc treatment (Day 0)
Operculum	78.85 ± 15.99[a]	17.85 ± 4.94[a]
Chelicerae	86.86 ± 11.94[a]	69.90 ± 21.84[a]
Leg	141.52 ± 9.75[ab]	127.58 ± 31.92[ab]
Digestive tract	319.82 ± 66.25[b]	229.50 ± 38.84[b]
Hepatopancreas	987.56 ± 133.83[c]	777.65 ± 101.55[c]
Carapace	37.15 ± 17.81[a]	23.35 ± 6.03[a]
P * value	**<0.001**	**<0.001**

[abc] shows the different groups of Zn concentrations among the other tissues of *T. gigas* without exposure (ANOVA, DMRT)

Table 2 shows there was no significant difference between gills 1-5 for Zn uptake in gills for the control tank (ANOVA, P>0.05). Since there was no significant difference between gills 1-5, the data for all gills 1-5 were pooled to represent the average of Zn concentrations in the gills.

Table 3 shows there was significant difference among the other tissues of horseshoe crab in the control tank (ANOVA, P<0.05). For the control tank, the highest concentrations of Zn was recorded in the hepatopancreas (987.56 ± 133.83 μg/g dw), followed by the digestive tract (319.82 ± 66.25 μg/g dw), (141.52 ± 9.75 μg/g dw) for the leg, (86.86 ± 11.94 μg/g dw) by the chelicerae, (78.85 ± 15.99 μg/g dw) by the operculum and (37.15 ± 17.81 μg/g dw) by the carapace. The results also showed there was significant difference among the other tissues the horseshoe crab in the Zn treatment tank Day 0 (ANOVA, P<0.05). For the Zn treatment tank Day 0, the highest concentration of Zn was recorded in hepatopancreas (777.65 ± 101.55 μg/g dw), followed by digestive tract (229.50 ± 38.84 μg/g dw), (127.58 ± 31.92 μg/g dw) for the leg, (69.90 ± 21.84 μg/g dw) by the chelicerae, (23.35 ± 6.03 μg/g dw) by the carapace and (17.85 ± 4.94 μg/g dw) by the operculum.

Zinc concentrations in horseshoe crab tissues with exposure

Table 4. Zinc concentrations among horseshoe crab tissues at different intervals

	Zn concentrations (μg/g; mean ± SE)				
Tissues	Day 0	Day 10	Day 20	Day 30	**P * value**
Operculum	17.85 ± 4.94[a]	353.89 ± 144.46[ab]	739.02 ± 97.30[b]	1903.00 ± 620.69[c]	**0.002**
Gills	63.27 ± 5.13[a]	504.66 ± 39.84[a]	4602.90 ± 907.46[b]	6398.60 ± 909.51[c]	**<0.001**
Chelicerae	69.90 ± 21.84[a]	644.44 ± 261.59[ab]	1343.40 ± 85.39[b]	4328.40 ± 1013.30[c]	**<0.001**
Leg	127.58 ± 31.92[a]	667.55 ± 227.89[a]	1792.20 ± 241.76[a]	4617.40 ± 1556.40[b]	**0.001**
Digestive tract	229.50 ± 38.84[a]	339.14 ± 9.91[a]	1175.30 ± 426.89[ab]	1697.90 ± 740.45[b]	**0.035**
Hepatopancreas	777.65 ± 101.55[a]	1417.30 ± 253.75[a]	3234.00 ± 328.41[b]	3762.90 ± 702.85[b]	**<0.001**
Carapace	23.35 ± 6.03[a]	138.57 ± 23.17[a]	296.15 ± 68.74[a]	888.66 ± 248.85[b]	**0.001**

[abc] shows the different groups of Zn concentrations in the tissues of *Tachypleus gigas* at different intervals (ANOVA, DMRT)

Table 5. Zinc concentrations among tissues of horseshoe crab

	Zn concentrations (μg/g; mean ± SE)			
Tissues	Day 0	Day 10	Day 20	Day 30
Operculum	17.85 ± 4.94[a]	353.89 ± 144.46[ab]	739.02 ± 97.30	1903.00 ± 620.69
Gills	63.27 ± 5.13[ab]	504.66 ± 39.84[ab]	4602.90 ± 907.46	6398.60 ± 909.51
Chelicerae	69.90 ± 21.84[ab]	644.44 ± 261.59[b]	1343.40 ± 85.39	4328.40 ± 1013.30
Leg	127.58 ± 31.92[b]	667.55 ± 227.89[b]	1792.20 ± 241.76	4617.40 ± 1556.40
Digestive tract	229.50 ± 38.84[c]	339.14 ± 9.91[ab]	1175.30 ± 426.89	1697.90 ± 740.4
Hepatopancreas	777.65 ± 101.55[d]	1417.30 ± 253.75[c]	3234.00 ± 328.41	3762.90 ± 702.8
Carapace	23.35 ± 6.03[a]	138.57 ± 23.17[a]	296.15 ± 68.74	888.66 ± 248.85
P * value	**<0.001**	**<0.001**	**0.054**	**0.053**

[abc] shows the different groups of Zn concentrations in the tissues of *Tachypleus gigas* (ANOVA, DMRT)

Table 4 shows different concentrations of Zn were accumulated in different tissues of *T. gigas*. In general, Zn concentrations in the tissues increased after the following exposure (20 mg/L) of Zn up to 30 days. The result shows there is significant difference for Zn uptake in the operculum at different intervals of exposure (ANOVA, $P<0.05$). The highest levels of Zn was recorded on day 30 (1903.00 ± 620.69 μg/g dw), followed by day 20 (739.02 ± 97.30 μg/g dw), (353.89 ± 144.46 μg/g dw) for day 10 and (17.85 ± 4.94 μg/g dw) for day 0. The result shows there is also significant difference for Zn uptake at different intervals of exposure (ANOVA, $P<0.05$) for the gills. The highest Zn concentrations in the gills were found on day 30 (6398.60 ± 909.51 μg/g dw), followed by day 20 (4602.90 ± 907.46 μg/g dw), (504.66 ± 39.84 μg/g dw) for day 10 and day 0 (63.27 ± 5.13 μg/g dw).

The result shows there is significant difference for Zn accumulate in the chelicerae at different intervals of exposure (ANOVA, $P<0.05$). The highest concentrations of Zn in the chelicerae was found on day 30 (4328.40 ± 1013.30 μg/g dw), for day 20 (1343.40 ± 85.39 μg/g dw), followed by day 10 (644.44 ± 261.59 μg/g dw) and (69.90 ± 21.84 μg/g dw) for day 0. The result, (ANOVA, $P<0.05$) shows there is also significant difference of Zn uptake at different intervals of exposure for the leg. Zn concentrations were recorded highest on day 30 (4617.40 ± 1556.40 μg/g dw), followed by day 20 (1792.20 ± 241.76 μg/g dw), (667.55 ± 227.89 μg/g dw) for day 10 and for day 0 (127.58 ± 31.92 μg/g dw).

The result shows there is significant difference for Zn accumulate in the digestive tract at different intervals of exposure (ANOVA, $P<0.05$). The highest concentrations of Zn in the digestive tract was found on day 30 (1697.90 ± 740.45 μg/g dw), for day 20 (1175.30 ± 426.89 μg/g dw), followed by day 10 (339.14 ± 9.91 μg/g dw) and (229.50 ± 38.84 μg/g dw) for day 0. The result, (ANOVA, $P<0.05$) shows there is also significant difference in Zn uptake at different intervals of exposure for the hepatopancreas. Zn concentrations in the hepatopancreas was recorded highest on day 30 (3762.90 ± 702.85 μg/g dw), followed by day 20 (3234.00 ± 328.41 μg/g dw), (1417.30 ± 253.75 μg/g dw) for day 10 and for day 0 (777.65 ± 101.55 μg/g dw).

The result shows there is significant difference for Zn accumulate in the carapace at different intervals of exposure (ANOVA, $P<0.05$). The highest concentrations of Zn in the carapace was found on day 30 (888.66 ± 248.85 μg/g dw), for day 20 (296.15 ± 68.74 μg/g dw), followed by day 10 (138.57 ± 23.17 μg/g dw) and the lowest of Zn concentrations was found on day 0 (23.35 ± 6.03 μg/g dw).

According to Table 5, the result shows there is significant difference of Zn uptake among all the tissues for day 0 (ANOVA, $P<0.05$). The highest of Zn levels was recorded in the hepatopancreas (777.65 ± 101.55 μg/g dw), followed by the digestive tract (229.50 ± 38.84 μg/g dw), leg (127.58 ± 31.92 μg/g dw), chelicerae (69.90 ± 21.84 μg/g dw), gills (63.27 ± 5.13 μg/g dw), for the carapace (23.35 ± 6.03 μg/g dw), while the lowest concentrations of Zn were found in the operculum (17.85 ± 4.94 μg/g dw).

There is also significant difference of Zn uptake among all the tissues of horseshoe crab (ANOVA, $P<0.05$) for day 10. The highest accumulation of Zn were recorded in the hepatopancreas (1417.30 ± 253.75 μg/g dw), followed by the leg (667.55 ± 227.89 μg/g dw), (644.44 ± 261.59 μg/g dw) for the chelicerae, (504.66 ± 39.84 μg/g dw) for the gills, for the operculum (353.89 ± 144.46 μg/g dw), (339.14 ± 9.91 μg/g dw) by the digestive tract and the lowest concentrations of Zn were recorded in the carapace (138.57 ± 23.17 μg/g dw).

The results for day 20 shows there is no significant difference of Zn uptake among all the tissues of horseshoe crab (ANOVA, $P>0.05$). The highest levels of Zn were found in the gills (4602.90 ± 907.46 μg/g dw) followed by the hepatopancreas (3234.00 ± 328.41 μg/g dw), leg, chelicerae, digestive tract, operculum (739.02 ± 97.30 μg/g dw) and the lowest concentrations of Zn were possessed in the carapace (296.15 ± 68.74 μg/g dw).

There is no significant difference of Zn uptake among all of the tissues of horseshoe crab (ANOVA, $P>0.05$) for day 30. Gills possessed the highest levels of Zn as high as (6398.60 ± 909.51 μg/g dw) followed by the leg (4617.40 ± 1556.40 μg/g dw), chelicerae, hepatopancreas, operculum, digestive tract (1697.90 ± 740.45 μg/g dw), while the carapace possessed the lowest concentrations of Zn as low as (888.66 ± 248.85 μg/g dw).
From this study, different concentrations of metals were uptake in different tissues of horseshoe crab. This finding also supported with the study by Lakshmanan and Nambisan (1989) and Harris and Santos (2000), that metal accumulation vary with the different types of tissues. Besides, the ecological and behavioural of organisms may also affect metal uptake (Weimin et al., 1992) in their tissues as horseshoe crabs were used in this study. In this study, the males and females horseshoe crab were used. The reasons for using both sexes of horseshoe crab in metals exposure is because the metal concentrations do not significantly depend on the sex of the animal as also supported with the study by Emsley (2001).

The gills show the highest concentrations of Zn (6398. 60 ± 909.51 μg/g) compared to the other tissues. This finding was also supported with the study conducted by Yap et al., (2003a) that high metals found in the gills.

This study was anticipated the metals exposure, thus the highest of metals found in the tissues than the previous study which conducted by Yap et al., (2003a). This might be due to gills have large surface contact with the surrounding area. Besides, filtrations of animals also may influence uptake rate from dissolved metals in aqueous phase as only done by gills of horseshoe crab in this study which is similar with the study by Wang and Fisher (1999). The gills filter the water to undergo the respiration process, this may cause the gills to gain the oxygen and filter the metals as well. The gills also showed the active part of horseshoe crab organs as it also was used as the paddle during swimming process, thus the metals will distribute and spread over their gills.

The lowest concentrations of metals, essential, Zn (23.35 ± 6.03 μg/g) was recorded in the carapace in this study. The carapace accumulates the lowest levels of metals also supported with the study conducted by Yap et al., (2008). This finding might be due to sequestration process in this chitinous shield covering the horseshoe crab as the method of excretion (Burger, 1994). The leg and the chelicerae also contained high levels of metal. The leg acts as the first organ that reached to the ground as horseshoe crabs are the benthic feeder whereas the chelicerae is used as the feeding leg of horseshoe crab (Botton, 1984). It can manipulate food to gnathobases of the legs (Botton, 1984). This also could be the reasons for the metals found inside both of the tissues.

The hepatopancreas also recorded high levels of metals. The high concentrations of Zn were uptake in the hepatopancreas due to it was the soft tissues as also supported with the study by Yap et al., (2008). The essential metals like Zn was important for them due to uptake in appropriate amount while the rest that they did not need will be sequestered as the excretion process by the hard tissues like the carapace (Burger, 1994).

The metals were found in the digestive tract but low in the metals uptake compared to the gills, leg, chelicerae and hepatopancreas. This might be due to this tissues act as the possible way to predict digestive absorptions from the particles (Amiard et al., 2007). With this assumption, the particles as well as the metals tend to uptake inside this organ. Besides, the metals like Zn also can enter the food chain through the water. In this study, only Zinc was exposed in order to see the metals uptake into the horseshoe crab tissues. This can provide the primary source of heavy metals that can contribute to the metals found in the digestive tract of the organism tissues like horseshoe crab as also suggested by Burger (1997).

The operculum also showed low levels of metals. It was expected to have high levels of metal since it has large surface contact with their surroundings (Yap et al., 2008). The result from this study, not follow as the expected. It might be due to this organ only function as the part of reproductive organs. In this study, the pattern of Zn showed that the uptake of the metal in all the tissues increased from day 0 till day 30. Uptake from the dissolved phase is important for metals into the organism tissues that tend to be transported by a facilitated process as reported by Wang and Fisher (1999).

The levels Zn was high in the gills. This finding was also supported with the study by Yap et al., (2008). Metals found in this organ might be due to it act as the most active part for the horseshoe crabs. It helps in gaseous exchange process as well as the filtration process. Rhythmic movement of the gill plates circulate water over the surface of the book gills (Fourtner et al., 1971), with that action most of the metals would highly accumulate onto the gills compared to the other tissues.

The levels Zn was low in carapace. This finding also supported with the study by Yap et al., (2008), that essential and non essential metals were low in the carapace of horseshoe crab. Zn found in carapace also effects from modified by the inherent sensitivities of the species present in Zn exposed ecosystem (Blanck et al., 2003). Besides, Zn were found in the soft tissues like hepatopancreas might be due to Zn is an essential trace nutrient for the aquatic organism. It could be suggested that the trace nutrients like essential (Zn) are required in hemocyanin biosynthesis and necessary for the functioning in many enzymes as reported with the study by White and Rainbow (1985). The metals also found high in the digestive tract in this study that is also similar with the study conducted by (Bebiano et al., 1993; 1994). This finding might be due to this accumulate the excess metal concentrations acts as storage organ reflecting long term metal exposure (Bebiano et al., 1993; 1994).

CONCLUSION

Continuous exposure of *T. gigas* to Zn (20 mg/L) up to 30 days give effects to metal concentrations in their tissues. As the metals increased in the water with exposure, the concentrations in horseshoe crab tissues also increased. Horseshoe crab had indicated the metals in the surrounding water. Metals uptake in gills of *T. gigas* was higher compared to the other tissues. Thus, the horseshoe crab had high potential to become a suitable bio-indicator (especially their gills) to detect the metal pollution in the surrounding water.

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