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Effect of Malathion on Protein Profile of a Polychaete Worm from Lake Burullus, Egypt.

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ABSTRACT

The Polychaete Neridid, *Hediste (Nereis) diversicolor*, isone of the soft bottom worms that were widely used as a biomarker of pollution in the coastal habitat. The present study was designed to assess the effect of the widely used pesticide, Malathion, on the protein profile of *H. diversicolor*, existing in an Egyptian polluted lake (Lake Burullus) connected to the Mediterranean coast by the Burullus outlet. SDS-PAGE technique was applied to detect polymorphic protein bands under different concentrations of Malathion in both worms and sediments. The results indicated a positive correlation between increasing temperature and polymorphism percentage of worm's protein bands and the Malathion content. The highest protein polymorphism (42%) was detected at high temperatures (30°C), whereas the lowest polymorphism (7%) was detected at low temperatures (17°C). Similarly, the highest concentrations of Malathion in the worm and sediment were recorded at 30°C and the lowest concentrations at the lowest temperature. The present study revealed also that Malathion level in the worms (4.17 - 4.4 ppm) was higher than in the sediment (3.14 - 3.87 ppm).

INTRODUCTION

The pollution of aquatic ecosystems by pesticides and heavy metals is a pressing problem that has drawn great attention everywhere (**Awheda *et al.*, 2015**). Contamination by widely utilized organophosphorous (OP) insecticides in agriculture, such as Malathion, is a potential problem for aquatic ecosystem, particularly during the cultivation season (**Thenmozhi *et al.*, 2012**).

The toxic effect of pesticides on aquatic organisms has received great attention, particularly in experimental works. The effect of pesticides on polychaetes was an important goal for numerous experimental studies (e.g. **Scaps *et al.*, 1997; Hansen *et al.*, 1999; Scaps and Borot, 2000; Dumbauld *et al.*, 2001; Ernst *et al.*, 2001; Mendez, 2005; Ait Alla *et al.*, 2006a; Granberg *et al.*, 2008; LinLan *et al.*, 2010; Willming *et al.*, 2013**). Malathion is a widely used pesticide in agriculture and ultimately reaches the aquatic habitat through water drainage. Because Polychaetes are the most dominant invertebrates in the contaminated aquatic habitats, particularly soft bottoms, they have

long been used to assess the health of benthic communities and as biomarkers of aquatic habitat contamination (**Dean, 2008; Croquer *et al*., 2016**).

Several experimental studies have been done on the toxicity of Malathion to polychaetes (e.g. **Buratti *et al*., 2005; Capkin *et al*., 2006; Singh and Mishra, 2009; Gulfer *et al*., 2009**). The nereidid *H. diversicolor* is abundant in brackish waters and coastal areas and has high tolerance to extremely stressed conditions (**Ait Alla *et al*., 2006b; Bouraoui *et al*., 2015**). Therefore, this species was intensively used as pollution biomarker (e.g. **Sun and Zhou, 2008; Tairova *et al*., 2008; Solé *et al*., 2009; Bouraoui *et al*., 2010; 2015; Buffet *et al*., 2011; Catalano *et al*., 2012; Ghribi *et al*., 2019**). Moreover, *H. diversicolor* is considered a potential candidate for monitoring the effects of organophosphate pesticide pollutants in an estuarine ecosystem (**Scaps *et al*., 1997**).

Lake Burullus is one of the Egyptian lakes occurring on the south eastern side of the Mediterranean Coast, and receives a huge amount of agricultural waste waters, including Malathion. *H. diversicolor* is a dominant invertebrate in the bottom of Lake Burullus, and therefore was selected to be used as biomarker to assess the pollution condition of this lake.

The aim of the present paper was to evaluate the employment capability of Polychaete as an environmental stress biomarker, through the relation between the total soluble protein fractions polymorphisms with the accumulated Malathion in the Polychaete and sediments in the environmentally-stressed Lake Burullus.

MATERIALS AND METHODS

The study area

Lake Burullus is one of the Egyptian lakes on the Mediterranean coast lying under anthropogenic stress (**El-Kafrawy *et al*., 2015**). It receives about 3904 million m^3/year of different waste waters (**El-Zeiny and El-Kafrawy, 2017**) without any pre-treatment (**Nafea and Zyada, 2015; El-Amier *et al*., 2017**).

Sampling methods

The present study was carried out in the periods from September 2018 to September 2019. The sediment samples and their contents of *H. diversicolor* were collected from a fixed site in Lake Burullus, kept in an ice box till reaching the laboratory, where they were kept in the fridge for further analysis. The concentration of Malathion was estimated in both the sediment and the worm, and the total soluble proteins and specified related Malathion degradation protein fractions polymorphisms were evaluated. The activity levels of glutathione S transferase Omega 1 (GST-O), a Malathion biodegradation key role enzyme, was estimated and compared among the different worms during the experiment.

Analytical methods

18S rRNA molecular identification marker

18S rRNA gene sequences were amplified and sequenced to identify the worm used in the present study. Total genomic DNA was extracted from the specimen through E.Z.N.A.® Mollusc DNA Kit (D3373-00) according to the manufacturer protocol. 18S rRNA genes were amplified using modified primers, as shown in Table (1) (**Medlin *et***

***al.*, 1988**). PCR was performed using peqSTAR gradient thermal cycler with the following conditions: 95°C for 3 min; 35 cycles with 94°C for 35 s, 45–55°C for 45 s, and 72°C for 1 min; final extension at 72°C for 10 min (**Sung-Rhee *et al.*, 2007**). After detecting amplicon via Agarose gel electrophoresis, PCR products were purified with E.Z.N.A.® Gel Extraction Kit, (D2500-01, Omega BIO-TEK, and USA). Sequence analysis was employed, using the ABI PRISM® 3100 (Genetic Analyzer, Micron-Corp. Korea). CLUSTAL W software analysis was employed to align 18S rRNA sequence (**Thompson *et al.*, 1994**). Moreover, PAUP* software (version 4.0b8) was applied to perform Maximum likelihood analysis (**Swofford, 2001**).

Determination of Malathion concentrations in sediment and worms

The Malathion in sediment was measured by USEPA method 3542B (**EPA Standard Methods, 1997**), using Malathion-(diethyl-d10) reference material R1057-10MG, supplied from Honeywell Specialty Chemicals Seelze. Standard Malathion solution (l ml= 400/ flg) was prepared in methanol medium. Malathion in sediment sample (0.5 g) was extracted in a mixture of hexane and acetone (1:1 v/v) for 12 hrs and the extract was concentrated in a rotary evaporator. Malathion colorimetric reaction was measured using T80+ double beam spectrophotometer (PG, Instrument).

For extraction of Malathion from the polychaete tissue, samples 1–3 g of the worm was placed in 100 ml of CH_3CN, the mixture was centrifuged at 3,000 r/min for 5 min, and 10 ml of the extract was decanted. The solution was loaded into a solid phase extraction cartridge (Florisil) and extracted with 5 ml acetone: hexane (2:8 V/V), and the residue was dissolved in 1 ml acetone (**Ho *et al.*, 2013**). Malathion was quantified via modified colorimetric method using T80+ double beam spectrophotometer (PG, Instrument) was used.

Polychaetes Electrophoretic analysis

To evaluate the influence of Malathion on the Polychaete worm, total soluble protein was purified through Tri-Fast solution (Peqlab, VWR Company) according to the manufacturer protocol and fractionated via SDS Polyacrylamide gel electrophoresis (SDS-PAGE) with 10% according to (**Lammli, 1970)**. Gel documentation system (Geldoc-it, UVP, England) was applied to evaluate protein fractions variations using Totallab analysis software (www.totallab.com, Ver. 1.0.1).

Colorimetric method for determination of glutathione S transferase Omega 1 (GST-O)

Equal amounts of protein (40 µg) from the clarified homogenate were fractionated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Loaded *Hybond*™ nylon membrane (GE Healthcare) with the worm's transferred protein was immunologically detected via incubated overnight at 4°C in Antibody Solution containing Anti-Glutathione S Transferase theta 1 primary antibodies (175418, Abcam, Cambridge, U.K.). Membrane with Anti-Glutathione S Transferase theta 1 and anti-actin was visualized using Gel documentation system (Geldoc-it, UVP, and England) and the density was analyzed with ImageQuant 5.2 (GE Healthcare, Buckinghamshire, U.K.).

Depuration experiments

To assess the ability of *H. diversicolor* to release the Malathion accumulated in its body, some experiments were carried out in the laboratory. A number of 10 – 15 worms with approximately similar size were placed in triplicates in small glass jars containing clean water with salinity similar to that in the original habitat of the worm. The concentrations of Malathion in the tested worms were measured directly after bringing them from their habitat. Two sets of experiments were carried out in laboratory; in the first set of experiments, the Malathion content was measured frequently every three days, while in the second set of experiments the Malathion was determined after one week from the beginning. These experiments were repeated on worms collected at different times from Lake Burullus.

RESULTS

Identification of the polychaete worms

Hediste diversicolor was identified based on morphological characteristics and 18S rRNA gene sequences. As shown in Figure 1A and B and Table (1), alignment of 18S rRNA gene sequences was identified as *H. diversicolor* (Accession number: LC381864.1) with high homology of 100% of identity.

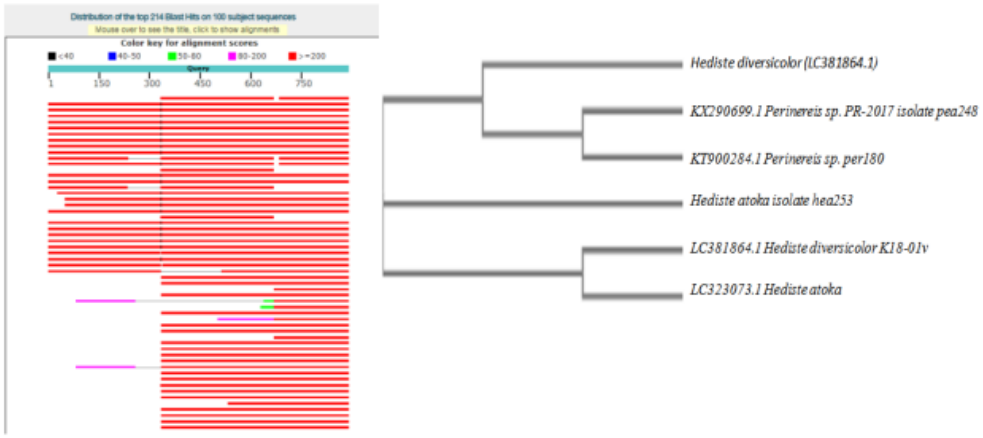

Figure (1): (A) Alignment data and (B) phyllogenetic tree for specific 18S rRNA nucleotide sequence.

Table (1): identified worm 18S rRNA nucleotide sequence with highest homology

High homology	GenBank	Identity %
Hediste diversicolor	LC381864.1	100
Hediste atoka haplotype: K18-02	LC323073.1	100
Hediste atoka haplotype: K18-01	LC323072.1	100

Electrophoretic Protein fingerprinting results

Figure (2) and Table (2) illustrate the relationship between the protein polymorphism and temperature variation. The highest protein polymorphism (42%, 41% and 33%) was observed during the periods of the highest temperature (30, 29 and 28°C, respectively). Meanwhile, the lowest temperature time periods with 17 and 19°C reflected decreasing of protein polymorphism to 7 and 8%.

Malathion level

As shown in Table 3, the Malathion level in the worm varied between 3.44 and 4.82 µg gdw^{-1}. Minimum values were recorded at low temperatures (17-20^{o}C respectively), whereas high protein concentrations were recorded at the highest temperature (25-30°C). In sediments, Malathion levels were lower concentrations than worms and fluctuating between 3.14 and 3.88 µg gdw^{-1}. However, the high concentrations in sediment were also recorded during the warm season and the low values appeared in cold season (Table 4).

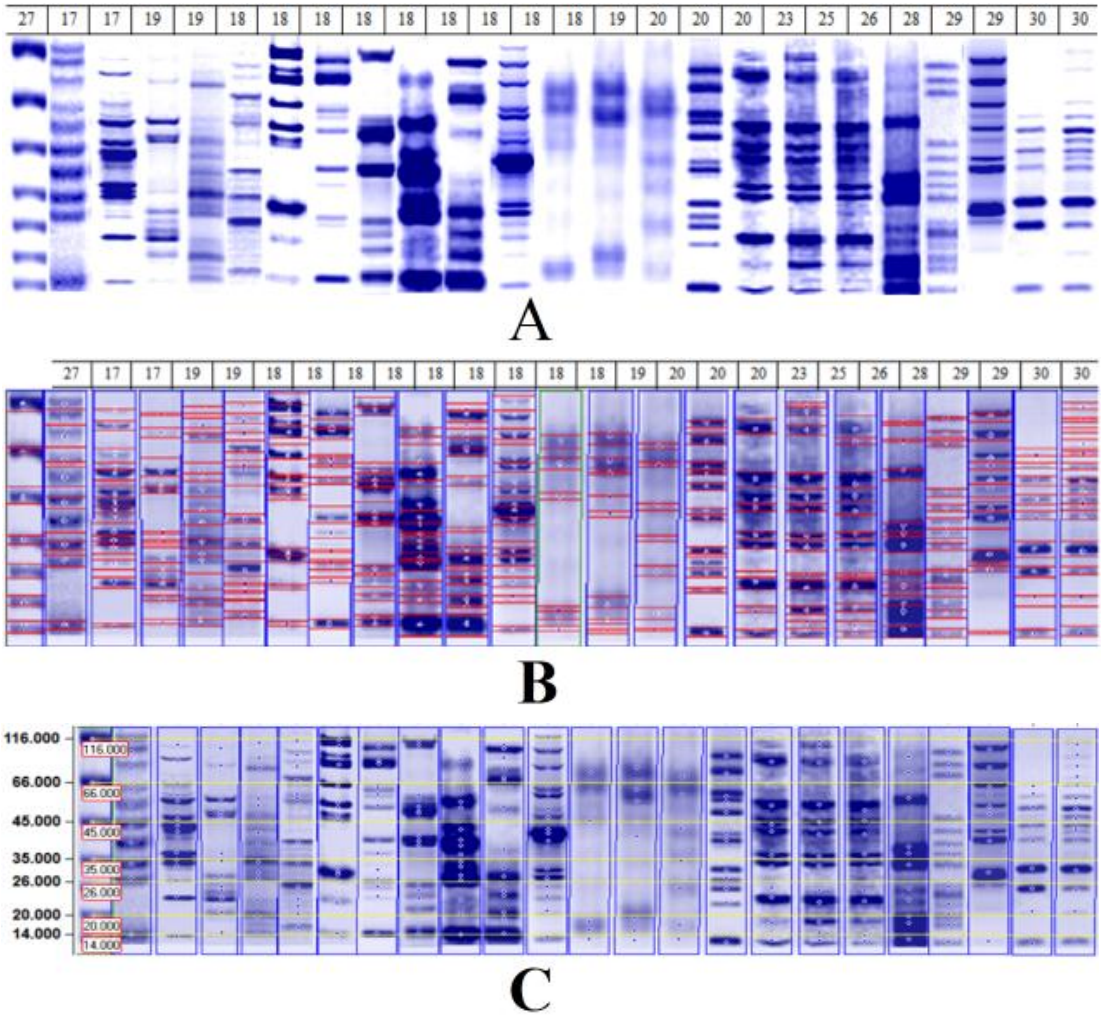

Figure (2): Protein electrophoretic patterns (A), computerized analysis (B) and molecular weight calculation (C) in different times of the year.

Table (2): total, polymorphic and polymorphism % for electrophoretic protein patterns of worm during temperature variation.

°C	Total fractions	Polymorphic fractions	Monomorphic fractions	Polymorphism %
27	10	3	7	30
17	15	1	14	7
17	13	2	11	15
19	14	3	11	21
19	13	2	11	15
18	13	2	11	15
18	15	5	10	33
18	16	3	13	19
18	16	3	13	19
18	15	2	13	13
18	17	4	13	23
18	6	1	5	17
18	8	1	7	12
18	7	1	6	14
19	12	1	11	8
20	10	2	8	20
20	13	3	42	23
20	12	3	9	25
23	11	2	9	18
25	14	2	12	14
26	11	2	9	18
28	9	3	6	33
29	15	5	10	33
29	12	5	7	41
30	13	5	12	40
30	14	6	8	42

Table (3): Average of accumulated Malathion level in worm body at different temperatures.

°C	Malathion (ppm)	°C	Malathion (ppm)
17	3.60	17	4.82
17	4.17	22	3.92
19	4.2	23	4.38
19	4.28	25	4.32
18	4.37	26	4.30
18	3.98	28	4.41
18	4.02	29	4.37
19	3.49	28	4.7
18	4.17	30	4.52
20	3.44	29	4.31
18	3.98	30	4.40
18	4.10		

Table (4): Average of accumulated Malathion level in sediment at different temperatures.

°C	Malathion (ppm)	°C	Malathion (ppm)
17	3.24	17	3.29
17	3.56	22	3.46
19	3.14	23	3.50
19	3.20	25	3.49
18	3.18	26	3.46
18	3.73	28	3.80
18	3.66	29	3.81
19	3.58	28	3.79
18	3.61	30	3.88
20	3.62	29	3.81
18	3.69	30	3.87
18	3.67		

First experiments showed higest recovery influence with increasing degradation rate (35%) comparing with fifth experiment which reflected the lowest recovery effect with decreased degradation rate (0.2%) (Table 5, 6).

Varied degradation rate were arranged descendingly (11, 8.1, 4.9, 2.5, 0.7, 0.7, 0.4, 0.3 and 0.2) for eighth, sixth, tenth, seventh, second, third, ninth, fourth and fifth recovery experiments respectively.

Our obtained results indicated positive correlation between degradation rates % and Average of Polymorphism % of protein patterns for ten recovery experiments. On one hand, first recovery experiment reflected highest degradation rates % (35) and highest average of Polymorphism % of protein patterns (59). On the other hand, lowest degradation rates % (0.2) which characterized twelfth recovery experiment was corresponded to the most decreased average of Polymorphism % of protein patterns (0).

Table (5): Total, polymorphic, monomorphic, polymorphic fragments and average of Polymorphism % of protein patterns for ten recovery experiments

	Experiment 1 (1)					Experiment 2 (2)		Experiment 3 (4)			Experiment 4 (6)		Experiment 5 (12)		Experiment 6 (13)		Experiment 7 (14)		Experiment 8 (16)						Experiment 9 (17)		Experiment 10(19)	
	D1	R1	R2	R3	R4	D2	R2	D3	R1	R2	D4	R4	D5	R5	D6	R6	D7	R7	D	R1	R2	R3	R4	R5	D9	R9	D10	R10
ion	3.24	2.80	2.65	2.15	2.11	3.21	3.19	3.28	3.24	3.22	4.41	4.40	4.21	4.16	3.98	3.66	4.02	3.92	4.17	4.08	3.84	3.81	3.77	3.72	3.92	3.76	4.32	4.11
ion rate	35					0.7		0.7			0.3		0.2		8.1		2.5		11						0.4		4.9	

Table (6): degradation values and degradation rates % for ten recovery influence experiments.

	Experiment 1 (1)					Experiment 2 (2)		Experiment 3 (4)			Experiment 4 (6)		Experiment 5 (12)		Experiment 6 (13)		Experiment 7 (14)		Experiment 8 (16)						Experiment 9 (17)		Experiment 10(19)	
	D1	R1	R2	R3	R4	D2	R2	D3	R1	R2	D4	R4	D5	R5	D6	R6	D7	R7	D1	R1	R2	R3	R4	R5	D9	R9	D10	R10
Degradation Value	3.24	2.80	2.65	2.15	2.11	3.21	3.19	3.28	3.24	3.22	4.41	4.40	4.21	4.16	3.98	3.66	4.02	3.92	4.17	4.08	3.84	3.81	3.77	3.72	3.92	3.76	4.32	4.11
Degradation rate %	35					0.7		0.7			0.3		0.2		8.1		2.5		11						0.4		4.9	

DISCUSSION

Malathion is a phosphorodithioate organophosphorus pesticide, soluble in water to 145 mg/L at 25°C (**Tomlin, 1994**). It degrades more rapidly in alkaline water, persisting in river water from 52% to 21% over 11 to 14 days but its bioaccumulation was not significant (**HSDB, 1996**). Bacteria could degrade more than 90% of the initial Malathion concentration within 4 days (**Hamouda *et al*., 2013**).There is no actual estimation of Malathion concentration reaching the aquatic habitat from the agriculture. However, **Mastrota et al. (2010)** estimated 0.614 - 89.8 µg/l, with a maximum of 1120 µg/l.

Due to lack information about Malathion accumulation in the aquatic biota, it was not possible to compare our results with other studies. The present study revealed significant correlation between temperature and Malathion concentration in both the worm's body ($r = 0.4615$, at $p< 0.05$) and in the sediment ($r = 0.6101$, at $p< 0.05$). Temperature affects the pesticides toxicity through alteration of Acetylcholinesterase (AChE) activity (**Hamza Chaffai *et al*., 1998**) and physiological mechanisms (**Cairns *et al*., 1975; Ward and Stanford, 1982**). The toxicity of agricultural chemicals including malathion to seems to be different with the species and with the temperature variation (**Johnson and Finley 1980; Willming *et al*., 2013**). This because temperature may modify the ability of organism to detoxify contaminants or their biotransformation rates (**Noyes *et al*., 2009; Rohr *et al*., 2011; Hooper *et al*., 2013**). Significant increase of AChE activity was indicated in *H. diversicolor* at a temperature of 12°C (**Scaps and Borot, 2000**). Furthermore, several organophosphate insecticides exhibited more toxicity at high temperatures, while other insecticides increased toxicity at lower temperature (**Coats *et***

***al.*, 1989; Lydy *et al*., 1999; Harwood *et al*., 2009**). By contrary, other studies supposed that poisonous effect of pesticides is not dependent upon environmental conditions such as temperature, pH, oxygen content and presence of residue molecules (**Capkin *et al*., 2006; Singh and Mishra, 2009; Gulfer *et al*., 2009**).

During the present study, the toxic effect of malathion was assessed by the variation of protein profile at different malathion concentrations, whereas noticeable polymorphism was observed. Malathion displayed LC50 for different fish species at widely variable concentrations 0.06 - 39,600 µg/L (**Newhart, 2006; Ahmad, 2012**). Although malathion concentration of 200 - 1,250 ppb reduced AChE activity by 70 – 90 % in different fish species (**Coppage and Matthews, 1974**), it had no effect on some invertebrate tissues (**Galgani and Bocquene, 1990**). Meanwhile, the AChE activities to Malathion were greatly sensitive in *H. diversicolor* than in *Mytilus edulis* and *Palaemon serratus* (**Scaps *et al*., 1997**).

The Malathion concentrations in lake sediment during the present study (3200 - 3880 ppb) appeared to be within the range recorded by several previous studies as toxic for different fish and invertebrates, including polychaetes. These concentrations were about 13 folds that detected by **Scaps et al. (1997)** as inhibitory dose for AChE activity in *H. diversicolor*. The accumulation of Malathion in *N. diversicolor* (3440 - 4820 ppb) from Lake Burullus caused up to 42% polymorphism in the protein profile of during summer. This may be in consistent with **Hai *et al*. (1995)** who stated that the oxidative stress of organophosphate compounds may cause protein degradation besides their inhibitory effect on AChE. It is to be noted that Malathion concentration in *H. diversicolor* was higher by 1.1- 1.5 than in the surrounding sediments. This indicates the ability of this worm to accumulate higher malathion concentration than the sediment.

Benthic polychaetes are relatively resistant to pesticides and they do not accumulate them (e.g. **Dumbauld *et al*., 2001; Granberg *et al*., 2008**). These observations contradict with our results which indicated that *H. diversicolor* accumulated Malathion 1 – 1.5 folds that found in the surrounding sediments.

CONCLUSION

The present study revealed that the protein profile of *H. diversicolor* experienced different patterns of polymorphism relative to the Malathion concentration in the sediment of Lake Burullus. These patterns were significantly correlated with temperature variation.

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