

TOXICITY EFFECTS OF DIFLUBENZURON, CYPERMETHRIN AND DIAZINON ON THE DEVELOPMENT OF *ARTEMIA SALINA* AND *HELIOCIDARIS TUBERCULATA*

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ABSTRACT

The toxicity of diflubenzuron (DFB) on the brine shrimp, *Artemia salina*, and the sea urchin, *Heliocidaris tuberculata*, was evaluated in the laboratory. DFB was toxic to *A. salina* embryos and larvae at $0.13 \mu\text{g.L}^{-1}$. The LC50 was $0.37 \mu\text{g.L}^{-1}$ for *A. salina* larvae after 48 h. Synergistic mortality effects after 48 h were found for the following combined insecticides: DFB and cypermethrin (LC20 + LC20 = LC85), DFB and diazinon (LC20 + LC20 = LC60) and cypermethrin and diazinon (LC20+LC20 = LC55). Antagonistic mortality effects to *A. salina* larvae occurred when DFB was combined with both cypermethrin and diazinon (LC20+LC20+ LC20 = LC40). We also show that DFB is toxic to the larvae of the sea urchin *H. tuberculata* at concentrations as low as $2.69 \mu\text{g.L}^{-1}$ during a 72 h EC50 experiment.

Key words: *Artemia salina*; cypermethrin; diazinon; diflubenzuron; *Heliocidaris tuberculata*.

INTRODUCTION

Diflubenzuron (DFB) and other insecticides such as cypermethrin and diazinon are agricultural insecticides used in commercial farming operations to eradicate pest arthropods. DFB is a widely used preventative, primarily in sheep, for the body louse *Bovicola ovis* and the blowfly *Lucilia cuprina* (Savage 1998; Savage and Russell 1999).

Although these insecticides are useful, there has been growing concern among environmental organizations and farming industries about the fate of these persistent insecticides once discharged into the environment (Kingsford and Battershill 1998). It is known that at low concentrations these insecticides pose a risk to non-target crustaceans, and this risk may be linked to the food chains of these organisms (Fischer and Hall 1992).

Early life stages of marine crustacean zooplankton and other marine invertebrates, e.g. sea urchin larvae, are more susceptible to a variety of insecticides than later life stages (Sanchez-Fortun et al. 1995). Greater susceptibility to insecticides of early life stages has ecological significance; successful fertilisation and embryonic and larval development have direct consequences on recruiting potential to succeeding generations within natural communities. Recruitment, in turn, affects the survival and propagation of target species and community structure in the area that is exposed to the insecticides and/or other pollutants (Abel 1989).

Many Environment Protection Agencies/Authorities, e.g. New South Wales Department of Environment and Conservation (NSW DEC), most commonly use sensitive larval stages of freshwater daphnids to assess insecticide toxicity in effluent samples (Coler and Rockwood 1989; NSW EPA 1999). Previous studies have shown that the toxicity of various insecticides to the crustaceans; *Daphnia* spp., marine copepods and marine decapods, could be predicted from toxicity data obtained using *Artemia salina* (L., 1758) (Abernethy et al. 1986; Sanchez-Fortun et al.

1995). Although *A. salina* is not used by government and toxicity testing laboratories, due to its insensitivity to a range of insecticides, it is convenient and economical to use in certain instances. For example, the use of *A. salina* in the present study made it possible to predict the potential toxic effects of the insecticides used on larval crustacean species with greater sensitivity.

Although DFB has been documented to affect reproductive fitness (e.g. cyst hatching and reproductive life span) in *A. salina* (Cunningham 1986), the LC50 of DFB over 48 h has hitherto not been established. Extrapolating the LC50 for *A. salina* from the literature is not possible as there is substantial variation in the effects of DFB on the different developmental stages and on different species of crustaceans. For Grass shrimp, the DFB LC50 for the larval stage was found to be 1.44 mg.L^{-1} data whereas the adult stage is considerably more tolerant at $>200 \text{ mg.L}^{-1}$ (Wilson and Costlow 1987). The LC50 of adult Mysid shrimp assessed over the same period as above was found to be two orders of magnitude lower than that of Grass shrimp. Further, lower DFB concentrations achieve LC50 over longer exposure periods than the LC50 of higher concentrations but comparisons prove difficult given the range of organisms used, developmental stage and the parameter of effect measured (Table 1).

Most toxicity studies have focused on the toxicity of single chemicals (Abel 1989). The biological effects and toxicity of two or more toxicants in combination are often considerably different from the effects of the toxicant alone, and are not always equivalent to the simple summation of individual effects (Wang 1987). The effects of interactions between chemicals within a mixture on biota are complex and are dependent on the components of the mixture and the characteristics of the chemical, the test organism, and the concentrations tested (Wang 1987). For organisms that are exposed to combinations of toxicants simultaneously there are three possible types of responses: additive, synergistic, or antagonistic (Wang 1987).

Table 1. Review of published DFB concentrations to inflict either lethal or sublethal effects to marine crustaceans.

Effect parameter	Species	Life stage	[DFB] ($\mu\text{g.L}^{-1}$)	Exposure time	Reference
Lethal concentration	<i>Mysidopsis bahia</i> (Mysid shrimp)	adult	1.24	21 day	Nimmo et al. 1979
		nauplii	1.97	96 h	
		adult	1.97	48 h	
LC50	<i>Palaemonetes pugio</i> (Grass Shrimp)	post larvae	> 200	96 h	Wilson and Costlow 1987
		larvae	1.44		
Lethal concentration	<i>Rhithropanopens harrisi</i> (crab)	brachyuran	10	48 h	Christiansen et al. 1978
	<i>Sesarma reticulatum</i> (crab)	brachyuran	3	12 day	
		larvae	10	5 day	
	<i>Rhithropanopens harrisi</i> (crab)	larvae	10	48 h	
Survival reduced	<i>Rhithropanopens harrisi</i> (crab)	brachyuran	>1		Christiansen et al. 1978
	<i>Artemia salina</i>	nauplii	> 10	72 h	
Total mortality	<i>Palaemonetes pugio</i>	larvae	> 2.5		Wilson and Costlow 1987
	<i>Menippe mercenaria</i> (stone crab)	larvae	0.5 – 6		
Development	<i>Artemia salina</i>	adult	1 – 10		Cunningham 1986
		larvae	0.5 – 6		
Development	<i>Artemia salina</i>	adult	1 – 10		Cunningham 1986
		nauplii	0.25 – 0.75		
Reproduction	<i>Mysidopsis bahia</i> (Mysid shrimp)	adult	0.075	28 day	Nimmo et al. 1979
		nauplii	0.25 – 0.75		
Reproduction	<i>Tigriopus californicus</i>	adult	0.1 – 100		Antia et al. 1985
		adult	0.1 – 100		

The specific objectives of this paper were: (1) to investigate the lethal toxicity of DFB to the larval development of *A. salina*; (2) to determine whether DFB combined with other agricultural insecticides, cypermethrin and diazinon, results in a synergistic, additive or antagonistic effect to *A. salina* larvae; and (3) to investigate whether the development of the non-crustacean, *Heliocidaris tuberculata* (Lamarck 1816), is sensitive to DFB.

METHODS

Insecticide stock solutions for toxicity tests were prepared by taking 0.01000 g of technical grade DFB, cypermethrin and diazinon (obtained as a gift from Schering-Plough Pty. Ltd., Baulkham Hills, Sydney), and initially dissolving in 2 mL of AR grade acetone. Required test concentrations were obtained after further dilutions with seawater (filtered through 0.2 μm filters). Seawater was collected from Clovelly Beach, NSW. Control solutions contained the same concentration of acetone as the treatments.

Cysts of *A. salina* (Californian Salt Lakes variety) were hatched and reared at room temperature in filtered seawater (0.2 μm filter) pH of 7.8 to 8.0, to the second larval stage over 48 h using continuous gentle aeration, 16 h light:8 h dark light cycle. This method for rearing *A. salina* followed that of Cunningham (1976) and Cunningham and Myers (1986).

For the developmental tests, *Artemia salina* cysts were exposed to four DFB nominal concentrations: 0, 0.13, 1, and 10 $\mu\text{g}\cdot\text{L}^{-1}$. Concentrations for each treatment were measured using a 5-place digit balance. Treatments were replicated three times and each replicate contained 0.3 g cysts (approximately 80 000 cysts) in 2 L of medium. Large cyst numbers were required as many cysts do not hatch, and large larval numbers were needed for representative sub-sampling to determine hatching success and survival (NSW EPA 1999). All test vessels (3-L Erlenmeyer flasks) were placed randomly under 40 W fluorescent lights (approximately 180 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Three 5-mL sub-samples were taken from each flask and the numbers of live *A. salina* larvae were counted after 48 h. Larvae were noted as being immobilised if there was no observed movement of appendages. In addition to counting numbers of live *A. salina* larvae after 48 h, the numbers of *A. salina* larvae that matured to 2nd instar stage were also counted.

In order to properly assess the effects of combinations of insecticides on adult *A. salina*, it was important to establish LC20 for each insecticide individually over a standard time period. LC20 was chosen as combining the LC20 for each of the three insecticides (LC20 x 3) would still detect a potentially synergistic result by having the additive mortality remaining under 100%, or LC100. *A. salina* larvae were exposed to the following concentrations of i) DFB: 0, 1, 2, 5 and 10 $\mu\text{g}\cdot\text{L}^{-1}$; ii) cypermethrin: 0, 2, 5, 10 and 20 $\mu\text{g}\cdot\text{L}^{-1}$, and iii) diazinon: 0, 2, 5, 10 and 20 $\mu\text{g}\cdot\text{L}^{-1}$. Each treatment concentration had a final volume of 40 mL and was replicated 4 times; 5 larvae of *A. salina* were exposed for 48 h per replicate. After 48 h the mortality of *A. salina* in each replicate was assessed and the data used to calculate the LC20 (lethal concentration causing 20% mortality) of each insecticide individually as well as to estimate the effect of combinations of insecticides: DFB and

Table 2. Summary of test conditions adapted from Simon and Laginestra (1996) for *H. tuberculata* fertilisation tests and from Doyle et al. (2003) for larval development tests.

Test type	Static
Control medium	0.2 μm filtered seawater
Temperature	20
Salinity	30 - 32 ppt
pH	7.8 - 8
Replicates	4
Fertilisation test	
Test stage	sperm
Test duration	80 min (60 min sperm + 20 min egg/sperm exposure)
Sperm: egg	100:1
Culture density	5 nauplii.50 mL ⁻¹
Test volume	5 mL
End point	fertilised eggs
Test acceptability	> 70% fertilisation in controls
Larval development test	
Test stage	embryos < 2 h post fertilisation
Test duration	72 h
Sperm: egg	50:1
Culture density	30 embryo.mL ⁻¹
Test volume	5 mL
End point	normal development to 2-arm plutei
Test acceptability	> 75% normally developed larvae in controls

cypermethrin, DFB and diazinon, cypermethrin and diazinon, and DFB, cypermethrin and diazinon. To determine the LC20 of insecticide combinations experimentally, *A. salina* larvae (5 larvae per 40 mL combined insecticide treatment replicated 4 times) were exposed for 48 h to each insecticide combinations as described above.

To examine the potential effects of DFB on a non-crustacean, the sea urchin *Heliocidaris tuberculata*, fertilisation and larval development tests were performed. Male and female sea urchins, *H. tuberculata*, from the Sydney Aquarium (Australia) were induced to spawn by injecting approximately 2 mL of 0.5 M KCl through the peristomal membrane on the oral surface. Sperm and eggs obtained from the sea urchins were used for the fertilisation and larval tests. Fertilised gametes and 72 h old larvae were exposed to concentrations of DFB (0, 0.11, 0.85, 8.5 $\mu\text{g}\cdot\text{L}^{-1}$), which were prepared as described above for *A. salina*. A more detailed summary of test conditions/parameters used in the sea urchin fertilisation test (Simon and Laginestra 1996) and the sea urchin larval development test (Doyle et al, 2003) is given in Table 2.

To assess the effect of DFB on fertilisation in comparison to larval development, the larval development test and the fertilisation test utilised the same *H. tuberculata* sperm suspension. For the fertilisation test, 30 μL of the sperm suspension was added to 5 mL test solutions ($n = 4$ for each concentration) after which 1 mL of egg stock was added to each test solution. Twenty minutes was allowed for fertilisation to occur and the test was terminated by the addition of 0.5 mL of 5% formalin to each tube. The first 100 eggs in each test tube were counted and scored as fertilised (clear film of sperm around egg) or unfertilised.

Table 3. Differences between mortality after 48 h assessed by ANOVA of: (a) *A. salina* embryos exposed to four DFB treatments: control, 0.13, 1 and 10 µg.L⁻¹, (b) *A. salina* larvae exposed to four treatment combinations (DFB and cypermethrin; DFB and diazinon; cypermethrin and diazinon; and DFB, diazinon and cypermethrin; and control), and (c) abnormalities after 72 h for *H. tuberculata* exposed to four DFB treatments: 0, 0.13, 1 and 10 µg.L⁻¹.

Test organism	Source of variation	df	MS	F	P
(a) <i>A. salina</i> embryo	DFB treatment	2	7 000 000	13.3	***
	Residual	24	527 778		
	Total	26			
(b) <i>A. salina</i> larvae	Treatment	4	3 470	12.7	***
	Residual	15	273.3		
	Total	19			
(c) <i>H. tuberculata</i> larvae	DFB treatment	3	4 252.8	120.2	***
	Residual	12	35.4		
	Total	15			

Raw data for experiments (a), (b) and (c) were homogenous (Cochran's test was non-significant), therefore transformations were not necessary. *** = P<0.001.

Table 4. Comparison of mortality using SNK tests for (a) *Artemia salina* embryos, (b) *A salina* larvae exposed to LC20 combinations: DFB and cypermethrin; DFB and diazinon; cypermethrin and diazinon; and DFB, diazinon and cypermethrin; and comparison of abnormality for (c) *Heliocidaris tuberculata* larvae.

Toxicity test description	Insecticide treatment (µg.L ⁻¹)	Statistical mean	SNK summary of significance
DFB			
(a) <i>A. salina</i> embryos at 48 h	10	2222	2222 > * 889 and 556
	1	889	
	0.13	556	
	0	0	
DFB, Cyp and D			
(b) LC20 <i>A. salina</i> larvae at 48 h	DFB + Cyp	85	85 > * 40 85, 60, 55, 40 > * 5
	DFB + D	60	
	D + Cyp	55	
	DFB + Cyp + D	40	
	Control	5	
DFB			
(c) <i>H. tuberculata</i> larvae at 72 h	10	86.8	86.8 > * 33.8, 21.5, 15 33.8 > * 21.5, 15
	1	33.8	
	0.13	21.5	
	0	15	

DFB = diflubenzuron, Cyp = cypermethrin and D = diazinon.
SNK = Student-Newman-Keuls, only significantly different means are shown.

To assess the effect of DFB on sea urchin development, 30 µL of sperm suspension was incubated at 20°C for 1 h, after which 1 mL of egg stock was added (n=4). Test tubes containing fertilised eggs from *H. tuberculata* were incubated at 20°C for 72 h. Tests were terminated by the addition of 0.2 mL of 5% formalin to each test tube. The first 100 larvae in each test tube were examined and the number of larvae that had developed normal 2-arm plutei was counted, indicating healthy development.

One-way ANOVA and SNK tests were used to determine which treatment means were significantly different. Homogeneities of variance were determined using Cochran's C-test and no transformations were necessary. US Environmental Protection

Agency provided a copy of the statistical software Toxstat 3.3 which was used to calculate EC50 (effect concentration causing 50% abnormality) values using Trimmed Spearman Karber Tests, and calculated LC20 values using probit estimates. Both these methods are considered suitable for determining estimates of mortality and abnormality in toxicity tests (NSW EPA 1999). LC50 values were calculated using regression equations for those experiments, which determine the effects of DFB on *A. salina* larvae, due to the data not fulfilling the requirements of Toxstat 3.3 (NSW EPA 1999).

RESULTS AND DISCUSSION

DFB had a strong inhibitory effect on the development of *A. salina* after 48 h of treatment. No dead embryos were found in controls after 48 h; although there were larger numbers of dead embryos found at $1 \mu\text{g.L}^{-1}$, compared to $0.13 \mu\text{g.L}^{-1}$, differences were not significant (Figure 1; Table 3 and 4). The numbers of dead embryos found at an insecticide concentration of $10 \mu\text{g.L}^{-1}$ were significantly higher than at concentrations of $0.13 \mu\text{g.L}^{-1}$ and $1 \mu\text{g.L}^{-1}$ (Table 3 and 4).

DFB also had a strong inhibitory effect on the larvae that progressed to the 2nd instar stage. The percentages of 1st instar stage larvae that progressed to 2nd instar stage larvae, compared to controls, for 0.13, 1 and $10 \mu\text{g.L}^{-1}$ DFB treatments was 70%, 16 % and 2 %, respectively (Figure 2a). The 48 h LC50 for *A. salina* larvae when exposed to DFB was $0.37 \mu\text{g.L}^{-1}$ (Figure 2b).

After finding the 48 h LC20 of *A. salina* larvae when exposed to DFB, cypermethrin and diazinon, insecticides were combined in order to determine if there were any synergistic or antagonistic effects on *A. salina* larvae mortality caused by chemical interactions. The 48 h LC20 to *A. salina* when exposed to DFB, cypermethrin and diazinon was 4.33, 6.88 and DFB was $8.33 \mu\text{g.L}^{-1}$, respectively (Figure 3). Where two insecticides were combined, the effects were synergistic (Figure 4): DFB and cypermethrin = LC20 + LC20 = LC85, DFB and diazinon = LC20 + LC20 = LC60, and cypermethrin and diazinon = LC20 + LC20 = LC55. The combined effect of all three insecticides, DFB, cypermethrin and diazinon, was antagonistic with LC20 + LC20 + LC20 (DFB, cypermethrin and diazinon, respectively) = LC40 (Figure 4).

DFB had no effect on the fertilisation process of *H. tuberculata* ova and sperm (Figure 5), but DFB did cause abnormalities to *H. tuberculata* larvae. After 72 h larval abnormalities at 1 and $10 \mu\text{g.L}^{-1}$ were significantly higher compared to larvae exposed at $0.13 \mu\text{g.L}^{-1}$ and controls (Figure 5, Table 3 and 4).

DFB has been known to prevent moulting of larval stages in a variety of insects (Antia et al. 1985), and this effect on larval stages in insects also applies to crustaceans in general. A potential concern is that DFB residues from run-off and discharge will persist in the environment and affect viabilities of natural crustacean populations (Savage 1998; Savage and Russell 1999).

We have shown that DFB causes lethal effects to *Artemia* larvae at DFB concentrations of 0.13 to $1 \mu\text{g.L}^{-1}$. It is important to note that concentrations as low as $0.13 \mu\text{g.L}^{-1}$ were found at the point of discharge from Black Rock Sewage Outfall during a study by Russell (1999). The 48 h LC50 of $0.37 \mu\text{g.L}^{-1}$ for *A. salina* larvae exposed to DFB was up to 30-fold lower than LC50 results for other crustacean larvae, namely *Rhithropanoplys harrisii* larvae (Christiansen et al. 1978; Table 1).

Because chitin synthesis does not occur during the onset of embryonic development in *A. salina* (Browne et al. 1991), the mortality of *A. salina* embryos is unlikely to have been due to the DFB acting as an inhibitor of chitin synthesis

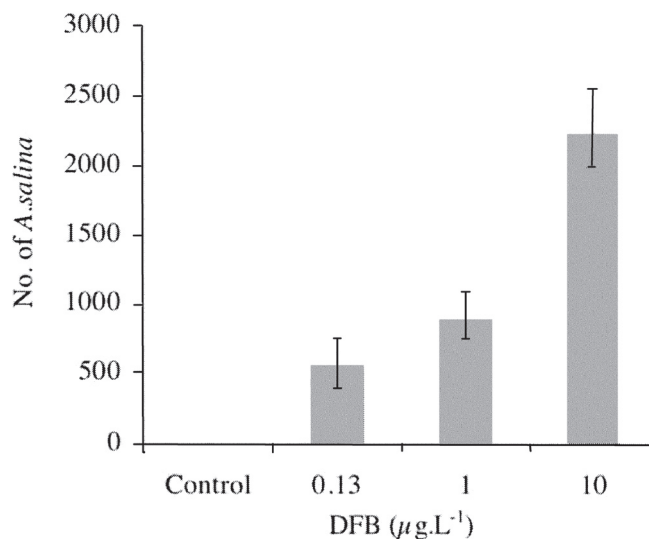


Figure 1. Mortality of hatched *Artemia salina* embryos exposed to diflubenzuron concentrations for 48 h. Three replicates per concentration were used; each contained 80 000 cysts. *Post-hoc* analyses show statistical differences between the $10 \mu\text{g.L}^{-1}$ treatment with the 1 and $0.13 \mu\text{g.L}^{-1}$ treatments.

(Cunningham 1976, 1986; Fischer and Hall, 1992). During *A. salina* embryonic development no chitin is synthesised as all energy is directed towards initial metabolic conversions that occur prior to chitin synthesis or the formation of the first instar stage (Browne et al. 1991).

Therefore, instead of embryo death resulting from chitin inhibition, DFB may have caused some other impairment to the maturation of embryos. At the present time, the exact mechanisms and biochemical steps involved in early embryonic development in *A. salina* are largely unknown (Browne et al. 1991). Further studies would need to be performed; firstly to understand more clearly the physiology and biochemistry of *A. salina* embryonic development, and then, the effects of DFB on certain aspects of the biochemical pathways.

Few studies have been published on the effects of interactions of insecticides on developmental stages of marine invertebrates including crustacean zooplankton. Here we show that DFB in combination with either cypermethrin or diazinon, and cypermethrin with diazinon cause synergistic mortality to *A. salina* larvae. This is an important finding because these combinations of insecticides are likely to be present in agricultural effluents, e.g a wool scouring residues (Russell 1999). Such a result raises concern when considering the discharge of multiple contaminants into the environment, and the risks to biota associated with these discharges. There is a need not only for testing combinations of toxicants but also whole effluent toxicity studies where test organisms are exposed to complex effluent mixtures (Schimmel et al. 1989). Although the toxicity of individual chemicals tested from the field may meet effluent limitations set by government agencies, the potential risk for these discharged chemicals to interact and cause synergistic responses to sensitive biota in the environment is likely (Abel 1989) and should be taken into consideration.

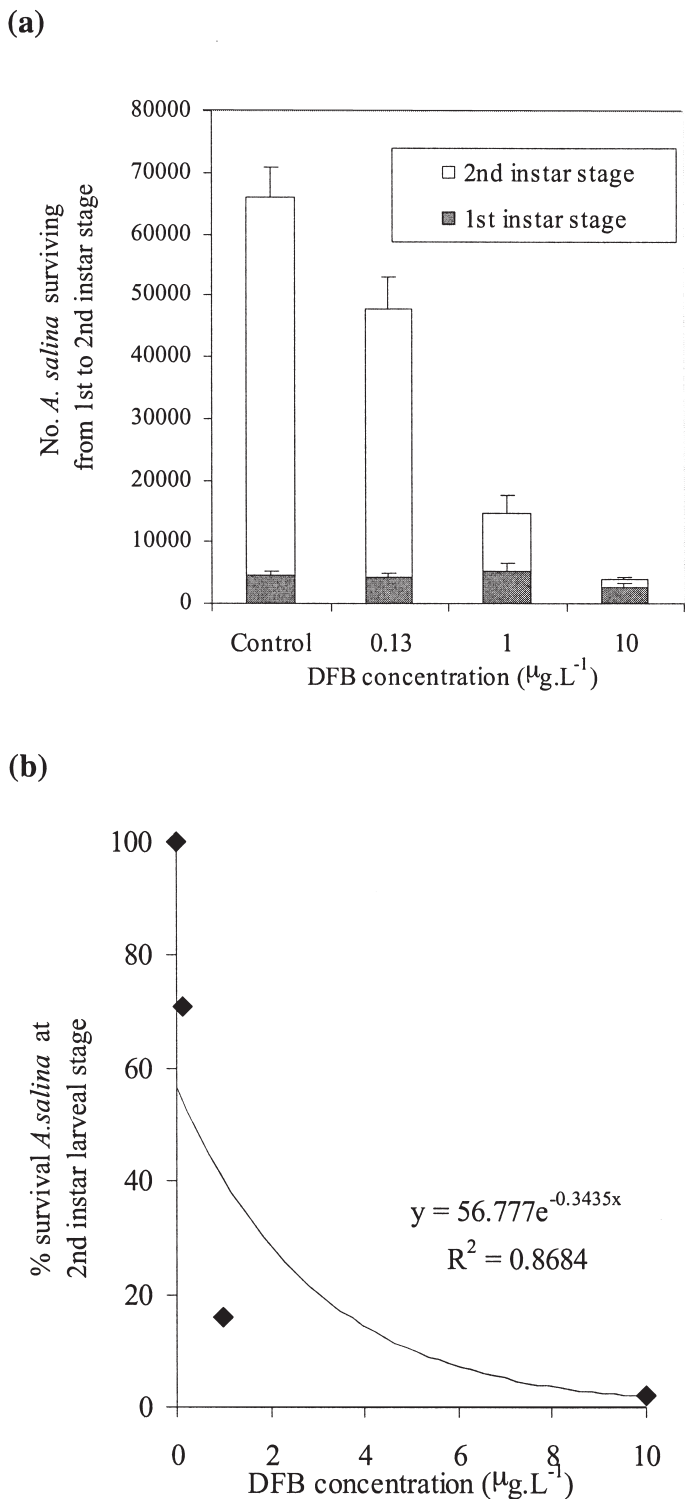


Figure 2. (a) Percentages of *Artemia salina* larvae that survived to the 2nd instar at the end of 48 h exposure to DFB treatments - 0, 0.13, 1 and 10 $\mu\text{g.L}^{-1}$ (n = 3). (b) A probit curve was used to estimate the 48 h LC50 of 0.37 $\mu\text{g.L}^{-1}$ for *Artemia salina* exposed to diflubenzuron (DFB)

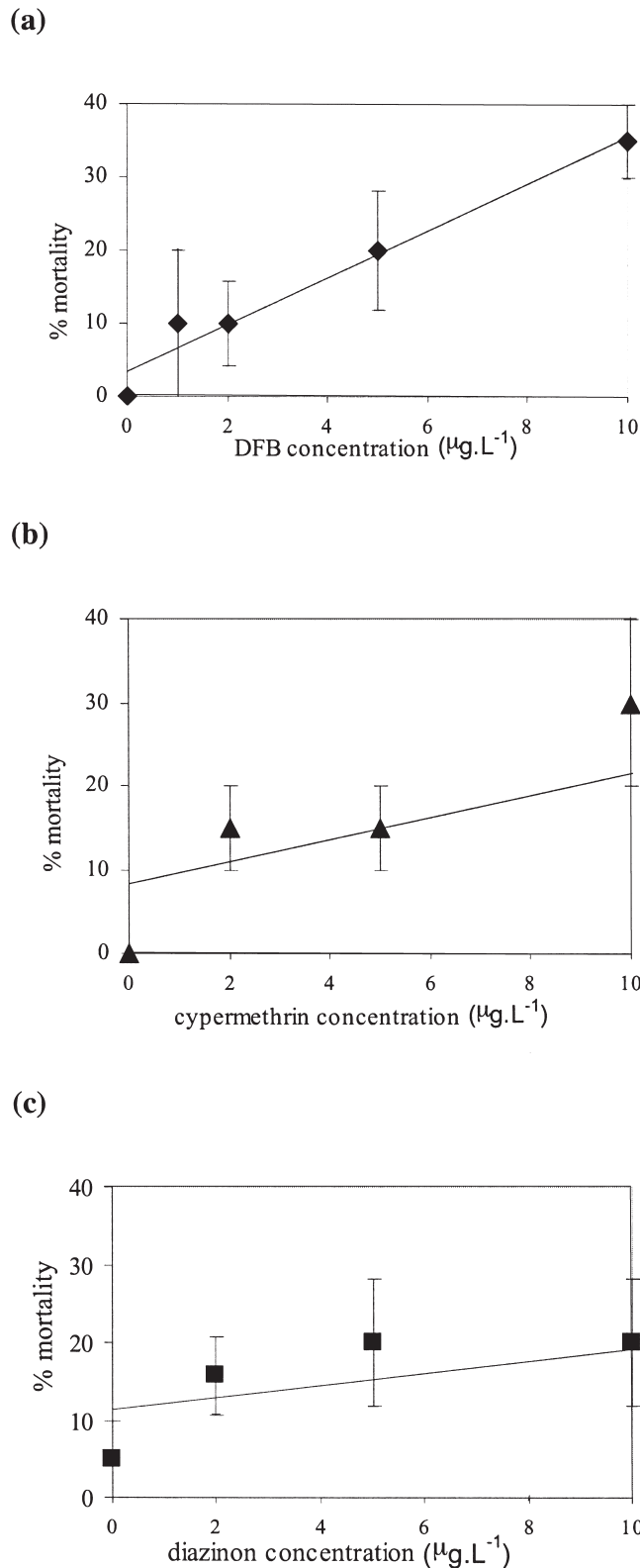


Figure 3. Percentage mortality for *Artemia salina* at 48 h when subjected to insecticides: (a) diflubenzuron (0, 1, 2, 5 and 10 $\mu\text{g.L}^{-1}$), (b) cypermethrin (0, 2, 5, 10 and 20 $\mu\text{g.L}^{-1}$) and (c) diazinon (0, 2, 5, 10 and 20 $\mu\text{g.L}^{-1}$). Each insecticide concentration had 4 replicates. The LC20 and confidence intervals shown were calculated using probit estimates with log transformation.

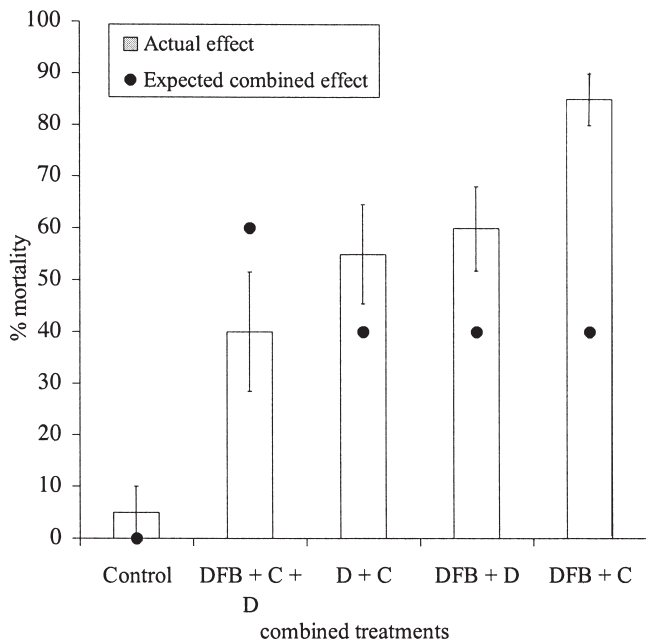


Figure 4. Mortality of *Artemia salina* larvae when exposed to combined 48 h LC20 for the insecticides: diflubenzuron (DFB) diazinon (D) and cypermethrin (C). Each insecticide combination had 4 replicates.

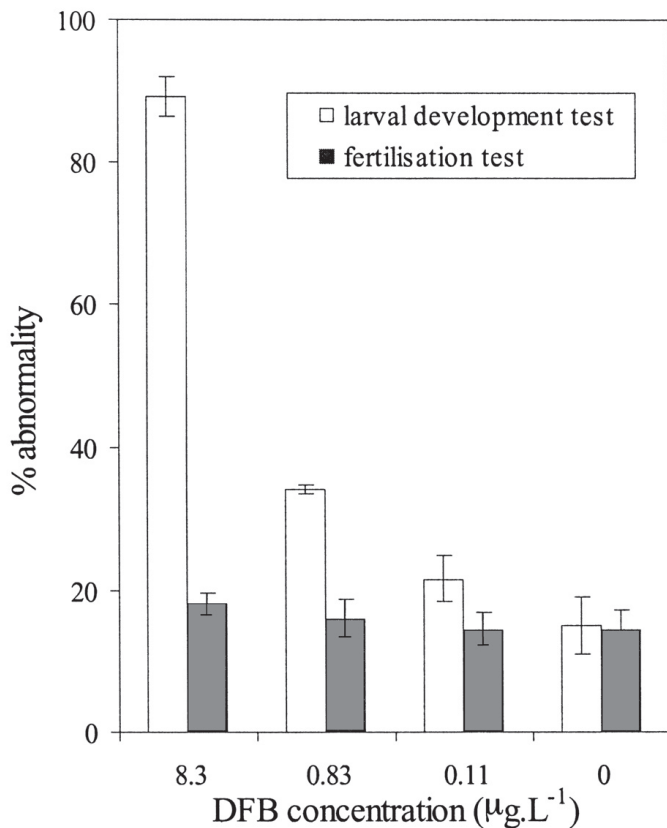


Figure 5. Percent abnormality of *Heliocidaris tuberculata* during fertilisation and larval development when exposed to diflubenzuron (DFB) concentrations: 0, 0.13, 1 and 10 µg.L⁻¹. The fertilisation and larval tests each had 4 replicates.

DFB was found to be non-toxic to the fertilisation of *H. tuberculata* sperm and eggs for DFB concentrations of up to 8.3 µg.L⁻¹, but DFB caused significant abnormalities to *H. tuberculata* larvae after 72 h at 1 and 10 µg.L⁻¹. The 72 h EC50 for *H. tuberculata* was 2.69 µg.L⁻¹. This level of toxicity has been found to be lower than many EC50 and LC50 toxicity values for crustacean larvae during previous studies (Table 4). The fact that DFB caused abnormalities to *H. tuberculata* larvae at comparable concentrations found to cause toxicity to crustacean larvae contradicts previous assertions that DFB targets only crustacean larvae at low concentrations (Antia et al. 1985; Cunningham 1986; Fischer and Hall 1992). Nevertheless, other marine animals such as fish and diatoms have been proven to be non-sensitive to DFB (Tasheva 1996). In fact, larval crustaceans have been shown to be more than 25 000 times more sensitive to DFB than fishes (Fischer and Hall 1992). The LC50 values for fish when exposed to DFB in the laboratory have been reported to be 150 mg.L⁻¹ (Tasheva 1996). Fish kills have never been reported in DFB field tests (Tasheva 1996). Thus, there is a need to define the range of organisms, particularly marine organisms, where DFB is toxic at environmental levels.

This study has two environmental implications which demand further research: (1) DFB together with cypermethrin, DFB and diazinon, and cypermethrin and diazinon caused synergistic mortality to *A. salina* larvae; however, when all three insecticides were combined together mortality to *A. salina* was antagonistic. These insecticides are known to be discharged into the marine environment as agricultural residues, yet studies of their synergistic and/or antagonistic effects have not previously been performed; (2) DFB has been shown to be toxic to not only crustacean larvae but also to the larvae of the sea urchin, *H. tuberculata*. Further studies are needed in order to determine the effects of DFB on a wider variety of marine biota to determine whether these and similar organisms may also be at risk to DFB and other chemicals when exposed in the environment.

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