World Applied Sciences Journal 33 (8): 1334-1340, 2015
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2015.33.08.15230

Selected Morphological Changes in Nauplii of Brine Shrimp (*Artemia salina*) after Tributyltin Chloride (TBTCL) Exposure

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Abstract: Early life stage of organisms is a critical period because it is very sensitive to changes that occur in the surrounding. The present study aimed to determine the lethal concentration 50 (LC_{50}) and identify morphological changes of brine shrimp (*Artemia salina*) nauplii exposed to tributyltin chloride (TBTCl) after 24hr exposure. Results showed the LC_{50} of TBTCl for *A. salina* nauplii was 469.08 $ng.L^{-1}$. Significant differences were discovered in the morphology of nauplii in control and those exposed. The nauplii underwent prominent abnormal growth in total length, head width, abdominal width and tail width. Other abnormalities included improper development of mandibles, underdeveloped endopod and endite, as well as swimming site in the second pair of antenna. These results indicated that TBTCl is an environmentally toxic substance with negative effects on non-target organism. Therefore, further in-depth investigation should be conducted to establish *A. salina* as a bioindicator for TBTCl contamination.

Key words: Antifouling Biocide · Tributyltin · *Artemia salina* · Early Life Stage · Abnormal · Morphology · Ecotoxicology

INTRODUCTION

Chemical pollutions do occur in the environment due to tin-based antifouling substances [1]. Besides, many organic compounds have also been introduced in antifouling paint formulations as booster biocides [2]. These antifoulants have been introduced in paint formulations to increase their performance against a wider spectrum of fouling organisms [3]. During the early 1960s, the extensive use of tributyltin (TBT)-based paints began and due to its great antifouling potency, it rapidly dominated the world market. However, it was only in the 1980s, when several impacts of TBT paints on aquatic organisms started to be described (e.g., abnormal growth, poor and reproductive failure in cultivated oysters *Crassostrea gigas*, imposex in female neogastropods, etc.) [4, 5]. Furthermore, TBT consists of organotin compounds with three alkyl groups attached to the tin atom, containing the element tin (Sn) that belongs to Group IV of the periodic table. Besides, TBT is found in wide applications as an antifouling agent in marine paint formulations, wood and stone preservatives, disinfectants, bactericides in cooling water, as well as agricultural fungicides [6]. TBT gained widespread use in antifouling paints in the 1970s and to date, it is the most effective antifouling agent in the market; saving commercial and military shipping over a billion dollars each year [7]. Furthermore, the measurements of TBTCl concentration in U.S. water since1991 indicated that there has been no risk of acute toxicity to aquatic organisms since 1994 and risk of chronic toxicity was considered as low as at 1996 levels [8]. Nevertheless, the results of the present study might add some information towards this direction, clarifying the higher magnitude of TBTCl toxicity.

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Crustaceans of the genus *Artemia* are in the phylum Arthropoda of the class Crustacea. The species of the genus *Artemia* (brine shrimps) are characterized by its adaptability to hypersaline environments [9]. *Artemia salina* is an example of a bisexual species. For taxonomic purposes, some criteria, such as morphology of adults, specific numbers of chromosomes, genetic distance and crossbreeding experiments, have been used extensively. Plus, *Artemia* species are considered as sensitive as other screening instruments or organisms [10], as they have some important advantages, including constant commercial availability during the year round, cost efficiency, easy to culture, short life-cycle, no feeding required during the assay and great offspring production [11, 12]. These advantages have led to a wide range of *Artemia*-based bioassays. Moreover, the determination of LC_{50} in nauplii (instar II-III stage), the hatchability of the cysts [13], the different age specimens [14] and the disruptions on an enzyme property [15] are only some of the *Artemia* endpoints that have already been examined as evidence of toxicity [16]. Nonetheless, the present study determined the lethal concentration 50 (LC_{50}) as well as the effects of tributyltin chloride (TBTCl) on the morphological development of brine shrimp (*A. salina*) nauplii.

MATERIALS AND METHODS

The hatching procedure adhered to the one described in ARC-test, a standardized short-term toxicity test with *Artemia* nauplii [17]. The hatching medium, artificial seawater of normal seawater salinity (35 $g.L^{-1}$), was prepared on site according to Dietrich and Kalle [18]. As for tests, approximately 0.5 g of cysts from brine shrimp *A. salina* was incubated in 500 ml of seawater in a cylindroconical tube at a temperature of 25±1 °C and with lateral illumination by a light tube (1000 Lux) for 24hr. All the cysts were kept in continuous suspension by aeration provided by a small air tube extending to the bottom of the hatching device. Over 18 up to 24hr, the aeration was stopped and the hatched larva (Instar I) was transferred to new petri dishes as they were sucked out via pipetting for subsequent manual distribution to the test petri dishes, whereby each petri dish had ten samples of nauplii and they were incubated at 25 C° for 24hr. After 24hr from the start of the test, all larvas mounted to the instar 2-3 stages. These youngsters had, in several papers, been shown to be the most sensitive stage [17, 19] and were accordingly used for toxicity tests [20].

The test was carried out in small petri dishes. Ten nauplii were transferred with a Pasteur pipet to each dish. The volume of seawater carried over with the nauplii was minimal. After that, the toxicant dilutions were prepared. Each toxicant dilution was transferred to the petri dishes. The distribution of the test solutions was carried out starting with the control (-ve) towards the highest concentration (+ve: increasing concentrations of toxicant). The dishes were filled with 10 ml of the respective concentrations of the toxicant and they were incubated at a temperature of 25±1 °C for 24 hours. Then, the petri dish was placed on the stage of the dissection microscope and the estimated mortality of 10 larvas was transferred and recorded. The nauplii were considered dead if no movement of the appendages was observed within 10 Sec. After that, the percentage of mortality was calculated from the total number of dead larvae for each concentration. The survivors were used to study the effect of toxicant on their morphological abnormalities, as depicted in Table 1. The morphological abnormalities of exposed *A. salina* nauplii in each toxicant was observed under magnification (10x) using a Leica M 205 stereomicroscopy attached to a camera with the aid of software (Easy-Grab; Noldus Information Technology).

RESULTS AND DISCUSSION

Based on the experimental protocol, a range finding test for TBTCl toxicants was conducted. The concentrations of TBTCl dilutions used were 400 $ng.L^{-1}$, 450 $ng.L^{-1}$, 455 $ng.L^{-1}$, 460 $ng.L^{-1}$, 465 $ng.L^{-1}$, 470 $ng.L^{-1}$, 475 $ng.L^{-1}$, 480 $ng.L^{-1}$ and 485 $ng.L^{-1}$. The mortality results showed that the toxicity range for 0% to 100% mortality was between 200 $ng.L^{-1}$ and 900 $ng.L^{-1}$ for TBTCl. Based on these data, a definitive test was designed, while the concentrations used for TBTCl toxicants are shown in Table 1.

Table 1: *A. salina* exposed to different concentrations of TBTCl ($ng.L^{-1}$) for 24-hr.

Concentration ($ng.L^{-1}$)	Mortality (%)	Survived (%)
450	20	80
455	23	77
460	30	70
465	46	54
470	53	47
475	56	44
480	66	34
485	83	17
490	86	14

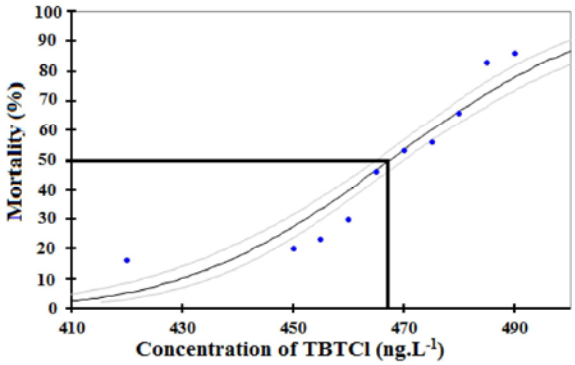

Fig. 1: Relationship between the mortality rates and increasing concentrations of tributyltin.

The respective 24hr mortality values of *A. salina* nauplii for TBTCl are shown in Fig. 1, which indicate the relationship between mortality rates and increasing concentration of TBTCl respectively. LC_{50} value was determined by the Probit Analysis using a statistical program XLSTAT-Pro (Version 2014.5.03). In this study, the minimum and the upper limits of LC_{50} values of TBTCl were 450 ng.L^{-1} and 490 ng.L^{-1}, while thecalculated LC_{50} value for TBTCl on *A. salina* nauplii determined in the present study had been 469.08 ng.L^{-1} (Fig. 1). In the present study, the LC_{50} value for TBTCl had been 469.08 ng.L^{-1}; similar to the result obtained in a study conducted by [21], which found a TBT dose value at 41.41 ng.L^{-1} with a bioassay method carried out to examine acute toxicity of TBT on *A. salina* by dissolving TBT in 0.01 ppt xylene. Besides, this study revealed that the system of TBTCl was indeed toxic and it was proved to be an environmentally toxic substance. However, acute toxicity alone does not give enough information about the environmental impact of using such an antifouling agent.

Besides, the toxicity of TBTCl was tested on nauplii of brine shrimp *A. salina* and their morphology were observed to change by undergoing certain developmental in terms of prominent growth, as well as total length, head width, abdominal width and tail width. Further formation of a pair of mandibles and two pairs of antennas occurred, whereby the second pair consisted of exopod, endopod, endite, swimming setae, etc., within 24hr.

Furthermore, the toxicity concentrations varied obviously at different concentrations in *A. salina* nauplii and the variation had been due to the increase in dose of TBTCl, which increased the effect on *A. salina*. Moreover, the one-way ANOVA indicated a significant difference at $p<0.01$ between the average mean values of toxicity concentrations due to the increased dosage of TBTCl that resulted in a decrease in the general growth of *A. salina* nauplii. Besides, the minimum and the maximum toxicity concentrations showed variations in total length (TL), width of head (WH), the width of the abdomen (WA) and width of the tail (WT) at a range of 0.0-932.51 ng.L^{-1},

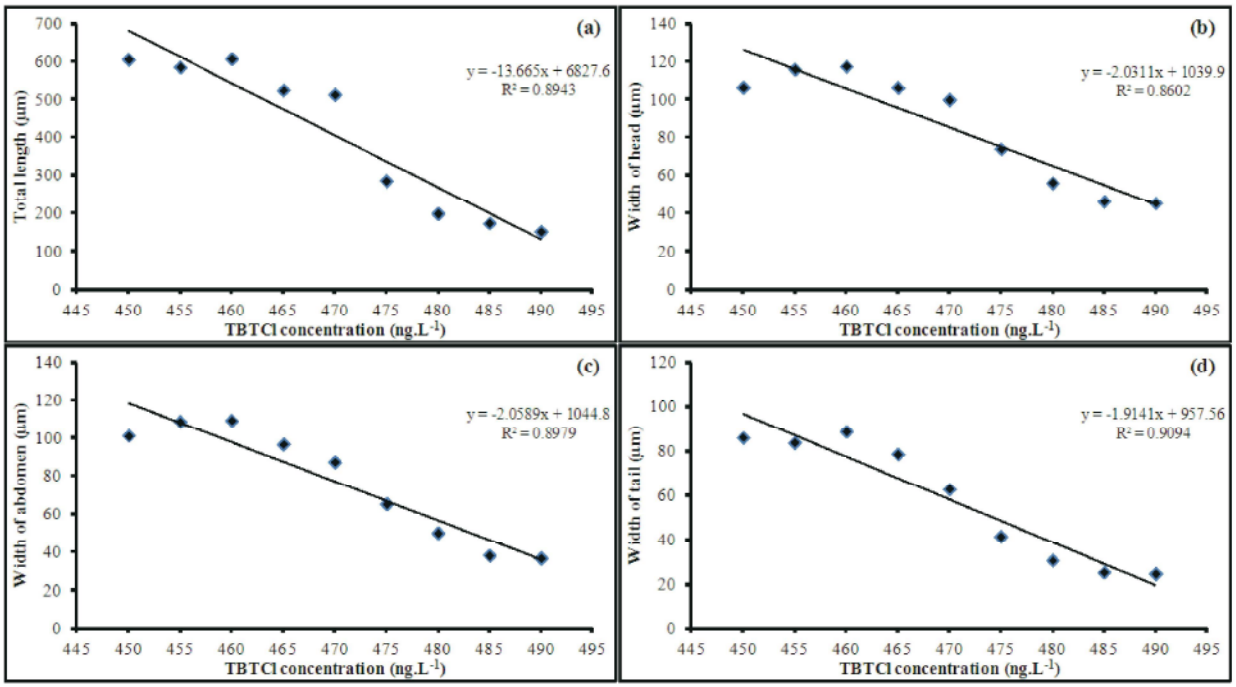

Fig. 2: Relationship of TBTCL concentrations with selected morphological measurements of *A. salina* nauplii after 24hr exposure [Remark: (a) total length; (b) width of head; (c) width of the abdomen; (d) width of tail].

Fig. 3: Morphological condition of *A. salina* exposed to TBTCl. [Remark: (a) negative control; (b) 455ng.L^{-1}; (c) 465ng.L^{-1}; (d, e) 475ng.L^{-1}; (f) 485ng.L^{-1}; Bar scale = 200 µm].

0.0-234.49 ng.L^{-1}, 0.0-259.51 ng.L^{-1}, and 0.0-828.66 ng.L^{-1}, respectively. These demonstrated that TBTCl had been responsible for the change in total length and width of body for.

A. salina after 24-hour of exposure. Generally, TL, WH, WA and WT decreased when TBTCl concentrations were increased (Fig. 2). High R^2 values demonstrate a strong inverse relationship between morphological measurements with the increase of TBTCl concentrations. Apart from that, significant differences in morphology were observed in all survivors of *A. salina* nauplii exposed to all toxicant concentrations of TBTCl (Fig. 3). The abnormalities discovered included improper development of mandibles, underdeveloped endopod and endite, as well as swimming setae in the second pair of antenna, which resulted in an imbalanced and irregular swimming pattern.

Therefore, the toxicity of TBTCl was tested against *A. salina* nauplii and the morphological changes in the nauplii of brine shrimp that underwent certain developments in prominent growth, as well as total length, head width, abdominal width, tail width, formation of a pair of mandibles and two pairs of antennae were recorded. In addition, the second pair of antenna consisted of exposed, indeed, endite etc., within 24hr. Furthermore, the one-way ANOVA employed indicated a significant difference at $p<0.01$ between the average mean values for total length, width head, width abdomen, as well as width tail and the different toxicity concentrations.

This indicated that the morphological development decreased when the doses of TBTCl were increased. This was also reported by Rosser [22] as the study showed the effect of alcohol percentage on the development rate of *A. salina* and found that alcohol did affect the development of brine shrimp, whereby the group of brine shrimp with 0.025% of alcohol displayed the slowest development. These results indicated that alcohol is a teratogen and harmful to the human fetus. Furthermore, Rao *et al.* [23] revealed acute toxicity in four organophosphorus insecticides when LC_{50}-exposed nauplii after 24hr indicated a maximum decrease in their swimming speed, while significant morphological alterations were noticed in CPP-exposed brine shrimps. Moreover, Zulkifli *et al.* [12] determined the suitability of *A. salina* for heavy metals acute toxicity test and the results indicated that *A. salina* nauplii is a sensitive bioindicator for heavy metals contamination. However, the effect of the toxicity of TBTCl and it is differences in sensitivity could be expected on marine species. It is obvious that TBTCl residues constantly seep into marine water, which cause adverse effects to marine organisms [5, 24, 25] with various mechanisms such as described by [26]. TBTCl may not posing threat to marine lifes, but also to freshwater organisms [27-30] that exposed to point- and non-point TBTCl sources. Therefore, simple, reliable and Inexpensive bioassay with suitable biomarkers are essential to detect the adverse effects of TBTCl on marine organisms. Hence, this present study proved that the brine shrimp assay could be a simple and accurate biomarker to assess the marine aquatic toxicity profile for any toxicant. It also demonstrated that TBTCl altered the morphology of brine shrimp, *A. salina*. Between the toxicants, TBTCl exhibited prominent adverse effects on the tested organism.

Therefore, further experiments are warranted to study the effects of extensively used TBTCl against different marine aquatic organisms. Based on these findings, there is a strong concern to use TBT and therefore, the alternatives provided should be less hazardous compared to TBT. Apart from that, the alternatives must be thoroughly evaluated to ascertain that they are less hazardous. With that, the results of the present study might contribute some information towards this direction, clarifying the higher magnitude of TBTCl toxicity.

The present study supports the idea of using brine shrimp (*A. salina*) as a simple and accurate bioassay organism to assess the marine aquatic toxicity profile for any toxicant. The present study showed that the LC_{50} value for TBTCl was 469.08 $ng.L^{-1}$ and this indicated that TBTCl is indeed toxic and it is definitely an environmentally toxic substance. Nevertheless, limited studies have looked into the significant morphological differences that occur and its toxicity on marine species. However, the effect of toxicity of TBTCL and the differences in sensitivity could be expected on marine species. As mentioned earlier, acute toxicity alone does not provide enough information pertaining to the environmental impact of using such an antifouling agent. Instead, further long-term toxicity studies and synergistic effects investigations would permit the complete evaluation of TBTCl as a hazardous chemical to aquatic organisms. Nonetheless, the results retrieved from this study strongly proved that the system of TBTCl is indeed acutely toxic.

ACKNOWLEDGMENT

This study was jointly supported by the special Fundamental Research Grant Scheme (FRGS) (Reference No. KPT.P.(S)400-7/2/29-4(65)) for matching fund research between JSPS Asian CORE Program and Universiti Putra Malaysia (UPM) and FRGS (Reference No. FRGS/1/2014/STWN01/UPM/02/4) from the Ministry of Higher Education Malaysia.

REFERENCES

1. Mohamat-Yusuff, F., K.W. Sien, W.L.W. Johari, A. Ismail, S.Z. Zulkifli and M. Mustafa, 2014. Potential tributyl-tin (TBT) biodegradation agent in contaminated sediment. Malayan Nature Journal, 66(1 and 2): 81-93.
2. Cima, F., M. Bragadin and L. Ballarin, 2008. Toxic effects of new antifouling compounds on Tunicatahaemocytes I. Sea-Nine 211 TM and chlorothalonil. Aquatic Toxicology, 86(2): 299-312.
3. Bustos-Obregon, E. and A. Vargas, 2010. Chronic toxicity bioassay with populations of the crustacean *Artemia salina* exposed to the organophosphate diazinon. Biological Research, 43(3): 357-362.
4. Alzieu, C., 1998. Tributyltin: Case study of a chronic contaminant in the coastal environment. Ocean and Coastal Management, 40(1): 23-36.
5. Mohamat-Yusuff, F., S.Z. Zulkifli, A. Ismail, H. Harino, M.K. Yusoff and T. Arai, 2010. Imposex in *Thais gradata* as a biomarker for TBT contamination on the southern coast of Peninsular Malaysia. Water, Air, and Soil Pollution, 211(1-4): 443-457.

6. Idris, E.I.M., F. Mohamat-Yusuff, H. Juahir, S.Z. Zulkifli and A. Ismail, 2012. Source apportionment of organotin compound based on spatial variation using multiple linear regression (MLR) and hierarchical agglomerative cluster analysis (HACA) in Southern Coast of Peninsular Malaysia. In Malaysia International Biological Symposium 2012: Sustainable Management of Bioresources, pp: 100-104.
7. Champ, M.A., 2000. A review of organotin regulatory strategies, pending actions, related costs and benefits. Science of the Total Environment, 258(1): 21-71.
8. Cardwell, R.D., M.S. Brancato, J. Toll, D. DeForest and L. Tear, 1999. Aquatic ecological risks posed by tributyltin in United States surface waters: Pre-1989 to 1996 data. Environmental Toxicology and Chemistry, 18(3): 567-577.
9. Dvoøøk, P., M. _d'ørský, K. Beòovø and M. Špalek, 2012. Selected morphological changes in Artemia franciscana after ionizing radiation exposure. Bulletin of Environmental Contamination and Toxicology, 89(2): 286-291.
10. Nunes, B.S., F.D. Carvalho, L.M. Guilhermino and G. Van Stappen, 2006. Use of the genus *Artemia* in ecotoxicity testing. Environmental Pollution, 144(2): 453-462.
11. Vanhaecke, P., G. Persoone, C. Claus and P. Sorgeloos, 1981. Proposal for a short-term toxicity test with *Artemia* nauplii. Ecotoxicology and Environmental Safety, 5(3): 382-387.
12. Zulkifli, S.Z., F.Z.A. Aziz, S.Z.M. Ajis and A. Ismail, 2014. Nauplii of brine shrimp (*Artemia salina*) as a potential toxicity testing organism for heavy metals contamination. In From Sources to Solution, Eds., Aris, A.Z., T.H. Tengku Ismail, R. Harun, A.M. Abdullah and M.Y. Ishak. Singapore: Springer, pp: 233-237.
13. MacRae, T.H. and A.S. Pandey, 1991. Effects of metals on early life stages of the brine shrimp, *Artemia*: a developmental toxicity assay. Archives of Environmental Contamination and Toxicology, 20(2): 247-252.
14. Barahona, M.V. and S. Sønchez-Fortún, 1996. Comparative sensitivity of three age classes of *Artemia salina* larvae to several phenolic compounds. Bulletin of Environmental Contamination and Toxicology, 56(2): 271-278.
15. Varó, I., R. Serrano, E. Pitarch, F. Amat, F.J. López and J.C. Navarro, 2002. Bioaccumulation of chlorpyrifos through an experimental food chain: Study of protein HSP70 as biomarker of sublethal stress in fish. Archives of Environmental Contamination and Toxicology, 42(2): 229-235.
16. Kokkali, V., I. Katramados and D. Jeffrey, 2011. Monitoring the effect of metal ions on the mobility of *Artemia salina* nauplii. Biosensors, 1(2): 36-45.
17. Vanhaecke, P. and G. Persoone, 1981. Intercalibration exercise on a short-term standard toxicity tests with *Artemia* nauplii, final report. EEC-ENV-396 (N), pp: 30.
18. Calleja, M.C. and G. Persoone, 1992. Cyst-based toxicity tests: IV. The potential of ecotoxicological tests for the prediction of acute toxicity in man as evaluated on the first ten chemicals of the MEIC program. Alternatives to Laboratory Animals, 20: 396-405.
19. Sønchez-Fortún, S., F. Sanz and M.V. Barahona, 1996. Acute toxicity of organophosphorous insecticides and protection by cholinergic antagonists and 2-PAM on *Artemia salina* larvae. Archives of Environmental Contamination and Toxicology, 31(3): 391-398.
20. Svensson, B., L. Mathiasson, L. Martensson and S. Bergström, 2005. *Artemia salina* as test organism for assessment of acute toxicity of leachate water from landfills. Environmental Monitoring and Assessment, 102(1-3): 309-321.
21. Panagoula, B., M. Panayiota and J. Iliopoulou-Georgudaki, 2002. Acute toxicity of TBT and IRGAROL in *Artemia salina*. International Journal of Toxicology, 21(3): 231-233.
22. Rosser, N.W., 2010. The effect of alcohol percentage on the development of *Artemia salina*. Central Virginia Governor's School 3020 Wards Ferry Road Lynchburg, VA 24502.
23. Rao, V.J., P. Kavitha, M.N. Jakka, V. Sridhar and K.P. Usman, 2007. Toxicity of organophosphates on morphology and locomotor behavior in brine shrimp, *Artemia salina*. Archives of Environmental Contamination and Toxicology, 53(2): 227-232.
24. Ismail, A., M.Y. Ferdaus and Z.Z. Syaizwan, 2004. Imposex in *Thais* sp. along the Straits of Malacca. In Proceedings of the First Joint Seminar on Coastal Oceanography, pp: 189-196.

25. Mohamat-Yusuff, F., S.Z. Zulkifli and A. Ismail, 2011. Imposex study on *Thais tuberosa* from port and non-port areas along the west coast of Peninsular Malaysia. Journal of Tropical Marine Ecosystem, 1(2): 1-9.
26. Mohamat-Yusuff, F., S.Z. Zulkifli, T. Otake, H. Harino and A. Ismail, 2014. Study on a new mechanism of sterilization in imposex affected females of tropical marine neogastropod, *Thais* sp. Journal of Environmental Biology, 35(5): 995-1003.
27. Jagtap, J.T., K.B. Shejule and D.P. Jaiswal, 2011. Acute toxicity study of tributyltin chloride on the freshwater bivalve, *Lamellidens marginalis*. World Journal of Fish and Marine Sciences, 3(2): 100-103.
28. Taha, H.M., H.A. Said, N.H. Abbas and A.F.M. Khaleafa, 2009. Biosorption and biodegradation of the antifouling compound tributyltin (TBT) by microalgae. American-Eurasian Journal of Scientific Research, 4(1): 1-6.
29. Kharat, P.S., T.S. Pathan and K.B. Shejule, 2014. Histopatholocal changes in hepatopancreas of freshwater prawn, *Macrobrachium kistnensis* Exposed to TBTCl. Middle-East Journal of Scientific Research, 22(9): 1396-1400.
30. El-Din, T.A.S., A.K. El-Sayed, H.A. Hamid and B.M. Mahmoud, 2012. Mode of action of some molluscicides on neurons of *Biomphalaria alexandrina* snail. Middle-East Journal of Scientific Research, 12(10): 1413-1419.