

Physiological and behavioural responses of *Ruditapes decussatus* to roundup and reldan

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ABSTRACT

The clam *Ruditapes decussatus* was used as a bioindicator for pollution to determine the toxicity of a herbicide (48% glyphosate, 'roundup') and an insecticide (50% chlorpyrifos-methyl, 'reldan'). After estimating LC_{50} ($\mu\text{L L}^{-1}$) and LT_{50} (days) of both organophosphorous pesticides over 60 days, the impact of $\frac{1}{2} LC_{50}$ of roundup ($1.1 \mu\text{L L}^{-1}$) and reldan ($0.6 \mu\text{L L}^{-1}$) was measured. After two months of treatment, reldan reduced valve activity more than roundup; the rest period increased with increasing time of exposure. There was direct relationship between valve movement and metabolic heat output of respiration and excretion. The decrease in respiration rate was associated with a decrease in ammonia excretion. Thus valve-movement responses of *R. decussatus* to $\frac{1}{2} LC_{50}$ of the two pesticides are type-dependent. Both pesticides reduced physiological condition, but reldan is more potent than roundup and its effect is time-dependent. Respiration and excretion rates were components of the energy expenditure that accounted for the decline in metabolism and excretion with increasing the time of exposure to pesticides. This study highlights the potential use of behavioural and physiological response of a sentinel organism for monitoring the pesticides in the marine environment.

KEYWORDS: LC_{50} , glyphosate, chlorpyrifos-methyl, valve movement, oxygen consumption, ammonia excretion, energy lost.

INTRODUCTION

Exposure to pesticide formulations, either through the course of work with pesticides, or due to unintentional exposures to environmental contamination, or even through residues in food, can cause a range of adverse human health impacts. Most environmental pesticide contamination is a result of agricultural, commercial and household application to control insects. Pesticides washed by rain into streams, ponds or other wetlands can harm aquatic animals, and cause serious ecotoxicological problems mainly due to their persistence and high toxicity (Howard 1991).

The herbicide glyphosate, usually formulated as an isopropylamine salt, is the most popular herbicide widely used in the United States and elsewhere (Tominack *et al.* 1991). Glyphosate is the active ingredient, manufactured for a range of non-selective post-emergence herbicides and weedicides (eg. roundup and rodeo). It has been trialled for control of macroalgae (*Ecklonia radiata*) and aquatic vegetation (Farnsworth & Meyerson 1999). It is useful on essentially all annual and perennial plants including grasses, sedges, broad-leaved weeds and woody plants (Tominack *et al.* 1991).

The insecticide chlorpyrifos-methyl is a non-systemic organophosphorothioate commonly used in households (Whitehead 1997), and recently introduced to Egyptian agriculture: its usage is significant (El-Sebae 1989). It is one of the 12 major environmental pollutants in Japan (Gamo *et al.* 2003). It is also widely used as an ectoparasiticide for dairy cattle, in crops used for animal feed, and in homogenized and pasteurized Mexican milk samples (Salas *et al.* 2003). The toxicity of chlorpyrifos to the fish *Oreochromis mossambicus* was determined by Rao *et al.* (2003).

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Bivalves have been considered mainly because of the need to find suitable organisms for monitoring purposes (El-Shenawy *et al.* 2001a,b); the Mussel Watch Programmes use mussels for monitoring chemical contamination in coastal areas throughout the world (Widdows & Donkin 1992). Bivalves in general appear to be promising candidates for indicator organisms for several reasons. Many bivalve species have wide geographical distribution (Burky 1983), which facilitates comparison of results obtained in different areas. As sedentary organisms, bivalves reflect local conditions; as efficient filter feeders, they accumulate measurable contaminant body burdens from environmental concentrations that are near or below the limit of detection in chemical analysis (Ullvén 1993); and they have a long life span of up to several years (Perret *et al.* 1996). In addition, their ability to biotransform accumulated toxicants is generally lower than in many other aquatic organisms (Borchert *et al.* 1997). Bivalves are often among the dominant species in benthic ecosystems (Burky 1983), which makes the collection of material easy. The clam, *Ruditapes decussatus* is a filter-feeding marine bivalve satisfying many criteria required for a biological indicator (El-Shenawy 2002). It is edible and widely distributed in the Mediterranean Sea along the north coast of Egypt and along the Lake Timsah in Ismailia (Mostafa 2002).

Acute lethal toxicity tests provide useful data on the comparative toxicity of chemical contaminants to aquatic biota but are of limited value for environmental management, particularly when the LC₅₀ values are higher than the toxicant concentrations measured in the natural environment. Consequently, there is an increasing need to use tests which measure sublethal responses to toxicants at environmentally realistic concentrations that may be used to predict environmental impact (Roast *et al.* 1999). Chronic lethal toxicity is more useful than an acute toxicity test (El-Shenawy *et al.* 2003). Recording the activity of mussels (opening and closing of the valves) is used for ecotoxicological testing of chemicals under laboratory conditions (El-Shenawy *et al.* 2001b). Several physiological responses have also been used to quantify the sub-lethal effects of contaminants on bivalves, such as respiration and excretion (El-Shenawy 1999). Measurement of changes in physiological energetics of individual animals form an important component of any toxicological or environmental monitoring programme, for it represents an integration of the wide variety of possible cellular responses (both detrimental and beneficial) (Widdows & Johanson 1988)

Therefore the general aim here has been to study the toxicity of the organophosphate herbicide glyphosate (roundup) and the insecticide chlorpyrifos-methyl (reldan) on *R. decussatus*. Firstly, marginal concentration and time effects on *R. decussatus* (LC₉₀, LC₅₀, LC₁₀, LT₉₀, LT₅₀ and LT₁₀) were estimated for roundup and reldan using probit analysis. Secondly, responses to toxicants at sublethal levels were studied using behavioural valve activity and physiological changes (respiration and excretion) under standard laboratory conditions.

MATERIALS AND METHODS

Roundup, the isopropylamin salt of N-(phosphonomethyl)glycine (purity 48%, 52% inert ingredients) was obtained from Monsanto Company U.S.A. Reldan, chlorpyrifos-methyl [O,O-dimethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] (purity 50%, 50% inert ingredients), was obtained from Dow Agroscience Agrochem.

R. decussatus with shell sizes of 50-60 mm were collected from Timsah Lake, Ismailia Egypt. They were acclimatized to laboratory conditions for 2 weeks in tanks prior to experimentation. The tanks were supplied with water from Timsah Lake. The temperature was 19 ± 1 °C, and a natural photoperiod was maintained. The clams were given no food other than that occurring naturally in water surrounding them.

A static system with daily renewal of solutions was used in chronic exposures (El-Shenawy *et al.* 2003). The determination of the LC₅₀ for roundup and reldan were carried out at different concentrations for a period of 2 months. 10 clams were placed in a glass tank with 2.5 l of sea water taken directly from Timsah Lake. Duplicate tanks acted as controls and others contained different concentrations of toxicants. A wide range of concentrations was used in order to determine the tolerance distribution in long-term experiments. Clams with valves opened and not responding to touch by closing their valves were considered to be dead.

Valve movement behaviour of *R. decussatus* was measured using the method described by Xie & Burnell (1995). The activity of the animal (n = 15-20) was monitored at 15-minute intervals as scores (0-4) for the various states of activity rather than ordinal scale data and the mean score over the whole incubation period was calculated.

Rates of oxygen consumption were measured individually on 7-8 clams in "closed" glass respirometers (*ca.* 500 ml Quickfit flasks). (Soberal & Widdows 1997). After one hour of incubation, the rate of decline in oxygen within the respirometer chamber was measured by a micromodification of the Winkler technique (Aminot & Chaussepied 1983). The respiration rate was expressed as a weight-specific rate ($\mu\text{M O}_2 \text{ h}^{-1}\text{g}^{-1}$).

Ammonia excretion rate is usually closely coupled to the respiration rate. Ammonia excretion was determined using the phenol-hypochlorite method described by Solorzano (1969). The ammonia excretion rates ($\mu\text{M NH}_4 \text{ h}^{-1}\text{g}^{-1}$) were calculated as described by Widdows (1985). Following oxygen and ammonia sampling, dry tissue weight was determined by drying the soft tissues in an oven to constant weight (24 h) at 90 °C and wet-to-dry weight ratio was calculated.

The calculation of energy lost was done in two parts. First, from the oxygen consumption rate using the equation $R = (\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}) \times 0.456$ where the heat equivalent of oxygen uptake is 0.456 J $\mu\text{mol}^{-1} \text{ O}_2$ (Widdows & Johenson 1988). All rates of oxygen uptake were converted to heat dissipation by applying a generalized oxycaloric equivalent of 20.3 J ml^{-1} (equivalent to 455 kJ mol^{-1} , where 1 ml $\text{O}_2 = 44.6 \text{ mole O}_2$). Second, from the ammonia excretion rate, $U = (\mu\text{mol NH}_4 \text{ g}^{-1} \text{ h}^{-1}) \times 0.349$ where the excretion of 1 $\mu\text{mol NH}_4 \text{ g}^{-1} \text{ h}^{-1}$ is equivalent to an energy loss of 0.349 J $\text{g}^{-1} \text{ h}^{-1}$ (Widdows & Johenson 1988).

The data were analyzed using probit analysis to determine LC₅₀, and LT₅₀ of both pesticides. Data were expressed as mean \pm SE and significant differences were established at the $p < 0.05$ level using Student unpaired t-test. Spearman's rank correlation coefficient was used to test for associations between behavioural and physiological changes of the clams. All processing of data were conducted with the software packages Microsoft Excel XP (for data storage) and SPSS version 11.0, for statistical evaluation.

RESULTS

In a sub-chronic toxicity test, one tank acted as control and the others were treated with the following concentrations of roundup; 2.5, 5, 10, 20, 40 and 60 $\mu\text{l l}^{-1}$. During a test, death was indicated by a failure to close and dead animals were removed immediately. The LC₅₀ at 7 days was 16.9 $\mu\text{l l}^{-1}$; at 35 days, 12.1 $\mu\text{l l}^{-1}$; at 39 days, 2.9 $\mu\text{l l}^{-1}$; at 45 days, 2.2 $\mu\text{l l}^{-1}$ until the end of the experiment (60 days). Limits in time (minimum time in which 50% mortality can be produced) and concentration (median lethal threshold concentration) were determined (Tables 1 and 2, Fig. 1). Concentrations of more marginal effects (LC₉₀ and LC₁₀) were also estimated for roundup using probit analysis (Table 2). The median period of survival (LT₅₀) and slope function were recorded at each roundup concentration (Table 3, Fig. 2). LT₉₀ and LT₁₀ for roundup were also calculated (Table 4).

Table 1: Concentration-response analysis of roundup. Median Lethal Concentrations (LC₅₀) (µl l⁻¹) and slope function at a range of exposure time (days) of clams (5-6 cm).

	Exposure Time (days)							
	1	3	6	18	30	36	45	60
LC ₅₀	45.4	32.7	16.9	16.9	16.9	8.9	2.2	2.2
Slope	23.3 ± 0.5	6.7 ± 0.2	3.7 ± 0.4	3.7 ± 0.4	3.7 ± 0.4	3.3 ± 0.4	3.1 ± 0.6	3.1 ± 0.6

Table 2: Concentration-response analysis of roundup. Ten- and ninety-percent lethal concentrations (LC₁₀ and LC₉₀) (µl l⁻¹) at a range of exposure times (days) of clams (5-6 cm).

	Exposure Time (days)							
	1	3	6	18	30	36	45	60
LC ₁₀	40.0	20.13	7.6	7.6	7.6	3.7	0.8	0.8
LC ₉₀	51.5	53.2	37.8	37.8	37.8	21.0	5.7	5.7

Table 3: Time-response analysis of roundup treated clams. Median period of survival (LT₅₀-days) and slope function at a range of concentrations

	Concentration (µl l ⁻¹)					
	2.5	5	10	20	40	60
LT ₅₀	45.5	34.2	32.5	10.5	2.27	1.2
Slope	6.6 ± 1.1	2.4 ± 0.2	2.9 ± 0.8	1.8 ± 0.6	5.2 ± 0.5	5.2 ± 0.4

Table 4: Time-response analysis of roundup treated clams. Ten- and ninety-percent period of survival (LT₁₀ and LT₉₀-days) at a range of concentrations.

	Concentrations (µl l ⁻¹)					
	60	40	20	10	5	2.5
LT ₁₀	1.2	1.29	1.95	11.7	10.2	29.1
LT ₉₀	3.99	4.0	56.7	90.5	114.5	170.9

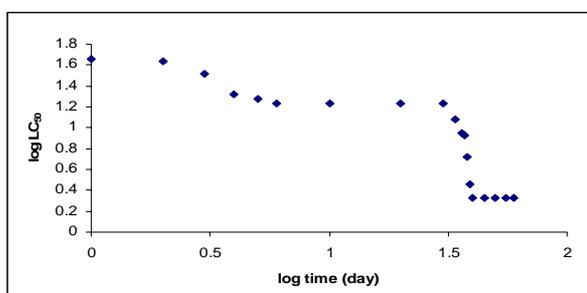


Figure 1: Concentration-response analysis of roundup in *R. decussatus*

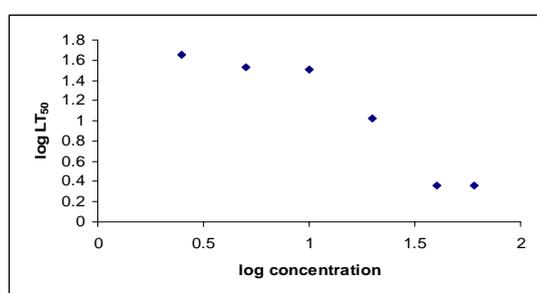


Figure 2: Time-response analysis of roundup in *R. decussatus*

Reldan toxicity was studied using different concentrations (2.5, 5, 10, 20 µl l⁻¹) and the time required to produce an adverse effect on clams is presented in Tables 5-8. The LC₅₀ as well as LC₉₀ and LC₁₀ of reldan significantly decreased with increasing time of exposure (Table 5-6 and Fig. 3). In the toxicity test (exposure for up to 2 months) the LT₅₀ was estimated as 10.5 days at 20 µl l⁻¹; and 2.27 days at both 40 µl l⁻¹ and 60 µl l⁻¹ (Table 7 and Fig. 4). Times for more marginal effects of reldan (LT₉₀ and LT₁₀) were calculated in Table 8. For roundup and reldan, there was a concentration or threshold level below which there was no observed effect (Figs.1, 3).

The effects of exposure to ½ LC₅₀ for 60 days of roundup or reldan on the activity and rest period of the clams valve movement were investigated (Fig. 5). The normal valve movement behaviour of *R. decussatus* was characterized by long periods in the open state, with siphons extending out to more than half of their full length; the valves re-open within a

few minutes of any closure. The score of normal valve activity ranged from 2.3 to 3.1 during the whole time of the study. As time elapsed following the addition of roundup or reldan, a gradual increase in the time spent with the valve closed was observed. After one week, the initial reaction to 0.6 $\mu\text{l l}^{-1}$ of reldan concentration was a significant decrease ($p < 0.004$) of the score activity compared with the control group. After 60 days of exposure to reldan, shell opening decreased markedly ($p < 0.001$) and clams remained gaping (partial isolated state) or closed completely. In contrast, the gape of opening was not affected with 1.1 $\mu\text{l l}^{-1}$ roundup in the first week, and only started to significantly decrease activity after 2 weeks of exposure ($p < 0.008$); this effect continued to the end of experiment ($p < 0.05$). There were significant differences between the effect of reldan and roundup on the clam valve movement after 2, 4 and 8 weeks ($p < 0.03$, 0.001 and 0.005, respectively). It is clear that the closure periods increased with increasing length of exposure.

Table 5: Concentration-response analysis of reldan. Median Lethal Concentrations (LC_{50}) ($\mu\text{l l}^{-1}$) and slope function at a range of exposure time (days) of clams (5-6 cm).

	Exposure Time (days)							
	1	4	6	12	25	28	45	60
LC_{50}	20.9	10.0	8.8	4	2.3	1.6	1.2	1.2
Slope	25.4 ± 0.5	3.7 ± 1.0	2.6 ± 0.7	2.5 ± 0.7	2.2 ± 0.7	1.9 ± 0.7	1.7 ± 0.9	1.7 ± 0.9

Table 6: Concentration-response analysis of reldan. Ten and ninety percent lethal concentrations (LC_{10} and LC_{90}) ($\mu\text{l l}^{-1}$) at a range of exposure times (days) of clams (5-6 cm).

	Exposure Time (days)								
	1	4	6	12	18	24	28	45	60
LC_{10}	18.7	4.5	2.8	1.3	0.9	0.6	0.2	0.2	0.2
LC_{90}	23.6	22.2	27.9	13.1	13.5	14.1	15.7	6.5	6.5

Table 7: Time-response analysis of reldan treated clams. Median period of survival (LT_{50} -days) and slope function at a range of concentrations.

	Concentration ($\mu\text{l l}^{-1}$)			
	20	10	5	2.5
LT_{50}	1.9	4.5	8.2	22.2
Slope	2.3 ± 0.9	2.2 ± 0.6	2.1 ± 0.4	2.1 ± 0.5

Table 8: Time-response analysis of reldan treated clams. Ten and ninety percent period of survival (LT_{10} and LT_{90} -days) at a range of concentrations.

	Concentration ($\mu\text{l l}^{-1}$)			
	20	10	5	2.5
LT_{10}	0.5	1.2	2.0	5.6
LT_{90}	7.1	16.8	33.1	88.8

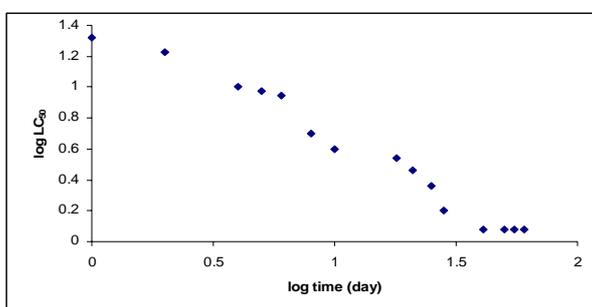


Figure 3: Concentration-response analysis of reldan in *R. decussatus*.

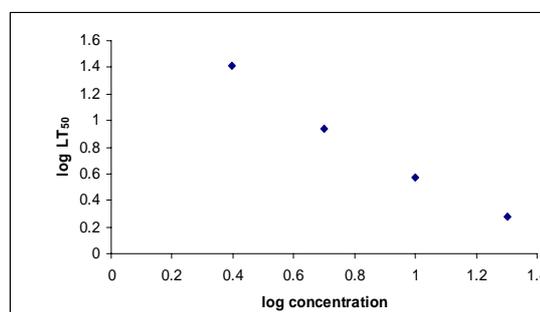


Figure 4: Time-response analysis of reldan in *R. decussatus*.

The metabolic rate in clams was evaluated by the rate of respiration, principally by the rate of oxygen consumption (Fig. 6). The rate of oxygen consumption of control clams during the experiment period (up to 2 months) was in the range from 11.5 ± 0.5 to $14.2 \pm 0.6 \mu\text{M g}^{-1} \text{h}^{-1}$. However, in clams treated with $0.6 \mu\text{l l}^{-1}$ reldan, O_2 consumption decreased

significantly ($p < 0.03$) after 2 weeks of exposure. By the end of the experiment, the O_2 consumption of reldan-treated clams was significantly decreased ($p < 0.001$) compared to the with control at the same time point, due to the partially closed state of the majority of the individuals in the respirometers. Roundup started to affect the metabolic rate of clams significantly after 4 weeks of treatment. Respiration decreased significantly with increasing the time of exposure to roundup ($p < 0.001$). There was a significant difference between reldan and roundup ($p < 0.04$) after 2 weeks of treatment. By extending the experiment for 2 months, the difference between roundup and reldan effect on respiration rate was decreased ($p < 0.09$ and 0.06 at 4 and 8 weeks, respectively).

The rate of ammonia excretion decreased significantly with increasing exposure to a sub-lethal concentration of reldan or roundup. Both pesticides decreased the ammonia excretion significantly ($p < 0.001$) after 4 weeks of exposure due to the partial closure of the clams. This effect continued till the end of experiment and it was observed that reldan decreased the ammonia excretion more than roundup by 28% (Fig. 7).

Metabolic heat output of *R. decussatus* in relation to pesticides was calculated to determine the effects of pesticides on metabolism at sub-lethal concentrations (Table 9). The metabolic heat output in *R. decussatus* varied depending on whether a high aerobic metabolism or a low anaerobic metabolism prevails. Short-term exposure to reldan depressed the percentage of the total expenditure energy represented by O_2 consumption. However, long-term exposure caused significant increase in the percentage of total maintenance energy represented by O_2 consumption to the same value as roundup. 60-days exposure to reldan significantly increased the energy lost through respiration as compared with control. However, roundup increased the percentage of total maintenance energy represented by O_2 consumptions. After 60 days of treatment, there was a significant difference between the effect of reldan and roundup ($p < 0.05$). The amount of total maintenance energy of clams decreased significantly under the influence of the pesticides (Table 9).

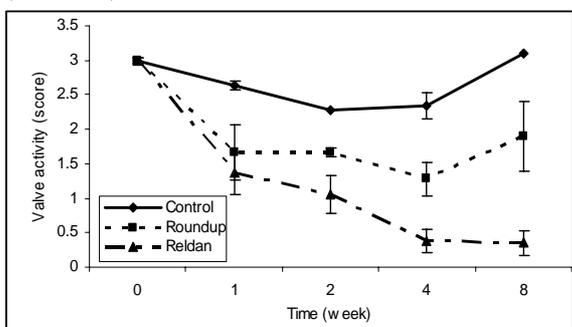


Figure 5: Effect of exposure to sub-lethal concentrations of roundup ($1.1 \mu l l^{-1}$) and reldan ($0.6 \mu l l^{-1}$) on the score of valve movement activity of the clams, *R. decussatus*.

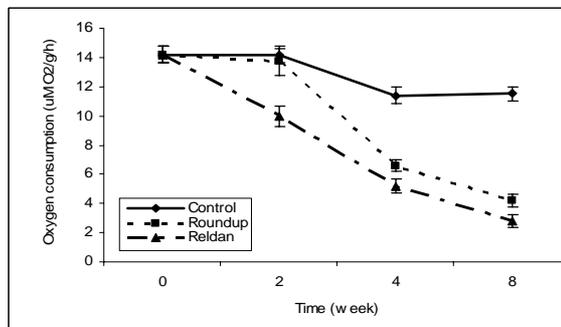


Figure 6: Effect of exposure for up to 60 days to a sub-lethal concentrations of roundup ($1.1 \mu l l^{-1}$) and reldan ($0.6 \mu l l^{-1}$) on oxygen consumption of the clam, *R. decussatus*

Figure 7: Effect of exposure for up to 60 days to a sub-lethal concentrations of roundup ($1.1 \mu l l^{-1}$) and reldan ($0.6 \mu l l^{-1}$) on ammonia excretion of the clam, *R. decussatus*

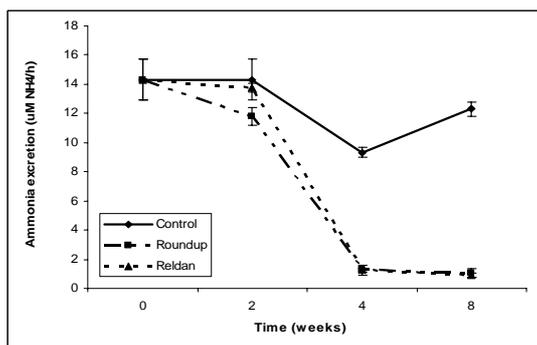


Table 9: Effect of median lethal concentrations of roundup and reldan on the total maintenance energy ($\text{J h}^{-1} \text{g}^{-1}$) of *R. decussatus*. R is the rate of energy lost due to respiration, and U is the rate of the energy lost due to excretion.^a Mean \pm S.E of total maintenance energy ($\text{J h}^{-1} \text{g}^{-1}$), n = 6-8.; ^b Significantly different as compared with the corresponding control; P < 0.05.; ^c Significantly different as compared with the other-treated group; P < 0.05

Time (week)	Total maintenance energy ($\text{R}+\text{U}, \text{J h}^{-1} \text{g}^{-1}$)			O ₂ consumption % of Total maintenance energy			ammonia excretion of % Total maintenance energy		
	Control ^a	Roundup ^a	Reldan ^a	Control	Roundup	Reldan	Control	Roundup	Reldan
0		11.4 \pm 0.8							
14	11.4 \pm 0.5	10.3 \pm 0.5	9.4 \pm 0.5	56.2	60.2	48.9	43.8	39.8	51.1 ^b
30	8.6 \pm 0.4	3.4 \pm 0.2 ^b	3.4 \pm 0.2 ^b	60.5	87.1 ^b	87.1 ^b	39.5	12.9 ^b	12.9 ^b
60	8.4 \pm 0.3	2.2 \pm 0.7 ^b	1.9 \pm 0.3 ^b	54.8	89.1 ^{b,c}	69.5 ^{b,c}	45.2	10.9 ^b	30.5 ^b

DISCUSSION

Organophosphorus compounds have the potential to causing acute toxicity, indicated by signs of cholinergic poisoning and delayed neuropathy that may develop days and weeks after exposure (Abd El-Baky *et al.* 1993). The need for collective information on the toxicity of organophosphorus pesticides in the environment and their correlation with any deleterious effects are still of considerable importance (Hasheesh *et al.* 2002). Thousands tonnes of formulated materials containing organophosphorous insecticides have been imported into Egypt (El-Sebae 1989).

In earlier years, toxicologists used to base their choice of doses or concentrations for subacute and chronic tests on the outcome of LD₅₀ or LC₅₀ determinations (El-Shenawy *et al.* 2003). The purpose of toxicity testing is to decide whether a compound is safe or not. Safety evaluation studies are based on two assumptions: first, that there is an appropriate animal model; and second, that dose-response and time-response relationships can be demonstrated. The LC₅₀ and LT₅₀ are best estimated with probit analysis (El-Shenawy *et al.* 2003; Kalyanasundaran *et al.* 2003). *R. decussatus* was selected as the target bioindicator organism for study because it is one of the most ubiquitously distributed bivalves and highly resistant to toxins (El-Shenawy 2002).

It is clear that the median lethal threshold concentrations for roundup and reldan are approached at the same time (45-60 days), but that for reldan ($1.1 \mu\text{l L}^{-1}$) was less than roundup ($2.2 \mu\text{l L}^{-1}$) at the end of the chronic experiment. In the present study, not only the LC₅₀ and LT₅₀ were estimated, but also other concentrations and response times (LC₁₀, LC₉₀, LT₁₀ and LT₉₀). USEPA (1987) found that in fish the formulated product, roundup, was more toxic than glyphosate. In rainbow trout, for instance, the 96-hour LC₅₀ was 8.3 ppm with roundup and 38 ppm with glyphosate. For bluegill the 96-hour LC₅₀ was 5 ppm for roundup and 78 ppm for glyphosate. Feei (1987) evaluated the acute toxicity (LC₅₀) of glyphosate for carp (*Cyprinus carpio*), mosquitofish (*Gambusia affinis*) and daphnids (*Scapholeberis kingi*), and reported that LC₅₀ values varied according to species.

The LC₅₀s for *Tilapia sp.* after 24 h and 96 h at 27-31°C for roundup (glyphosate 30.5%) were 7.4 and 1.0 ppm, respectively (Liong *et al.*, 1988). Salmonid fish have also been used to determine the acute lethal (96-h LC₅₀) and behavioural threshold effect of concentrations of forest-use glyphosate (Vision) with 10% and 15% surfactant formulations. Nominal concentrations of the herbicides eliciting threshold avoidance reactions in test fish were 150 ppm (Vision with 10% surfactant) and 54 ppm (Vision with 15% surfactant). These values represent twice the nominal 96-h LC₅₀ values. Definite nominal concentrations of the herbicides causing threshold changes in fish behaviour were 37.5 ppm Vision-10% surfactant and 13.5 ppm Vision-15% surfactant (Morgan *et al.*

1989). The 96 h-LC₅₀ for Rainbow trout at 12 °C was 10.42 mg l⁻¹ glyphosate in the Vision formulation (Morgan & Kiceniuk 1992). Moreover, Kumar & Gupta (1997) reported that LC₅₀ values at 24, 48, 72 and 96 h for the Indian catfish *Heteropneustes fossilis* ranged between 146.0 and 124.5 mg l⁻¹ for glyphosate. Wan *et al.* (1989) noticed significant variation of 96-h LC₅₀ values is recorded for the same fish species when different water types are used in the bioassay of glyphosate. Hardness of water and pH appear to be key factors causing variation of 96-h LC₅₀ values.

Kalyanasundaran *et al.* (2003) determined the susceptibility of larvae of mosquito vector species to reldan and dursban, calculating the LC₅₀ values (mg l⁻¹) by probit analysis. Dursban and reldan were effective against *Anopheles fluviatilis* larvae at lower LC₅₀ of 5.90 x 10⁻⁷ and 1.07 x 10⁻⁹ mg l⁻¹, respectively as compared with other species of mosquitoes. Dursban and Reldan were equally effective against *Aedes aegypti*, *An. stephensi* and *An. culicifacies*. Reldan was about nine times more effective than dursban against *Culex quinquefasciatus* with respective LC₅₀ of 1.17 x 10⁻⁴ and 1.34 x 10⁻³ mg l⁻¹ (Kalyanasundaran *et al.* 2003). Seagraves & McPherson (2003) reported that LC₅₀ of chlorpyrifos was 0.11 µg/vial for the Red Fire Ant, *Solenopsis invicta* Buren, using vial bioassay to determine the concentration-mortality responses. These contradictory results could be explained on the basis of species differences in relation to the effective concentration of the pesticide (Van Wezel *et al.* 1995).

Sub-lethal responses, such as changes in behaviour, metabolism or growth are usually more sensitive and often ecologically more relevant endpoints than death (Rand *et al.* 1995). Therefore, the second objective of the present study was to validate the possible use behavioural and physiological changes in marine organisms as biomarkers of pesticides contamination. The ½ LC₅₀-60 days of investigated pesticides was used to evaluate their effect on valve movement scores, respiration rate and ammonia excretion of the *R. decussatus*.

The great difficulty in quantifying the activity levels of experimental animals is believed to be one of the reasons that few studies have taken activity into consideration when measuring physiological rate (Xie & Burnell 1995). In the present study various parameters, including valve gape, siphon and foot extension, have been used in an attempt to score activity levels (El-Shenawy 2003). The adverse effects on clam activity varied from reldan to roundup. The percentage of valve activity score of treated clams with reldan fell to 11% of the control with increasing time of exposure. Roundup also decreased the score of valve activity during the same time interval. The effect of reldan on the behaviour of marine organism have been reported by Cooper & Bidwell (2003), who noticed that concentrations from 0.5 to 1.0 mg l⁻¹ chlorpyrifos, decreased the burrowing activity of the Asian clam, *Corbicula fluminea*. The present study indicates that the response of clams to the pesticides stress was characterized by a dramatic closure of the shell. The valve movement responses of *R. decussatus* to pesticides were depended on the type of pesticide used: after 15 days of exposure, roundup reduced valve activity more than reldan.

The integration of the basic biological processes into stress indices, such as respiration and excretion, provides not only a measure of the efficiency with which animals functions but also a method of quantifying the health or physiological condition of animals exposed to different environmental stress (Temblay *et al.* 1998).

In the present study, oxygen consumption by clams was significantly decreased by increasing exposure duration to reldan or roundup. Decreased rates of oxygen consumption have been reported previously for bivalve exposed to toxic contaminants. For example, oxygen-consumption of *Mytilus edulis* exposed to endrin or lindane decreased as compared with controls (El-Shenawy 1999). The reason for this difference may be linked, in part, with pesticide detoxification. Clams may be responding to the physiological stress of

chronic pesticide poisoning, and detoxification mechanisms may have been stimulated, resulting in more efficient metabolism and excretion of pesticides. Monooxygenase systems are important in the metabolism of foreign organic compounds and are believed to play a role in the metabolism of organophosphate pesticides (WHO 1989).

Oxygen consumption of reldan-treated clams had significantly decreased after 14 days and until the end of experiment (2 months), due to the partially or completely closed state of the majority of individuals, while the effect of roundup decreased only after 4 weeks. We conclude that a decreasing respiration rate of *R. decussatus* is an example of a sub-lethal response to reldan and roundup, reflecting a decreasing metabolic rate. In contrast, Markus *et al.* (2001) reported that glyphosate (720 and 960 ppm) increased oxygen consumption significantly of marine mollusc *Septifer bilocularis* (Bivalvia) after 2 h of exposure. These results are not in agreement with the present observation that $1.1 \mu\text{l l}^{-1}$ roundup (48% glyphosate) did not affect the oxygen consumption of *R. decussatus* during the first two weeks. The decreased oxygen consumption of *R. decussatus* following exposure to reldan may be coupled to the effects on clam valve activity. The result in the present investigation is contrary to that reported by Roast *et al.* (1999), where $0.038 \mu\text{g l}^{-1}$ chlorpyrifos increased the oxygen consumption of *Neomysis integer*. This difference could be due to the difference in the dose, duration of exposure and the species. Wang *et al.* (1994) reported that no significant variation was shown in the accumulation of the concentration of herbicide in carp and tilapia from 2 to 7 days. Bejarano *et al.* (2003) indicated that ^{14}C -chlorpyrifos (^{14}C -CHPY) was more likely to bioaccumulate through ingestion of contaminated particulate material than through filtration/ingestion of dissolved/colloidal material by *Mercenaria mercenaria*.

A decrease in ammonia excretion is usually associated with a decrease in respiration rate. However, these physiological processes do not always vary in the same direction, nor to the same extent, in response to toxicants (El-Shenawy 1999).

When the physiological rates were converted to energy equivalents using conversion factors, it was found that ammonia excretion accounted for 30.5% and 10.9% of the total metabolic energy loss for reldan and roundup respectively after 60 days exposure. The effect of roundup on the percentage of O_2 in total maintenance energy was greater than the effect of reldan. After 60 days of exposure, roundup and reldan had percentage of energy lost during respiration equivalent to 1.6-fold and 1.2-fold higher than control clams, respectively. The metabolic heat output in *Pisidium amnicum* varies naturally, depending on whether a high aerobic metabolism or a low anaerobic metabolism prevails (Holopainen & Penttinen 1993).

It is obvious that activity plays a significant role in influencing energy expenditure. The positive correlation between activity and physiological rates is expected as an increased activity would generally be associated with an increased oxygen consumption and excretion rate (Xie & Burnell 1995; El-Shenawy 2003). The heat-output signal was also used as an indirect measure of valve-openness. It is clear that roundup in the present study did not close the shell completely. The whole-animal rate of heat output (as a measure of metabolic rate) can be used in ecotoxicological studies as a sublethal response to toxicants (Penttinen 1996).

One final consideration is to assess which of the physiological responses measured in the present study offers the best potential for regulatory or environmental assessment purposes. Oxygen consumption was the most sensitive response. Valve movement was affected only following increasing the duration. All three responses were time-dependent and thus have potential as measures of pollutant effects on ecosystems. While oxygen consumption is affected by reldan, and thus can provide an earlier detection of a response to toxicants, it is difficult to interpret as it is an isolated physiological response. For

example, both increase and decrease can be regarded as beneficial (i.e. enhanced costs associated with energy consumption) or detrimental (i.e. metabolism of a toxicant or toxicant inhibition of metabolic pathway). Exposure to ½ LC₅₀-60 days of reldan also disrupted the valve behaviour of clams. Reldan is a neurotoxin (Roast *et al.* 1999) and disruption of valve behaviour is consistent with its mode of action on the nervous system.

In conclusion, this study provides evidence that physiology and behaviour respond to variation in pesticide exposure in ways that could be useful for biomonitoring purposes. Comparison of the two pesticides indicated that reldan was more toxic than roundup.

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