

Physiological and Histopathological Effects of Tributyletin (TBT) on *Lymnaea natalensis* and *Physa acuta*.

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Abstract:

Background: Two hermaphrodite snails *Lymnaea natalensis* and *Physa acuta*, the most abundant gastropods in Nile River was investigated to determine the effect of Tributyletin oxide (TBT) on some physiological parameters and histopathological changes in the ovotestis of both snails.

Methods: Nine plastic gars (six treated and three control sets) were used for each species of snails. Every aquaria contained 2 l. of dechlorinated water with concentration of 2 ppm of TBT. Samples of haemolymph and ovotestis tissues were taken after 2 and 4 weeks.

Results: The physiological data revealed that the concentration of glucose, cholesterol, calcium, total proteins, albumin, and globulin changed from one species to the other in different responses and according to time of exposure.

The histological data of the hermaphrodite gland or the ovotestis of both snails showed disturbances in differentiation and maturation process, cellular degeneration was also observed.

Conclusion: Due to the hazardous effects of TBT on the physiology and the histology of the ovotestis of both snail species, more research on the impact of TBT or related compounds has to be conducted. The use of TBT as antifouling agent has to be restricted in the developing countries and replace it by related compounds with less or no side effects.

Introduction

During the last decades, the scientists especially marine biologists have been concerned with new type of pollutants associated with antifouling paints known as organotin group (Meador, 1997; Gooding *et al.*, 1999; Hall *et al.*, 2000; Amr, 2004). Tributyletin TBT is one of the most powerful antifouling agents which have been found in sediments and surface water (Fent, 1996). Organotin compounds such as TBT and Triphenyletin (TPT) are used also as stabilizers in plastics, pesticides control of schistosomiasis and antifungal action in textiles and industrial water systems. (Amr, 2004).

Different species of water organisms were severely affected by TBT toxicity. TBT has been demonstrated to cause impairment in growth, development, reproduction and survival of many marine species (Haggera *et al.*, 2005). Recently, chronic toxicity, growth and reproduction in the freshwater gastropod *Lymnaea stagnalis* exposed to waterborne TBT over a range of four

concentrations in the range of 0–10 $\mu\text{g l}^{-1}$ has been investigated (Leung *et al.*, 2007). They observed that egg development was completely inhibited at 10 $\mu\text{g l}^{-1}$.

The major mode of action of TBT as other organotin group is that they act as hormonal disruptor for many marine organisms, especially mollusca (Gibbs and Bryan, 1996a). This hormonal disruption includes the development of male organs in gastropod females. This syndrome is known as imposex which can lead to sterilization and finally death. (Bech 2002; Marshall and Rajkumar, 2003 and Terlizzi *et al.*, 2004).

Imposex is thought to be irreversible and normal egg lying can be prevented and ultimately results in a population decline (Bryan *et al.*, 1986).

In some species, imposex is typically induced by TBT and TPT. Only a few reports, however, have presented evidence for population level effects of reproductive failure due to imposex.

Such evidence has been based on either morphological or histological methods (**Ide et al., 1997**).

In **2006**, **Toshihiro et al.**, suggested that reproductive failure (suppressed ovarian maturation and ovarian spermatogenesis) in adult *Babylonia japonica* accompanied with imposex induced by TBT and TPT which brought marked decline in the snail population.

Sex steroid hormones such as testosterone and 17 β estradiol are important physiologically in the development of sex organs and maturation of gonads in vertebrates. Thus, similar sex steroid hormones might also regulate the reproduction of invertebrates such as gastropods (**Le Blanc, 1999**).

Aromutase, the enzyme responsible for the conversion of testosterone to estradiol-17B (E_2), was found to be inhibited by TBT (**Heidrich et al., 2001**). Other studies showed other mechanisms of action in gastropods such as reducing of steroidogenesis or increasing of neuropeptide secretion (**Marcillo and Porte, 1999**).

In some publications TBT and TPT were used as biomarkers devised to measure molecular damage, developmental abnormality and physiological impairment combined with chemical analysis to determine the effects of pollution at different sites (**Galloway et al., 2004**).

Puccia et al. (2001) studied the relative impact of TBT chloride as one of the environmental pollutants using *Ciona intestinalis* ovary as a model system. They found that the effects of TBT exposure are concentration dependent and include a decrease of ATP levels, lipids content, and nucleic acid content. In contrast, a marked increase in calcium (Ca^{+2}) and glucose content was observed.

Since TBT and TPT act as hormonal disruptors, all the antistress responses start at the sub cellular level, and usually include the disruption of normal metabolic

Material and Methods:

Adult freshwater snails *Lymnea natalensis* and *Physa acuta* (> 20 mm in shell length) were collected from waters of irrigation channels and strains in Abou Rawash villages of Giza Governorate. The snails

pathway. These responses imply an energetic cost that interferes with the energetic budget for other vital process, such as growth and reproduction (**Widdows and Dankin, 1992**).

Glycogen level is one of the parameters that reflects the energetic and reserves status of organisms. Moreover, glycogen is used rapidly when organisms are under stress, and levels of this energy reserve have been suggested as useful biomarker of general stress (**Hugget, et al., 1992; and Vasseur and Cossu-Leguille, 2003**).

Lira et al. (2000) observed an increase in the total proteins concentration in the hemolymph of *B. similaris* at day 10 of starvation, when this value was 198% higher than that observed in the hemolymph of the fed snails and turning to values near to that of the control group at the end of the period of starvation analyzed (30 days).

Recently, it has become apparent that the growth of snails, due to their high-Ca requirements for shell formation, might be sensitive to metal exposure, especially when the metal interferes with Ca homeostasis (**Grosell and Brix, 2004**). They also suggested that the inhibition of Ca uptake by metals could potentially impair snail growth if Ca influx would become limiting for growth of the shell which consists almost entirely of $CaCO_3$.

Consequently, continued research of organotin compounds remains necessary due to the lack of toxicity data generated with fresh water organisms.

The present study was designed to determine the relative toxicity of TBT against two species of freshwater snails *Physa acuta* and *lymnaea natalensis*. Histopathological study was done on the gonads of both snails. Physiological study was also designed to determine the effect of TBT on the levels of glucose, cholesterol, calcium, total proteins, albumin and globulin in the heamolymph of both snails.

were kept in well aerated glass aquaria, each of them containing 3L of dechlorinated tap water for at least 96 h. to acclimatize them to laboratory conditions. The snails were fed on dried or fresh lettuce. The water was changed daily and dead snails were removed as soon as possible.

Preparation of stock solution of TBT:

The stock solution was prepared in the presence of absolute alcohol as primary solvent. 10 µl of tributyltin oxide TBT was taken and added to 10 ml of absolute alcohol and complete the solution to 1000 ml by adding tap water to give a concentration of 10 ppm. Prepared solution was stored in clean, dry and black bottle at room temperature.

Experimental design: Nine plastic gars (six treated and three control sets) were used for each species of snails. Every aquaria contained 2L of dechlorinated water and 2ppm of TBT for both snail sp. Samples of heamolymph were taken after two and four weeks for measuring various biochemical parameters. Glucose content was measured according to **Keilin and Hartree (1945)**. Cholesterol content was measured according to **Richmond (1973)**. Calcium was measured according to **Cowley et al. (1987)**.

Total proteins, globulin, and albumin heamolymph content were measured according to the method of **Doumas (1975)**. Ootestis of the treated and control groups of the two snail species were dissected after two and four weeks of treatment for histological study. After a 2-h Bouin's fixation, each piece of gonad was stored in 70% ethanol. Following dehydration through increasing alcohol concentrations, tissues were embedded in paraffin wax. Sections were cut and stained with haematoxylin and eosin as a basic stain (**Harris, 1990**).

Results:

Physiological Results:

Data in table,1 and figure,1 represent the results of the effect of TBT after two and four weeks on some physiological parameters. The results showed that, after exposure to two weeks the concentration of glucose decreased in both *Physa* and *Lymnea* snails to be 6.66 ± 1.500 mg/ml and increased in *physa* snails after exposure for four weeks to reach 20.0 mg/ml and increased also in *lymnea* to return to the control value with concentration of 13.330 ± 1.400 mg/ml

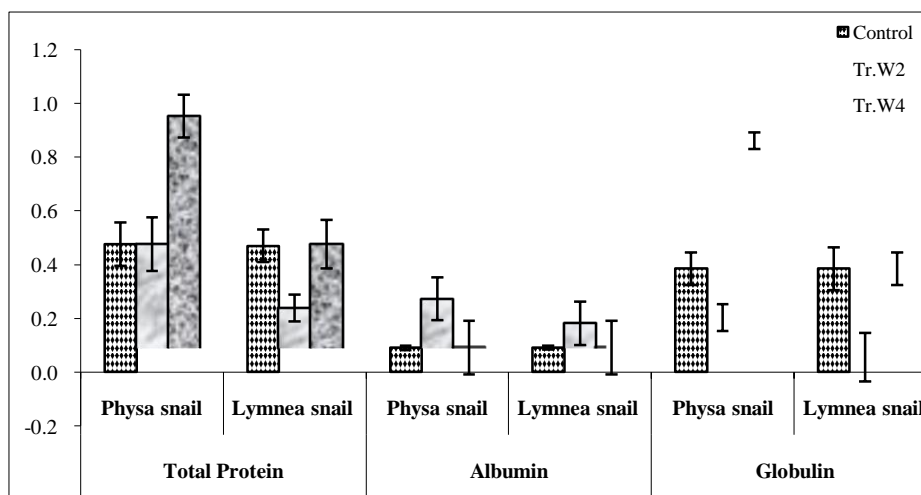
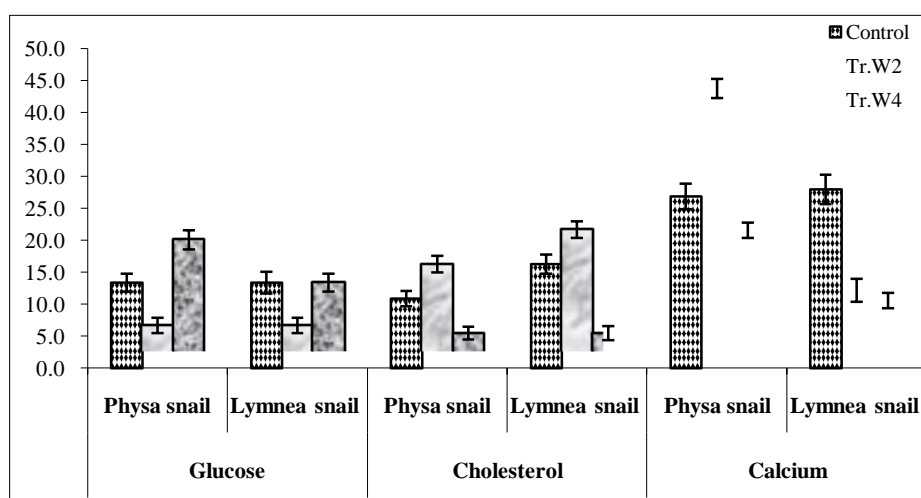
The concentration of cholesterol increased in both snails *Physa* and *Lymnea* after two weeks of exposure to reach 16.220 ± 1.300 mg/ml & 21.620 ± 1.300 mg/ml respectively, and then decreased after four weeks of exposure to 5.410 ± 1.000 in both snails.

The exposure of snails to TBT for 2 weeks showed a significant increase of calcium in *Physa* snail 43.680 ± 1.500 mg/ml after two weeks of exposure which decreased to 21.580 ± 1.200 mg/ml after four weeks of exposure compared with control. In the same time calcium concentration in *Lymnea* significantly decreased to 12.110 ± 1.800 mg/ml after two weeks of exposure and continues its decline after four weeks of exposure to be 10.530 ± 1.200 mg/ml.

The total proteins concentration of *Physa* snail have not changed compared with the control group 0.476 ± 0.080 mg/ml after two weeks of exposure and highly increased to 0.952 ± 0.080 after four weeks of exposure. In *Lymnea* snails a depletion of total proteins occurred after exposure to two weeks to be 0.238 ± 0.050 . concentration highly increased to 0.861 ± 0.030 in *Physa* and returned to the control value in *Lymnea* 0.385 ± 0.080 mg/ml.

Status		Control			Tr.W ₂			Tr.W ₄			T-test	
		Mean	±	SD	Mean	±	SD	Mean	±	SD	P ₁	P ₂
Glucose	<i>Physa snail</i>	13.330	±	1.400	6.660	±	1.200	20.000	±	1.500	0.003	0.005
	<i>Lymnaea snail</i>	13.330	±	1.700	6.660	±	1.200	13.330	±	1.400	0.003	1.000
Cholesterol	<i>Physa snail</i>	10.810	±	1.200	16.220	±	1.300	5.410	±	1.000	0.006	0.003
	<i>Lymnaea snail</i>	16.220	±	1.500	21.620	±	1.300	5.410	±	1.100	0.009	0.001
Calcium	<i>Physa snail</i>	26.840	±	2.000	43.680	±	1.500	21.580	±	1.200	0.000	0.018
	<i>Lymnaea snail</i>	27.900	±	2.300	12.110	±	1.800	10.530	±	1.200	0.001	0.000
Total Proteins	<i>Physa snail</i>	0.476	±	0.080	0.476	±	0.100	0.952	±	0.080	1.000	0.002
	<i>Lymnaea snail</i>	0.470	±	0.060	0.238	±	0.050	0.476	±	0.090	0.007	0.928
Albumin	<i>Physa snail</i>	0.091	±	0.008	0.273	±	0.080	0.091	±	0.100	0.017	1.000
	<i>Lymnaea snail</i>	0.091	±	0.007	0.182	±	0.080	0.091	±	0.100	0.121	1.000
Globulin	<i>Physa snail</i>	0.385	±	0.060	0.203	±	0.050	0.861	±	0.030	0.016	0.000
	<i>Lymnaea snail</i>	0.385	±	0.080	0.056	±	0.090	0.385	±	0.060	5.268	1.000

Table 1: Effect of TBT on glucose, cholesterol, calcium, total proteins, albumin, and globulin after two and four weeks



Histological results

The ovotestis of *Lymnea natalensis* and *Physa acuta* consists of large number of vesicles known as acini. Each acinus is enveloped in a sheath of squamous epithelium and thin connective tissue. Large vascular connective tissue lies between the acini and the covering epithelium of the mantle. In each acinus both male and female reproductive gametes are produced. Plate 1,2(A) are showing the ovotestis of control *Lymnaea natalensis* and *Physa acuta*. Each acinus possesses a number of immature oocysts, a few mature ova at the periphery and bundles of sperms in the center of the acinus. Two weeks of exposure of the ovotestis of snail *Lymnaea*

resulted in degeneration of most of the sperms beside fine observable histopathological changes in ova (Plate 1, b). After four weeks of exposure (Plate 1, c) marked degenerative effects in both sperms and most of the ova were showed, and the epithelial sheath was invaginated.

Plate (2 b) showed ovotestis of snail *physa* after two weeks of exposure where degeneration in both sperms and ova was observed. Four weeks of exposure (Plate 2,c) showed sever disruption of germ cells and more degenerative effect (tissue necrosis) in all the contents of acini. Epithelial tissue was invaginated and some acini seemed to be empty.

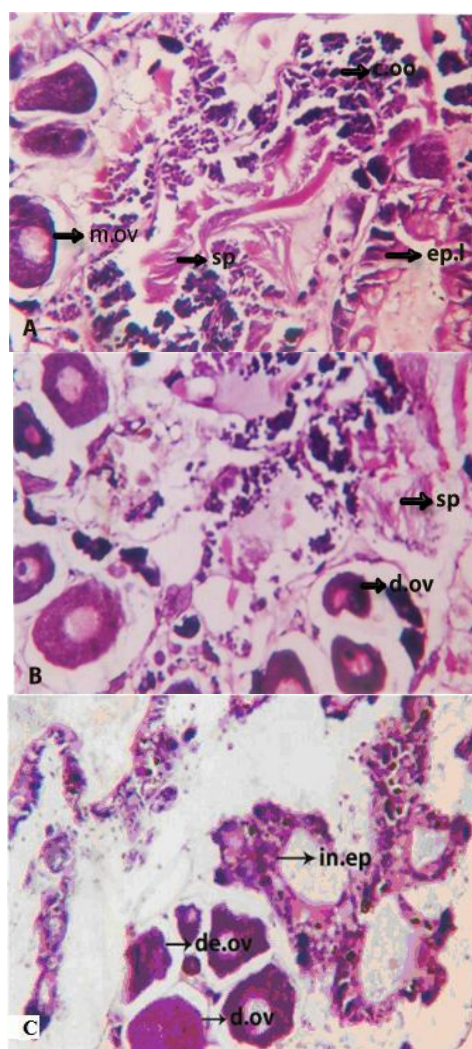


Plate 1: Sections of ovotestis of *Lymnaea natalensis*

- A- Control, showing mature ovum (m.ov), cluster of oogonia(c.oo), sperms(sp) , and epithelial lining (ep.l).
- B- Exposure to TBT for two weeks showed degenerated ovum (d.ov).

C- Exposure to TBT for four weeks showed invaginated epithelium (in.ep), degenerated and atrophied, ovum (de.ov.), and dead ovum (d.ov.).

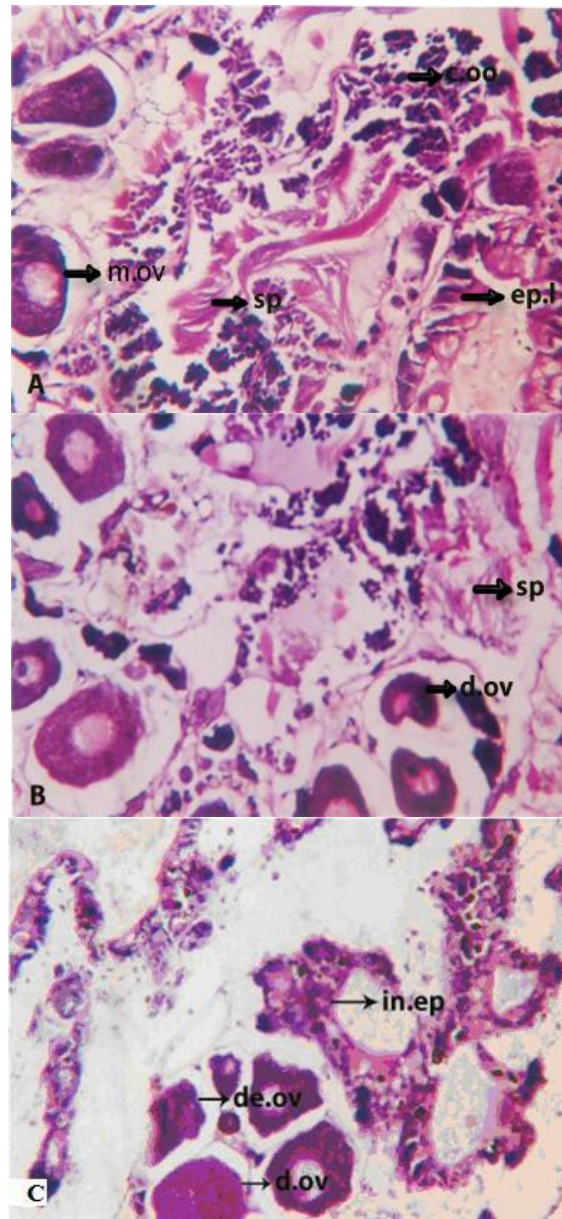


Plate 2: Sections of ovotestis of *Physa acuta* .

- A- Control showing mature ovum(m.ov),ovotestis acinus(ovt.ac.),squamous sheath(s.sq.),sperms(sp),cluster of oogonia(c.oo.), and epithelial lining(ep.l).
- B- Exposure for two weeks, showed degenerated sperms (d.sp.), degeneratd ovum (d.ov.) and invaginated epithelium (in.ep.).
- C- Exposure to four weeks, showing degenerated sperms (de.sp.), dead ovum (d.ov.) , and empty acinus (e.ac.).

Discussion

Tributyltin oxide TBT is an organotin compound used as preservative for wood, cotton, textiles, paper, and paints. It is also used as an antifouling agent in boats, ships quays, buoys, crab pots, fish nets, and cages, by protecting these surfaces and structure from the growth of mollusks and other marine organisms.

TBT has been used as an additive in paint and wood preservative to prevent the growth of mold and mildew, but was banned in 1988 from interior house paints because of the hazards it poses to human. Many countries have restricted the use of TBT as antifouling agent. In the developing countries like Egypt, it is still used as antifouling agent especially in pleasure boating activity and fish nets in Nile River. The banning of TBT compounds was not taken in consideration as it should be, so use of such materials may have a delayed effect on the fauna and flora of Nile River in the long run. The current study was designed to investigate the effect of TBT on two common species of freshwater snails (*Physa acuta* and *Lymnea natalensis*) inhabiting the River Nile and its branches, and the use of these invertebrates as bioindicators. The main source of TBT to the studied snails is the aquatic algae they feed on, beside the water source due to the life style of these two species of crawling on mud. Very little information is available on TBT effect on the physiological state of the invertebrates. However, the current work was trying to understand the effect of TBT on physiological status of these snails. The study focused on different physiological parameters which have an intimate relation to the development of gonads.

The depletion in glucose content in hemolymph of the exposing snails for two weeks indicates its rapid utilization as an energy reserve in a form of stored glycogen due to stress condition. This agrees the opinion of **Amr (2004)**. After exposure for a period of four weeks, glucose values tended to increase. This action may be due to under hypotoxic conditions, animals derive their energy from anaerobic

breakdown of glucose which was available to the cells by increased glycogenolysis (**Pankaj and Ajay, 2004**).

Arthur (1987) had suggested that catecholamine levels rise under stressful environmental conditions, enabling the increased utilization of glycogen for energy production, so glucose value appeared to increase in the snail hemolymph.

The concentration of hemolymph cholesterol of both *Physa* and *Lymnea* snail after exposure to TBT for two weeks significantly increased. This increase may be due to the change of the physiological adaptation under TBT stress. This is because in stress situation, the animal requires high energy where the tissue released its stored cholesterol to the circulating plasma. After exposure for four weeks the concentration of cholesterol was highly decreased due to its utilization by the animal under continuous stress (**Vink et al., 1995 and Amr, 2004**).

Both species had different behavior about the effect of TBT on the Ca^{+2} concentration of hemolymph. The results indicated that *Physa* snails showed highly increased level of Ca^{+2} after exposure for two weeks, then this concentration decreased after 4 weeks of exposure in the same snail. This indicated that TBT (hormonal disruptor) caused disruption of normal metabolic pathway as an antistress response (**Puccia et al., 2001**).

In the other hand, Ca^{+2} concentrations significantly decreased after exposure for two and four weeks in the case of *Lymnea* snails. The results of **Suzuki et al. (2006)** respected the same influences on *Physa* snails; they indicated that the plasma Ca and hypocalcemic hormones levels increased in Gold fish kept in water containing TBT.

Proteins are the most important and abundant macromolecules in the living beings, playing a vital role in the architecture and physiology of the cell and in cellular metabolism (**Mommsen and Walsh., 1992**).

Total proteins concentration of hemolymph of both species, *Physa* and *Lymnea* indicated confused results, where in *physa* its concentration remained constant after exposure for two weeks but it highly

increased after four weeks of exposure. *Lymnea* snails exhibited different reactions then increased to the same concentration as control after four weeks of exposure. **Ribeiro et al. (2001); and Amr (2004)** showed differed results where they indicated gradual decrease in the total proteins concentration after exposure to TBT.

In the other hand, **Ghosh and Chatter Jee (1989) and El-Emam and Ebeid (1989)** observed an increase of total protein concentration due to stress, they suggested that the increase in protein was most probably resulting from increase in lypolyses damage to cellular organization which give false indication coming from the damaged tissues within the snails.

On the other hand, the depletion of the protein fraction in the hemolymph of the snails in this experiment may be due to protein degradation for metabolic purposes. Under stress conditions, the dietary protein consumed by snails is not stored in the body tissue (**Baskaran and Palanichamy, 1990**) and hence the treated snails met their extra energy requirements from body proteins which are mobilized to produce glucose, the instant energy of which is made available for the snail by the process of gluconeogenesis (**Vasanthi et al., 1990**). Thus, the decreased protein content may be attributed to the destruction/necrosis of cells and consequent impairment in protein synthesis machinery (**Bradbury et al., 1987**).

Albumin concentration in the hemolymph of *Physa* showed a significant increase after two weeks of exposure to TBT then decreased to the control level after four weeks of exposure. In the same time, globulin concentration decreased after exposure for two weeks in both snails, then highly increased after four weeks of exposure in *Physa*, and still as control in *Lymnea* snail.

All these parameters influenced by the change of various hormones secreted by endocrine glands which already influenced by TBT (hormonal disruptor agent). These hormones alter the balance between the tissue and plasma protein.

The exposure of aquatic organisms to even very low levels of pesticides in their environment may result in various

where total proteins concentration significantly decreased after two weeks and biochemical, physiological and histological alterations in vital tissues of aquatic organisms (**Kulshrestha and Arora, 1984; Jonnalagadda and Rao, 1996; Bhavan and Geraldine, 2000 Cengiz et al., 2001; Cengiz and Ünlü, 2002; Çaliskan et al., 2003; Dutta and Arends, 2003**).

The two species of snails (*Physa acuta*, and *lymnea natalensis*) showed different changes in their ovotestis after exposure to TBT for two and four weeks period. The acini of ovotestis revealed marked disturbances in differentiation and maturation processes. After exposure for a long time, the ovotestis exhibited not only a severe disruption of germ cells formation but also reduction in the intensity of sperms and follicles within the acini. Cells degeneration also occurred, symptoms of atrophy appeared. The previous results showed complete agreement with the foundation of **Schulte-oehlmann et al. (2000); and Amr (2004)**.

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التأثيرات الفسيولوجية والهستولوجية لثلاثي بيوتيل القصدير على قوقعى ليمنيا ناتالينسس وفايزا أكويوتا

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أجرى هذا البحث لدراسة تأثير ثلاثي بيوتيل القصدير على نوعين من قواقع المياه العذبة الخنثى وهما قوقعى ليمنيا ناتالينسس وفايزا أكويوتا. وبعد ثلاثى بيوتيل القصدير من مجموعة من الملوثات تسمى (مربكة الغدد الصماء) وذلك لتدخلها فى عمل هذه الغدد مما يسبب إرباكا ملحوظا فى وظائفها. وتدخل هذه المادة فى الكثير من الصناعات فى صورة مبيدات فطرية أوبكتيرية وفى بعض أنواع الدهانات للمنازل والمراكب. وبمرور الوقت تم اكتشاف الآثار الجانبية الضارة لهذه المركبات مما دفع المنظمات الدولية للحفاظ على البيئة الى إعلان حظر استخدام مثل هذه المواد فى الدول المتقدمة وعلى الرغم من ذلك ما زالت مثل هذه المواد تستخدم فى الدول النامية ومنها مصر ومع إرتفاع نسبة هذه المواد الضارة وخاصة فى النيل والذى يعد من أهم مصادر مياه الشرب للإنسان و الحيوان بالإضافة الى العديد من الحيوانات المائية والاسماك التى تعيش فى مياه النيل كان لابد من إجراء بعض الدراسات مثل هذا النوع من الدراسة لتوضيح أثرها السىء ومن ثم الدعوة بحظر إستخدامها . ولقد أوضحت الدراسة أن هذا المركب له تأثير على نسبة تركيز بعض المواد الموجودة فى دم هذه القواقع مثل الجلوكوز، الكوليستيرول، الكالسيوم، البروتين، الألبومين، والجلوبيولين. وقد إختلف هذا التأثير من حيث الزيادة والنقص بالنسبة للمجموعة الضابطة على حسب نوع القوقع ومدة التعرض للمادة صوب الدراسة. وقد لوحظ أيضا وجود تأثير لهذه المادة على منطقة المناسل حيث حدث إرباك شديد فى عملية تكوين الخلايا التناسلية داخل المناسل وتحلل فى بعض هذه الخلايا وعند التعرض لفترة طويلة حدث موت شامل لهذه الخلايا مع وجود بعض الأماكن الخالية تماما من الخلايا التناسلية.