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Toxic Effects of Dimethoate and Carbaryl Pesticides on Reproduction and Related Enzymes of the Freshwater Snail *Lymnaea acuminata*

P. K. Tripathi, A. Singh

Natural Products Laboratory, Department of Zoology, D. D. U. Gorakhpur University, Gorakhpur 273 009 (U.P.), India

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Because of the cumulative nature of organochlorines in the natural environment (Cullen and Connell 1992), dimethoate (organophosphate) and carbaryl (carbamate) pesticides are frequently used for pest control in agricultural fields and in water bodies. These pesticides reach water bodies adjoining agricultural fields by rain or irrigation (Li 1975) or by their direct use there. Dimethoate and carbaryl pesticides caused disruptive effects on carbohydrate and protein metabolism of the freshwater snail *Lymnaea acuminata* (Tripathi and Singh 2002; 2003). Because of their electrophilic nature these pesticides also act on many other enzyme systems. Carbaryl pesticides have been found to have a significant inhibitory effect on the control mechanism of reproduction in fishes (Arunachalam et al. 1980). Reproduction is important in the life cycle of this organism. Fitness of organisms determines successful reproductive capability. Disruption of reproduction will affect the abundance and distribution of the species (Woin and Brönmark 1992). Effect of pollutants on natural ecosystems can be determined by understanding how pollutants affect the reproductive processes of key species. Therefore, laboratory test of long-term impact of sub-lethal pollutant concentrations to reproductive success is useful (Sheehan 1984).

Although dimethoate and carbaryl pesticides persist in the environment for only a few days (Matsumura 1985), it is possible that these pesticides affect enzymatic balance and reproduction in aquatic fauna. The snail *Lymnaea acuminata* is a cosmopolitan aquatic organism and important primary consumer in many freshwater bodies (Burris et al. 1990). These snails are hardy enough to be collected, transported and used in flow-through or static test chambers in the laboratory. They also reproduce normally under such conditions. So the aim of this study is to discern the effect of sub-lethal exposure of dimethoate and carbaryl pesticides on the reproduction, hatching, survival rate and some related enzymes in the nervous, ovotestis and hepatopancreas tissues of the snail *Lymnaea acuminata*.

MATERIALS AND METHODS

Adult freshwater snails (*Lymnaea acuminata*) of uniform size range (37.1±1.9 mm shell height and 20.6±1.4 mm shell width), were collected from non-contaminated waters of Gorakhpur district of Uttar Pradesh, India and kept in glass aquaria

Correspondence to: A. Singh

containing 30 L of dechlorinated tap water for at least 96h to acclimatize them to laboratory conditions. Water was changed every day. Dead snails were removed as soon as possible to avoid water fouling. The snails were fed daily on washed and dried *Nymphaea* leaves during the whole acclimatization period. Technical grade dimethoate [O, O- dimethyl S- (N-methylcarbamoylmethyl) phosphorodithioate] and carbaryl (1- naphthyl – N – methylcarbamate) were the organophosphate and carbamate pesticides, respectively. For these snails, the LC_{50} values for dimethoate are 19.7 mg/L and 10.8 mg/L for 24h and 96h, respectively, while LC_{50} values for carbaryl are 20.1 mg/L and 14.2 mg/L for 24h and 96h, respectively (Srivastava and Singh 2001). Nominal concentrations (i.e. 1.0 mg/L, 3.0 mg/L, 6.0 mg/L and 9.0 mg/L) of both the pesticides were used for biochemical experiments.

For the fecundity experiment the desired amount of pesticide was added to each glass aquarium containing 10 L dechlorinated tap water. Thirty snails were placed in the each aquarium. In control groups the water was pesticide free. Each aquaria set had six replicates. Water temperature was kept at 23±1°C during the whole experiments. *Nymphaea* leave was set to float at the top of the water surface for egg laying. Leaves were changed every day to avoid decay. No food was given to snails during the entire experiment. Lymnaeid snails attached their egg masses, containing large number of eggs, to the back surface of *Nymphaea* leaf when reproducing. The egg masses produced by the snails in the experiment were removed after every 24h and the number of eggs counted under a compound microscope. All the egg masses for each group were transferred into separate Petri dishes for hatching under the same exposure conditions as above. Numbers of hatched snails were counted and their survival rate was recorded for 28 days after hatching.

For enzymological analysis snails were removed from water after the fecundity experiment (i.e. after 96h exposure), dissected and nervous, ovotestis and hepatopancreas tissues were removed for analysis. Acetylcholinesterase (AChE) activity was measured by the method of Ellman et al. (1961). The homogenate (50 mg/mL, w/v) was prepared in 0.1M phosphate buffer (pH 8.0) in an ice bath and centrifuged at -4°C and the supernatant was kept for enzyme assay. Enzyme activity is expressed as μM SH hydrolyzed/min/mg wet tissue. Succinic dehydrogenase (SDH) activity was measured by the method of Arrigoni and Singer (1962). The homogenate (50 mg/mL, w/v) was prepared in 0.5M potassium phosphate buffer (pH 7.6) in an ice bath and centrifuged at 4°C. Enzyme activity is expressed as μmoles dye reduced/min/mg protein. Cytochrome oxidase activity was measured according to the method of Cooperstein and Lazarow (1951). The homogenate (50 mg/mL, w/v) was prepared in 0.33M phosphate buffer (pH 7.4) on ice and centrifuged at 4°C, with the supernatant used as the enzyme source. Enzyme activity is expressed in arbitrary units/min/mg of proteins (corresponding to the quantity of enzyme which catalyses an O_2 uptake by the oxidation of reduced cytochrome). Activities of phosphatases (acid and alkaline phosphatase) were measured according to the method of Andersch and Szcypinski (1947) as modified by Bergmeyer (1967). The homogenates (50 mg/mL, w/v) were prepared in ice cold 0.9% saline and centrifuged at 4°C, supernatant was used as the enzyme

source. Enzyme activities are expressed as the amount of p-nitrophenol formed/30 min/mg protein in supernatant. Total protein was measured according to Lowry et al. (1951). The homogenate (50 mg/mL, w/v) was prepared in 10% trichloro acetic acid (TCA).

Each assay was replicated six times, values are expressed as mean ±SE of six replicates and Student's 't' test was applied to determine significant ($P<0.05$) differences between treated and control groups

RESULTS AND DISCUSSION

Data for the fecundity experiment is given in Table 1. There was a reduction in reproductive output of the snails as a result of the exposure to dimethoate and carbaryl pesticides. Dimethoate and carbaryl pesticides significantly reduced fecundity and survival rates of the embryos of the snails. Fecundity was very low at higher doses of carbaryl, while for dimethoate treatment fecundity was reduced significantly. Some egg masses were laid without eggs at higher doses of carbaryl. No significant difference was found in the duration of hatching between the control and pesticides treated snails. The survival rate of snail embryos exposed to pesticide was significantly lower than the control value. The rate of surviving snails was significantly reduced 7 days after hatching and it was reduced to about 14% of the control after 28 days after hatching (Table 1).

Data for enzymological analyses are given in Tables 2 and 3. Both pesticides significantly inhibited the activities of AChE, SDH, cytochrome oxidase and phosphatases (acid & alkaline) in nervous, ovotestis and hepatopancreas tissues. Dimethoate and carbaryl pesticides are neurotoxic and they inhibit AChE activity. Carbaryl is well known to be a reversible inhibitor of AChE (Reiner, 1971), while dimethoate is considered to be a relatively irreversible inhibitor of AChE. The initial process of inhibition of AChE involves simple competition between the carbamate with acetylcholine for the active site on the enzyme surface. AChE inhibited by an organophosphate is less hydrolysed than AChE inhibited by a carbamate pesticide. Therefore, reactivation of inhibited enzyme takes place very slowly for an organophosphate in comparison to AChE inhibited by a carbamate (Kuhr and Dorough, 1976). At 20-70% inhibition of AChE, adverse effects become more subtle and can include reproduction problems and alterations in behaviour (Beyers and Sikoski, 1994) (Tables 2 and 3).

It has also been shown that alkaline phosphate, through the process of phosphorylation of carbohydrates and fats, plays an important role in the active transport of chemicals across cell membranes (Hugon and Bogers 1966). Pilo et al. (1972) demonstrated that alkaline phosphate is associated with protein synthesis. It also has been shown that this enzyme is involved in the synthesis of certain proteinaceous digestive enzymes (Ibrahim et al. 1974). Alkaline phosphatase also plays an important role in spermatogenesis (Pavlikova and Repas 1975). Acid phosphatase is a lysosomal enzyme and plays an important role in catabolism, pathological necrosis, autolysis and phagocytosis (Singh and Agarwal 1992).

Table 1. Number of eggs laid, egg masses, hatched eggs, hatching period and survivability of hatched young snails after 7, 14, 21 and 28 days after hatching of the freshwater snail *Lymnaea acuminata* after dimethoate and carbaryl exposure.

	Nominal concentrations (mg/L)				
	Control	**1.0**	**3.0**	**6.0**	**9.0**
			Dimethoate (Organophosphate)		
Number of eggs (after 96h)	152±2.4 (100)	98±1.6* (64)	84±1.6* (55)	65±1.9* (43)	42±0.8* (28)
Number of egg masses	8±1.2	6±1.0	5±0.6	3±0.4	3±0.3
Number of hatched eggs	151±2.4 (100)	76±1.2* (50)	64±1.4* (42)	55±1.9* (36)	38±0.5* (25)
Hatching period (in days)	10 – 13	10 – 13	10 – 13	10 – 13	9 –13
Survivability of hatchlings					
After 7 days after hatching	151±2.2 (100)	69±1.4* (46)	60±1.1* (40)	43±1.4* (28)	32±0.2* (21)
After 14 days after hatching	148±1.8 (100)	61±1.2* (41)	48±1.3* (32)	36±1.2* (24)	28±0.6* (19)
After 21 days after hatching	139±1.8 (100)	47±0.9* (34)	39±0.8* (28)	28±1.2* (20)	22±0.5* (16)
After 28 days after hatching	138±2.1 (100)	42±1.0* (30)	31±0.8* (22)	24±1.0* (17)	19±0.4* (14)
			Carbaryl (Carbamate)		
Number of eggs (after 96h)	152±2.4 (100)	84±3.4* (55)	53±1.9* (35)	-	-
Number of egg masses	8±1.2	6±1.0	4±0.6	2±0.4	-
Number of hatched eggs	151±2.4 (100)	65±1.7* (43)	40±1.6* (26)	-	-
Hatching period (in days)	11 – 13	10 – 13	10 – 13	-	-
Survivability of hatchlings					
After 7 days after hatching	151±2.2 (100)	56±1.7* (37)	33±1.1* (22)	-	-
After 14 days after hatching	148±1.8 (100)	46±1.5* (31)	26±1.1* (18)	-	-
After 21 days after hatching	139±1.8 (100)	37±1.2* (27)	19±1.0* (14)	-	-
After 28 days after hatching	138±2.1 (100)	24±0.9* (17)	16±0.8* (12)	-	-

* Significant ($P<0.05$) with Student's 't' test between control and treated groups. All the experiments replicated six times. Values are mean ±SE of six replicates. - No egg laying occurred. Values given in the parenthesis represent percent of control values.

Table 2. Acetylcholinesterase (μM SH hydrolyzed/min/mg tissue), succinic dehydrogenase (μmoles dye/min/mg protein), cytochrome oxidase (arbitrary units/min/mg proteins) and phosphatase (acid & alkaline) (p-nitrophenol/30 min/mg protein) activities in the nervous (NT), hepatopancreas (HP) and ovotestis (OT) tissues of freshwater snail *Lymnaea acuminata* after 96h dimethoate exposure.

	Tissue	Dimethoate dose				
		Control	1.0 mg/L	3.0 mg/L	6.0 mg/L	9.0 mg/L
Acetyl-cholinesterase	NT	0.086±0.02 (100)	0.071±0.02* (82)	0.064±0.01* (74)	0.052±0.01* (61)	0.039±0.01* (46)
	HP	0.074±0.02 (100)	0.067±0.01* (91)	0.058±0.01* (78)	0.047±0.01* (64)	0.038±0.01* (52)
	OT	0.068±0.01 (100)	0.053±0.01* (78)	0.048±0.01* (70)	0.039±0.01* (58)	0.027±0.00* (39)
Succinic dehydrogenase	NT	50.6±3.9 (100)	48.6±3.4 (96)	43.5±4.1* (86)	35.4±3.8* (70)	33.4±3.9* (64)
	HP	54.7±4.0 (100)	50.3±4.3 (92)	44.9±3.9* (82)	36.1±4.1* (66)	32.3±3.9* (59)
	OT	46.4±3.1 (100)	41.8±4.1 (90)	34.8±3.6* (75)	27.4±2.9* (59)	23.7±2.9* (51)
Cytochrome oxidase	NT	58.3±4.2 (100)	54.8±5.1 (94)	48.4±4.7* (83)	43.7±3.9* (75)	36.2±3.4* (62)
	HP	70.2±5.9 (100)	64.6±5.3 (92)	61.8±5.1* (88)	50.5±4.7* (72)	40.7±4.1* (58)
	OT	66.3±5.1 (100)	60.3±5.9 (91)	57.0±5.1* (86)	46.4±4.7* (70)	33.2±3.9* (50)
Acid phosphatase	NT	0.193±0.05 (100)	0.185±0.06 (96)	0.164±0.06* (85)	0.133±0.05* (69)	0.100±0.03* (52)
	HP	0.169±0.04 (100)	0.155±0.04 (92)	0.135±0.05* (80)	0.115±0.04* (68)	0.085±0.02* (50)
	OT	0.128±0.02 (100)	0.114±0.03* (89)	0.089±0.02* (70)	0.076±0.02* (59)	0.058±0.01* (46)
Alkaline phosphatase	NT	0.398±0.08 (100)	0.374±0.09 (94)	0.358±0.09* (90)	0.267±0.08* (67)	0.191±0.06* (48)
	HP	0.344±0.09 (100)	0.323±0.07 (94)	0.296±0.07* (86)	0.220±0.06* (64)	0.169±0.05* (49)
	OT	0.263±0.04 (100)	0.242±0.06 (92)	0.213±0.07* (81)	0.163±0.05* (62)	0.121±0.04* (46)

* Significant ($P<0.05$) with Student's 't' test between control and treated groups. Values are mean ±SE of six replicates. Values given in parenthesis represent percent of control values.

Table 3. Acetylcholinesterase (μM SH hydrolyzed/min/mg tissue), succinic dehydrogenase (μmoles dye/min/mg protein), cytochrome oxidase (arbitrary units/min/mg proteins) and phosphatase (acid & alkaline) (p-nitrophenol/30 min/mg protein) activities in the nervous (NT), hepatopancreas (HP) and ovotestis (OT) tissues of freshwater snail *Lymnaea acuminata* after 96h carbaryl exposure.

	Tissue	Carbaryl dose				
		Control	1.0 mg/L	3.0 mg/L	6.0 mg/L	9.0 mg/L
Acetyl-cholinesterase	NT	0.086±0.02 (100)	0.065±0.02* (76)	0.046±0.01* (54)	0.028±0.00* (33)	0.022±0.00* (26)
	HP	0.074±0.02 (100)	0.061±0.01* (83)	0.052±0.01* (59)	0.031±0.01* (36)	0.025±0.00* (29)
	OT	0.068±0.01 (100)	0.042±0.01* (62)	0.034±0.01* (40)	0.024±0.00* (28)	0.019±0.00* (22)
Succinic dehydrogenase	NT	50.6±3.9 (100)	48.1±4.1 (95)	39.9±4.1* (79)	34.9±3.9* (69)	30.9±2.9* (61)
	HP	54.7±4.0 (100)	49.8±3.9 (91)	41.6±3.7* (76)	34.5±3.2* (63)	31.7±2.9* (58)
	OT	46.4±3.1 (100)	38.9±3.4* (84)	31.5±3.4* (68)	25.1±2.9* (54)	21.3±2.1* (46)
Cytochrome oxidase	NT	58.3±4.2 (100)	53.6±5.1 (92)	46.1±4.1* (79)	40.8±4.4* (70)	34.9±3.7* (60)
	HP	70.2±5.9 (100)	65.3±5.9 (93)	58.9±5.1* (84)	44.9±4.9* (64)	38.6±4.1* (55)
	OT	66.3±5.1 (100)	59.9±5.0 (90)	53.8±4.7* (81)	41.2±4.7* (62)	34.6±3.9* (52)
Acid phosphatase	NT	0.193±0.05 (100)	0.183±0.05 (95)	0.160±0.04* (83)	0.119±0.03* (62)	0.077±0.01* (40)
	HP	0.169±0.04 (100)	0.155±0.04 (92)	0.137±0.03* (81)	0.099±0.02* (59)	0.074±0.01* (44)
	OT	0.128±0.02 (100)	0.114±0.03 *(89)	0.088±0.02* (69)	0.073±0.01* (57)	0.049±0.01* (39)
Alkaline phosphatase	NT	0.398±0.08 (100)	0.330±0.09* (83)	0.218±0.07* (55)	0.183±0.05* (46)	0.151±0.03* (38)
	HP	0.344±0.09 (100)	0.316±0.07 (92)	0.272±0.06* (79)	0.199±0.06* (58)	0.144±0.03* (42)
	OT	0.263±0.04 (100)	0.226±0.07* (86)	0.179±0.05* (68)	0.142±0.04* (54)	0.095±0.02* (36)

* Significant ($P<0.05$) with Student's 't' test between control and treated groups. Values are mean ±SE of six replicates. Values given in parenthesis represent percent of control values.

Reduction in the activities of both phosphatases could be due to the necrosis of cells after sub-lethal exposure (Tables 2 and 3).

It appears that the stress produced by an adverse effect on energy metabolism causes decreased succinic dehydrogenase activity. It seems that it is a compensatory effect. But the actual mechanism of decreased SDH activity is still unknown. The decrease in cytochrome oxidase activity might be either the result of reduced availability of oxygen, which in turns reduced the capacity of the electron transport system (ETS) to produce ATP molecules or could be due to the direct impact of the pesticide. Conney (1967) and Stevans et al. (1972) reported that anticholinesterase compounds are known to usually inhibit mitochondrial reactions such as the function of cytochrome oxidase in the ETS. Dimethoate and carbaryl poisoning can affect the Kreb's cycle, which disturbs the ETS and oxidative phosphorylation, resulting in less ATP synthesis.

In conclusion, dimethoate and carbaryl pesticides significantly altered the enzyme activities in body tissues of the snails. Thereafter, reproduction in the snails was reduced significantly, resulting in less egg production and production of some egg masses without eggs. The effect of carbaryl pesticide on reproduction was more severe than dimethoate pesticide. So, the use of carbamate pesticides near low lying areas should be banned especially during the rainy season.

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