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EFFECTS OF TRIBUTYLTIN CHLORIDE ON DIFFERENT LIFE STAGES OF BRINE SHRIMP, Artemia salina (Linnaeus, 1758)

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By

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Faculty : Science

Organotin is one of the most effective antifouling biocides ever invented to reduce the threats pose by fouling organisms. A previous risk assessment of this biocide has been inadequate, so that its possible effects on aquatic ecosystems are a matter of great concern. This study explored the responses of common marine water Anostraca *Artemia salina* to lethal concentration of a type of organotin, tributyltin chloride (TBTCl) under different concentration, similar environmental conditions and at various stages of the lifestyle. Endpoints of this study encompassed hatching inhibition of cyst stage, mortality percentage in different stages of the life cycle *A. salina*, morphological changes in different stages, histological study about the pathological lesion changes in epithelial layer in gut *A. salina*, and effects TBTCl on reproductive organs, ovaries and testes with used Caspase-3 and HSP70 antibody in the adult stage to enable a comprehensive analysis of the effects on the organism. Samples for this study were obtained from Aqua fauna Bio Marine (Hawthorne, California, U.S.A). *A. salina* cysts hatched under the recommended physicochemical parameters. In the first experiment, the cysts were exposed to different the concentrations of TBTCl (5, 10, 15, 20, 25, 50, 75, 100, 150, 200 ng.L^{-1}) for 24 hours and observed hatchability performance, and influenced the percent survival to determine the median effective concentration 50% (EC_{50}). Then, cysts hatchability performance of different stages of *A. salina* under different concentration of TBTCl in the artificial sea water was studied. Median lethal concentration (LC_{50}) of different life stages of *A. salina* under different concentration of TBTCl (1, 25, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500 ng.L^{-1}), were determined via acute toxicity test for 24 hours. LC_{50} bioassay method was carried out to study acute toxicity of TBTCl on different stages of *A. salina.* After that, we studied the morphological changes and histopathological lesions in the epithelial layer at different stages life cycle of *A. salina*. The samples for histological evaluation were placed in Boun's fixative and continued with normal steps in histology method with minor modification. The prepared samples were stained with hematoxylin and eosin method, examined under a light microscope and recorded some photomicrographs for documentations.

Immunohistochemistry (IHC) for the first time used to study reproductive organs males and females *Artemia* sp after exposed to TBTCl. In brief IHC used to see effects of TBTCl on gonad tissue, where IHC using specific apoptosis markers where they followed these steps start with primary antibody on the gonad tissue and the primary antibody it will be attachment with the antigen in tissue, and then add the secondary antibody which will be linked with primary antibody. After that, add AB enzyme complex (ABC) (Avidin/Biotinylated). Finally, add enzyme substrate (DAB-chomogen) and the results as positive immunostaining were observed as brownish deposits, indicating the location of the Caspase-3 or HSP70 antigen-antibody reaction.

Results showed TBTCl has the ability to inhibitor hatching process of *A. salina* cysts when increasing the concentration of TBTCl and affects morphological development (total length and body width) of active nauplii. LC_{50} values of nauplii, juvenile adult male and adult female are 469.08, 226.69, 146.99 and 94.72 respectively. LC_{50} results showing the most sensitive stage to TBTCl is an adult female stage compared to other stages. The morphological results showed higher regression values, thus demonstrate a strong inverse relationship between morphological measurements for total length and body width by increasing TBTCl in different stages of *A. salina*. This study revealed for the first time study effects TBTCl on *A. salina* cell structure and revealed the pathological changes. Based on the findings the effects of toxicity TBTCl on *A. salina* tissues were associated with pathological lesions, which cause the mortality following exposure. Besides that, IHC revealed the apoptosis marker and immune system stress of the *A. salina* reproductive gonads in adults. In conclusion, these results confirmed that the females are more sensitive to TBTCl toxic compared to males. All results of present study strongly agree that TBTCl can cause various biological effects to target and non-targeted organisms, especially in the TBT contaminated areas with intensive shipping and industrial activities. Furthermore, this study showed that *Artemia* sp. is a good model organism to be used as a bioindicator for marine pollution studies. Since there are no detail studies on effects of TBTCl on cellular structures of model organisms, including *Artemia* sp., this study also reconfirms the suitability of the *A. salina* for bioassay in assessing marine aquatic toxicity profile of any toxicant with simple and reproducible results.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

KESAN TRIBUTILTIN KLORIDA KEPADA PERINGKAT HIDUP BERBEZA UDANG AIR GARAM, *Artemia salina* (Linnaeus, 1758)

Oleh

NAJLA MOHAMED ABOU SHAALA

Februari 2017

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Organotimah adalah salah satu biosida antitempelan paling berkesan yang pernah dicipta untuk mengurangkan ancaman yang disebabkan oleh organisma tempelan. Penilaian risiko biosida sebelum ini tidak mencukupi, sehingga kemungkinan kesan mereka ke atas ekosistem akuatik menjadi perkara yang membimbangkan. Kajian kali ini adalah untuk mengkaji tindakabalas Anostraca yang biasa ditemui dalam air laut iaitu *Artemia salina* terhadap sejenis organotimah, tributiltin klorida (TBTCl) di bawah kepekatan yang berbeza dalam keadaan persekitaran yang sama tetapi pada pelbagai peringkat hidup. Kajian merangkumi perencatan penetasan, peratusan kematian, perubahan morfologi dan histologi, bagi membolehkan analisis yang komprehensif untuk menkaji kesan organotimah terhadap organisma. Sampel untuk kajian ini diperolehi daripada di Utah Aqua Fauna Bio Marin (Hawthorne, California, U.S.A). Kami telah mengkaji kesan TBTCl pada *Artemia salina*, yang telah dicadangkan untuk menjadi petunjuk biologi sebatian organotimah dalam persekitaran marin. Dalam eksperimen pertama, sista tersebut diletakkan pada kepekatan maut TBTCl (5, 10, 15, 20, 25, 50, 75, 100, 150, 200 ng.L^{-1}) untuk tempoh 24 jam, median kepekatan berkesan (EC_{50}) ditentukan berdasarkan pemerhatian prestasi penetasan, dan peratus survival. Sista *Artemia* yang telah ditetaskan di bawah parameter fizikokimia yang disyorkan. Kemudian, preastasi penetasan cyst dan semua peringkat tumbesaran *Artemia* telah dikaji di bawah kepekatan TBTCl yang berbeza di dalam air laut tiruan. Median kepekatan maut (LC_{50}) peringkat tumbesaran yang berbeza *A. salina* di bawah kepekatan TBTCl yang berbeza (1, 25, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500 ng. L^{-1}), telah ditentukan melalui ujian ketoksikan yang singkat iaitu dalam tempoh 24 jam. Kaedah bioasai LC_{50} telah dijalankan untuk mengkaji ketoksikan singkat TBTCl pada peringkat tumbesaran *A. salina* yang berbeza. Selepas itu, perubahan morfologi dan kesan histopatological pada lapisan epitelium pada berbeza kitaran peringkat kehidupan *A. salina* telah dikaji. Sampel untuk penilaian histologi telah diletakkan di dalam pengawet Boun's. Kemudian, sampel telah direndam kepada hematoxylin dan eosin, sampel histologi

telah diperiksa di bawah mikroskop cahaya (LM) dan kemudian beberapa gambar telah ditangkap.

Immunohistochemistry (IHC) pertama kali digunakan untuk mengkaji sistem pembiakan jantan dan betina pada *Artemia* sp selepas didedahkan pada TBTCl. Secara ringkasnya, IHC digunakan untuk melihat kesan TBTCl pada tisu gonad, dimana IHC mengunakan penanda apoptosis, ia dimulakan dengan antibodi pertama akan terpasang pada antigen di dalam tisu gonad. Selepas itu, enzim ABC kompleks (Avidin/ Biotinylated) di tambah. Akhir sekali, enzim substrat (DAB-chromogen) ditambah untuk memberikan result positive immunostaining dengan memperlihatkan tompok-tompok warna coklat, dimana ia menunjukkan kehadiran reaksi Caspase-3 atau HSP70.

Dapatan kajian menunjukkan TBTCL mempunyai keupayaan untuk menhalang proses penetasan *A. salina* cysts apabila berlaku peningkatan kepekatan TBTCl dan ia juga memberi kesan tumbesaran marpologi (jumlah panjang dan luas badan) pada nauplii yang aktif. LC_{50} untuk naupli, juvenile, jantan yang dewasa dan betina yang dewasa adalah 469.08, 226.69, 146.99 and 94.72. Keputusan LC_{50} mendapati, peringkat yang paling sensitif pada TBTCl adalah peringkat betina yang dewasa. Keputusan marfologi menunjukkan regrasi yang tinggi, oleh itu ia membuktikan perkaitan yang kuat diantara ukuran marfologi untuk jumlah panjang dan luas badan dengan peningkatan kepekatan TBTCL dalam peringkat *A. salina* yang berbeza. Kajian ini telah mendedahkan untuk pertama kali kesan TBTCl pada sel struktur *A. salina* dan perubahan patalogi.

Berdasarkan penemuan kesan ketoksikan TBTCl pada *A. salina* tisu dikaitkan dengan luka-luka patologi, yang menyebabkan kematian selepas pendedahan. Selain itu, IHC menunjukkan penanda apoptosis dan tekanan pada sistem imun gonad pembiakan salina *A. salina* yang dewasa. Kesimpulannya, keputusan ini mengesahkan bahawa yang berjantina betina adalah lebih sensitif kepada TBTCl toksik berbanding yang berjantina jantan. Semua keputusan kajian ini bersetuju bahawa TBTCl boleh menyebabkan pelbagai kesan biologi kepada organisma tertentu dan yang bukan disasarkan, terutamanya di kawasan TBT yang tercemar dengan penghantaran intensif dan aktiviti industri. Tambahan pula, kajian menunjukkan *Artemia* sp merupakan organisma model yang baik untuk digunakan sebagai petunjuk biologi untuk kajian pencemaran laut. Hal ini kerana, tidak ada kajian terperinci mengenai kesan TBTCl pada struktur sel model organisma, termasuk *Artemia* sp. Kajian ini juga mengesahkan kesesuaian *A. salina* untuk bioesei dalam menilai profil tahap ketoksikan marin dengan keputusan yang mudah dan tepat.

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I certify that a Thesis Examination Committee has met on 6 February 2017 to conduct the final examination of Najla Mohamed Abou Shaala on her thesis entitled "Effects of Tributyltin Chloride on Different Life Stages of Brine Shrimp, *Artemia salina* (Linnaeus, 1758)" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

OT	Organotin
BT	Butyltin
PT	Phenyltin
TBT	Tributyltin
TBTCl	Tributyltin chloride
et al.	et alia / and others
IPCS	International Programme on Chemical Safety
Mm	Millimeter
LC_{50}	Median lethal concentration
HSP70	Heat shock protein 70
%	Percent
$\mu g.L^{-1}$	Microgram per liter
US$	United States Dollar
$ng.L^{-1}$	Nanogram per liter
M	Meter
UK	United Kingdom
e.g.	For example
OTCs	Organotin compounds
USA	United States of America
$g.L^{-1}$	Gram per liter
°C	Degree Celsius
Ppt	Part per trillion
pers. com.	Personal communication
$mg.L^{-1}$	Milligram per liter
‰	Part per thousand
mL	Milliliter
L	Liter
EC_{50}	Median effective concentration
G	Gram
W	Watt
DO	Dissolved oxygen

CHAPTER 1

INTRODUCTION

Organotin compounds (OTs) were widely used in antifouling paints since decades ago. Butyltin (BT) and phenyltin (PT) are among the commonly used organotin species in many commercial products and activities. During the last few decades, the BT had been used widely as antifouling agents in paints for aquaculture nets, ships, and boats (Champ and Seligman, 1996; Fent, 1996; Ohji, 2009). Recently, it became clear that antifouling compound with biocide properties such as tributyltin (TBT) could cause negative impacts on the components of the marine ecosystem. These compounds have been detected in various marine organisms, frequently at concentrations higher than the chronic or acute toxicity levels (Bryan and Gibbs, 1991; Alzieu, 1996). The TBT has negative effects on numerous aquatic organisms, including delayed molting, retardation of the regenerative growth, deformities in the limbs of the fiddler crab, and reduction in the burrowing activity (Weis et al., 1987; Weis and Perlmutter, 1987; Weis and Kim, 1988), reduction in the larval growth in Silversidee (Hall, 1988), impairment of egg production in calanoid copepod (Ohji et al., 2003; Johansen and Møblenberg, 1987), and avoidance reactions in the Baltic amphipod (Laughlin et al., 1984). In line with this, Sudaryanto et al. (2004) opined that continuous studies are needed to examine the environmental effects and toxicity of these anthropogenic tin compounds, with particular emphasis on their effects on the aquatic biota in the Malaysian coastal environment.

Long earlier, the International Program on Chemical Safety (IPCS) (Mercury, 1990) documented the presence of TBT at high levels in seawater, estuaries, freshwater, and sediments as well as in aquatic organisms, such as bivalves, gastropods, and fishes. This issue needs to be carefully investigated because several recent studies have confirmed the coastal waters of Malaysia are widely contaminated with butyltin compounds (BTs), which consequently, can spread to a wide range of environmental media and biota (Tong et al., 1996; Hashimoto et al., 1998; Sudaryanto et al., 2002). Sudaryanto et al. (2004) also reported that high concentrations of TBTs were recorded in Malaysian coastal waters, suggesting that there are ongoing inputs of TBTs to the coastal waters in Malaysia. Logically, the high levels of contamination with BTs reflect the lack of regulations of TBTs in Malaysia (Sudaryanto et al., 2004).

Furthermore, it has become evident lately that antifouling chemicals with biocide characteristics such as TBT have passive impacts on the marine ecosystem environment and these impacts have become worrying environmental issue worldwide (Champ and Wade, 1996; Bosselmann, 1996). In order to prevent destruction of the marine ecosystems, application of BTs to fish-farming equipment and small boats has been regulated or banned in developing countries from the late 1980s (Champ and Wade, 1996; Bosselmann, 1996). However, a substantial accumulation of BTs has been observed at many trophic levels in the marine food chain, including algae, plankton, cetaceans, fish, and crustaceans, thus indicating that the effects of BTs in the marine ecosystems are long lasting. It has been reported that the tri-organotins and TBT are the most toxic compounds and that their toxicity is evident at the nanogram-per-liter level.

High concentrations of BTs have been detected in lower trophic animals such as caprellids. It appears that TBT accumulates specifically in caprellids in the marine ecosystem, irrespective of the trophic level in the food chain, and that it may establish breakpoint for disturbance in the natural food chain structure, therefore causing other organisms to accumulate BTs at elevated concentrations due to their lower metabolic capacities to degrade TBT (Ohji et al., 2002a). In addition, it seems that the BTs accumulate in a species specific manner. Hence, studying implications of the species specific accumulation and biological impacts of BTs on brine shrimp (*Artemia salina*) may provide clues for further understanding of the accumulation mechanisms in coastal ecosystems as well as of the mode of action of BTs in these organisms. *A. salina* is a small crustacean (0.2–12 mm in body length, depending on the life stage), and it is a primitive aquatic arthropod of the Artemiidae family that is found worldwide and which especially lives in salt water and mud (Abatzopoulos et al., 2002). *A. salina* has a lifespan of 1–3 months and a shorter average life span of 35- 45 days, including embryo incubation and the maturation time of the hatching nauplii.

The use of *A. salina* to monitor small spatial and temporal changes in the baseline concentrations of TBTs has been proposed as a crustacean watch (Ohji et al., 2002a). Nevertheless, little information is available at present about the biological effects of TBTs on such features as growth, survival, reproduction, and the sex ratio of *A. salina*. The determination of such effects is prerequisite to credible Biomonitoring of the coastal ecosystem state using *A. salina* as a model.

Several studies have recently been performed on the biological effects of TBT, including investigations of reproduction, the growth rate, and the sex ratio in response to exposure to TBT at the ambient water levels in caprellids (Ohji et al., 2003). It was found that the sex ratio dramatically changed after even brief exposure to TBT during the embryonic stage (five days) (Ohji et al., 2002b). However, differences between the two genders in risk of survival response of crustaceans to TBT exposure are still unknown. The mechanism of action of TBT can differ from organism to another and the effects of exposure to TBT may also vary according to the developmental stage. Based that, no complete study has been done before on effect TBTCl on different stages of the life cycle of marine organisms include *Artemia* sp.

Justifications of Study

1- Tributyltin Chloride (TBTCl) can cause various biological effects to target and non-targeted organisms, especially in TBT-contaminated areas with intensive shipping and industrial activities.

2- There is no study conducted before in Malaysia to use *Artemia* sp asa model organismfor toxicity test in the marine environment
3- There are no detailed studies on TBTCl effects on the life cycle *A. salina* on morphological changes, histological cell structures of epithelial layer in the gut and Immunohistochemistry study of model organisms, including *Artemia* sp.
4- *Artemia* sp is a good model organism to be used as indicators for pollution studies, and *Artemia* is low cost and can use it anywhere at any time.

The objective of the present study was to investigate the exposure of *A. salina* to TBTCl during its lifecycle, including the post-hatching and the embryonic stages, and to examine the biological effects of TBTCl on *A. salina*. This study also evaluated the effects of TBTCl on hatchability of *A. salina* cyst and attempts to determine the median lethal concentration (LC_{50}) of TBTCl for the different growth stages of *A. salina.* Population-level effects of chemical pollutants are evaluated in terms of the decrement of the mean extinction time of populations based on LC_{50}, and estimation of the extinction risk of populations is utilized for the conservation of wildlife (Tanaka and Nakanishi, 2000). Furthermore, the biological effects of exposure on *A. salina* to TBTCl in artificial sea water was evaluated via morphological changes and cell structures of the epithelium layer during different growth stages of *A. salina*. Moreover, the present study examined the differences between the two genders in terms of the acute toxicity of TBTCl to *A. salina*, and investigated whether or not the effects of contamination by TBTCl are associated with intersex quality with *A. salina*. In other words, this study attempts to observe whether sex-specific differences in TBTCl toxicities exist in *A. salina*. Finally, the study investigates the effects of TBTCl on the reproductive gonads of the adult stage (males and females) tissue staining with hematoxylin and eosin also demonstrate to possess important functions in apoptosis and immune system stress in *A. salina* uses Caspase-3 and heat shock protein (HSP70). The results of this study will be the basis for discussion of the fluctuation in abundance of this species in the coastal ecosystem as well as the biological impacts of TBTCl.

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