Bull Environ Contam Toxicol (2009) 82:290–295
DOI 10.1007/s00128-008-9614-3

Comparative Toxicities of Fungicide and Herbicide Formulations on Freshwater and Marine Species

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Received: 26 October 2007 / Accepted: 11 November 2008 / Published online: 2 December 2008

Abstract The estimation of the toxic effects of plant protection products on non-target aquatic organisms is essential for risk assessment evaluation. In this study the acute toxicity of two fungicide and two herbicide formulations was determined in four marine species in comparison with the toxicity assessed for the freshwater crustacean *Daphnia magna*. From the study it is indicated that the marine crustacean species are effectively protected when acute toxicity data on *Daphnia magna* are used as surrogate for risk assessment while the comparative sensitivity of the unicellular green algae may vary considerably, depending on the mode of action of the specific formulation.

Keywords Ecotoxicology · Aquatic organisms · Fungicides · Herbicides

Contamination of water bodies very often appears to be the unavoidable consequence of agricultural activities, mainly related to the use of plant protection products (ppps). For the protection of the aquatic ecosystems against adverse effects from the uses of ppps and for the performance of reliable risk assessment for the aquatic organisms, the Directive 91/414/EEC sets several data requirements. However, both the required information and the information available in the literature are mainly related to the toxicity of ppps on freshwater organisms (Alberdi et al. 1996; Canton 1976; Ma et al. 2006, 2005, 2002). For geographical areas like southern Europe where the freshwater bodies are not as abundant as in the northern part and very often the marine ecosystems have several uses such as fish farming, the effective protection of both fresh and marine waters is of great importance. Thus, information on the toxic potential of ppps on marine organisms and the comparative sensitivity to the freshwater organisms is needed for the reassurance of the same degree of protection for the marine as for the freshwater ecosystems.

The aim of the present study is to determine the toxicity of four widely used pesticide formulations (two fungicides and two herbicides) in four marine species; the bacterium *Vibrio fischeri*, the green alga *Nannochloropsis occulata* the crustacean *Artemia fransiscana* and the marine rotifer *Brachionus plicatilis*. These marine organisms have been widely used as indicators for the toxicity evaluation of various pesticides (Ferrando and Andreu-Moliner 1991; Ruiz et al. 1997; Sanchez-Fortun et al. 1995; Somasundaram et al. 1990; Varo et al. 1998). Furthermore, the toxicity of the same formulations is tested on the freshwater crustacean *Daphnia magna*, which is considered to be one of the most sensitive species of the freshwater ecosystems (EFSA–Q-2005-042 opinion (2005)). Thus, the obtained data will provide information for the comparative sensitivity assessment between the freshwater and the marine organisms to fungicides and herbicides.

Materials and Methods

Water dispersible granule (WG) formulations of the fungicidal compounds thiophanate methyl and thiram at concentrations of 70% and 80% respectively, were tested. The herbicidal compounds pendimethalin as WG formulation at 60% and a suspension concentrate, combination of

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S-metholachlor and terbuthilazine at 31.2% and 18.8% respectively, were tested. The test substances were dissolved and diluted prior to the experiments and all the used concentrations were nominal. The toxicity tests were performed on five aquatic organisms, four marine and one freshwater, i.e., the bioluminescent bacterium *Vibrio fischeri* (*Photobacterium phosphoreum*), the green alga *Nannochloropsis occulata*, the rotifer *Brachionus plicatilis,* the crustacean *Artemia fransiscana* and the freshwater crustacean organism, *Daphnia magna.* Table 1 summarizes the experimental design of the study.

The Microtox® Analyzer Model 500 (Strategic Diagnostics Inc., Delaware, USA) was used to conduct the acute toxicity assay for the pesticide formulations under study. The microtox test is an ASTM Standard Method (D5660-96) (2004) and ISO standard method (11348-3:1998). The assay is based on the inhibition of the natural luminescence emitted by the bacterium *V. fischeri* when exposed to toxic compounds. The bacterial suspension was exposed to the test formulations and the acute toxicity was expressed as inhibition of bioluminescence. The end-point for the assessment of toxicity was the median effective concentrations (EC_{50}) at 5 and 15 min. The pH of all formulated solutions was adjusted within the range of 6–8. Each concentration of the studied formulations was tested at three replicates. The tests were performed according to the manufacturer's (Strategic Diagnostics Inc., Azur Environmental) protocols.

The algal growth inhibition test was performed on the unicellular organism *Nannochloropsis occulata* based on an in house experimental protocol in line with the principles of OECD guideline 201 (2006) and "ALGALOTOXKIT™: 72 hrs growth inhibition test based on the green alga *Selanastrum capricornum*" adapted to specific marine organism. The stock algal culture was maintained in flasks containing artificial seawater (Instant Ocean) at salinity of 28 ppt supplemented with vitamins, under continuous illumination with cool-white fluorescent light of 10,000 lux at 20 ± 2°C. The cell density in the culture was determined with a Thoma haematocytometer and the cell counts were correlated to the optical density (OD) as measured on a Jenway spectrophotometer at a wavelength of 670 nm. A good linear relationship between cell numbers and OD_{670} was obtained so the growth of algal cells during the bioassay was calculated indirectly by measuring the OD at 670 nm. During the bioassay, the algal cultures were incubated in long cell test vials (1 × 10 cm) with various concentrations of the respective formulation for 24, 48 and 72 h at a temperature of 20 ± 2°C, under continuous sideway illumination of 10,000 lux or 3,000–4,000 lux of top illumination. Each concentration of the studied formulations was tested at three replicates. The appropriate control system without the tested formulation was included in each experiment. Control and treated cultures were incubated under the same temperature and illumination conditions. The toxicity end point, of EC_{50} was determined as the concentration of the tested formulation resulting in 50% inhibition of the algal growth after 24, 48 and 72 h of incubation.

The toxicity of formulations on the cladocera *Daphnia magna* was assessed using commercially available Daphnia ephippia. The test was performed according to the principles of OECD guideline 202 (2004). The Daphnia ephippia (chitinous capsuled eggs) were incubated in pre-aerated standard freshwater for 72 h, at 20–22°C under continuous illumination of 6,000 lux. The hatched neonates were fed with a suspension of Spirulina microalgae for 2 h and then they were transferred into a multi-well test plate with the respective concentrations of the tested formulations, prepared in standard freshwater. The test plates were incubated in the darkness at a constant temperature of 20–22°C. Mortality of the daphnids was registered after 24 and 48 h of incubation, under stereoscope. The daphnids were considered dead if they remained settled at the bottom of the multi-well test plate without moving for a period of 15 s of observation. The toxicity end point, LC_{50}, was determined as the concentration of the tested formulation resulting in 50% mortality of daphnids at 24 and 48 h of exposure.

The rotifer *Brachionus plicatilis* was obtained from commercially available rotifer cysts. The brachionus cysts were incubated in pre-aerated artificial seawater (Instant

Table 1 Experimental design table: concentration range tested on the organisms

Tested organisms	Concentration range (mg/L)			
	Thiophanate methyl 70% WG	Thiram 80% WG	Pendimethalin 60% WG	S-metolachlor 31.2% + terbuthilazine 18.8% SC
V. fischeri	7–1,800	0.07–180	7–1,800	1–1,800
A. fransiscana	25–675	0.0156–1	15.625–1,000	25–675
B. plicatilis	1.5625–400	0.0078–2	3.9–1,000	3.9–1,000
D. magna	0.5–50	0.005–0.5	2.5–250	2–200
N. occulata	0.5–50	0.005–0.5	1–100	1–100

Ocean) with reduced salinity of 20 ppt, at 25°C, for 28–30 h under continuous illumination of 3,000–4,000 lux. The neonates were collected within 2 h after hatching and transferred into a multi-well test plate with the formulation dilutions prepared in standard seawater, 35 ppt salinity. The test plates were incubated in the darkness at a constant temperature of 25°C and examined for mortality of the organisms at 24 h of exposure under stereoscope. The rotifers were considered dead if they remained settled at the bottom of the multi-well test plate without moving for the observation period of 15 s. The toxicity end point, LC_{50}, was determined as the concentration of the tested formulation resulting in 50% mortality of *Brachionus plicatilis* at 24 h of exposure.

The nauplii of the marine copepod *Artemia fransiscana* were hatched from commercially available cysts. The cysts were incubated in artificial seawater (Instant Ocean) at salinity of 35 ppt. The first nauplii usually appeared after 24 h of incubation at 25°C under aeration and illumination of 1,000–4,000 lux. They were transferred into new seawater and incubated for 24 h under similar conditions. Then, they were transferred into a multi-well test plate with the respective concentrations of the tested formulations, prepared in artificial seawater. The toxicity of the formulations on *Artemia fransiscana* nauplii was tested at 24 and 48 h of exposure at 25°C in the darkness. The toxicity end point, LC_{50}, was determined as the concentration of the tested formulation resulting in 50% mortality of *Artemia* nauplii at 24 and 48 h of exposure.

The toxicity tests performed on *Brachionus plicatilis* and *Artemia fransiscana* were in house methods based on the principles of the OECD 202 test for *Daphnia* sp. adapted for the marine crustacean.

For all test organisms, range-finding tests were performed for the determination of the appropriate concentrations, used in the definitive tests. The respective LC_{50} and EC_{50} values were calculated using linear regression analysis as natural logarithm of the concentration of formulation versus percentage of mortality or percentage of growth inhibition. All the effective concentration values based on the percent of total product (formulation).

Results and Discussion

The results from the Microtox® assay are presented as acute 5 and 15 min EC_{50} values in Table 2. Differences were observed between formulations with regard to EC_{50} values but also to the duration of exposure required for the complete inhibition of light emission from the bacteria. Thiram 80% WG formulation exhibited the highest toxicity and the EC_{50} values were determined at 0.45 and 0.32 mg/L for 5 and 15 min of exposure, respectively. These results are in agreement with previous study, where the EC_{50} of pure thiram (99%) in the bacterium *V. fischeri* was determined at 0.25 and 0.19 mg/L for 5 and 15 min of exposure, respectively (Twagilimana et al. 1998). The rest of the three tested formulations exhibited much lower toxicity.

The results of the toxicity assay on the alga *Nannochloropsis occulata* are presented in Table 2. Thiram 80% WG was the most toxic formulation tested without any significant change in EC_{50} values with increased duration of exposure. All other tested formulations exhibited much lower toxicity. The EC_{50} values of S-metolachlor 31.2% + terbuthilazine 18.8% SC formulation did not change significantly with the duration of exposure. In the case, of Thiophanate methyl 70% WG the *Nannochloropsis occulata* population recovered with the longer duration of exposure and the EC_{50} values were determined at 5, 11.3 and 93 mg/L at 24, 48 and 72 h, respectively. The toxicity of the active substance thiophanate methyl technical in different algae species had been examined in previous studies and the EC_{50} values ranged from 5.7 to 138 mg/L for *Chlorella pyrenoidosa* and *Scenedesmus obliqnus,* respectively (Canton 1976; Ma et al. 2002). In addition Pendimethalin 60% WG reached maximum toxicity on the first 24 h of the bioassay. The decline of toxicity observed at 48 and 72 h of exposure is mainly attributed to the degradation of the active substance and mainly photodegradation under the conditions of the specific assay followed by recovery of algae population (Weed Science Society of America 1994).

The toxicity of the formulations tested on the rotifer *Brachionus plicatilis* are presented in Table 3. Thiram 80% WG formulation was the most toxic formulation on this organism while pendimethalin 60% WG formulation was the least toxic. Thiophanate methyl 70% WG and S-metolachlor 31.2% + terbuthilazine 18.8% SC formulations exhibited moderate toxicity.

The toxicity on the crustaceans *Artemia fransiscana* and *Daphnia magna* are presented in Table 3. From the presented results it is concluded that the fungicide thiram 80% WG formulation is the most toxic while the herbicide formulations of pendimethalin 60% WG and S-metolachlor 31.2% + terbuthilazine 18.8% SC were the least toxic on both *Artemia fransiscana* and *Daphnia magna*. The LC_{50} values of thiophanate methyl 70% WG determined for *Daphnia magna* are in agreement with other studies where the LC_{50} value of pure thiophanate methyl on *Daphnia magna* was determined at 16 mg/L for 48 h of exposure (Canton 1976). All tested formulations exhibited higher LC_{50} values at 24 h than at 48 h of incubation. Moreover, the crustacean *Artemia fransiscana* is more resistant than *Daphnia magna* when the respective LC_{50} values are considered.

Table 2 Toxicity of the tested formulations on the marine bacterium *Vibrio fischeri* and on marine algae *Nannochloropsis occulata*

Formulation	Time of exposure	Regression equation	Coefficient correlation	EC_{50} (mg/L)
Vibrio fischeri				
Thiophanate methyl 70% WG	5 min	Y = 22.13X − 73.65	0.9865	267.0
	15 min	Y = 26.17X − 74.63	0.9729	117.0
Thiram 80% WG	5 min	Y = 22.18X + 67.53	0.9615	0.45
	15 min	Y = 23.06X + 76.39	0.921	0.32
Pendimethalin 60% WG	5 min	Y = 14.65X − 28.61	0.9785	215.0
	15 min	Y = 15.08X − 31.65	0.981	225.0
S-metolachlor 31.2% + terbuthilazine 18.8% SC	5 min	Y = 18.58X − 40.3	0.9627	130.0
	15 min	Y = 17X − 27.59	0.9159	96.0
Nannochloropsis occulata				
Thiophanate methyl 70% WG	24 h	Y = 22.886X + 13.408	0.9842	5.0
	48 h	Y = 21.843X − 2.945	0.9677	11.3
	72 h	Y = 12.353X − 5.9908	0.9287	93.0
Thiram 80% WG	24 h	Y = 23.596X + 124.99	0.9358	0.04
	48 h	Y = 23.043X + 125.16	0.9327	0.04
	72 h	Y = 24.27X + 125.97	0.939	0.04
Pendimethalin 60% WG	24 h	Y = 12.345X + 15.528	0.904	16.3
	48 h	–	–	>100
	72 h	–	–	>100
S-metolachlor 31.2% + terbuthilazine 18.8% SC	24 h	Y = 20.792X + 8.3652	0.9613	7.4
	48 h	Y = 22.66X + 6.7666	0.9473	6.7
	72 h	Y = 25.159X − 8.2512	0.9132	10.5

Y = percentage inhibition, X = natural logarithm of formulation concentration

From the above results it is indicated that, the most toxic formulation for all tested organisms was the fungicide thiram 80% WG formulation (Table 4). The most sensitive organisms to this formulation were the unicellular marine algae and the freshwater crustacean *Daphnia magna*. On the contrary, the marine crustacean *Artemia fransiscana* and the rotifer *Brachionus plicatilis* were less sensitive to thiram formulation although the difference in toxicity values was slight. The marine bacterium *Vibrio fischeri* was the most resistant organism.

Thiophanate methyl 70% WG formulation was less toxic than the respective thiram formulation (Table 4). Also in this case, the most sensitive organisms were the microalgae and *Daphnia magna* and slightly less sensitive was the rotifer *Brachionus plicatilis.* The marine crustacean *Artemia fransiscana* was more resistant to thiophanate methyl formulation and the bacterium *Vibrio fischeri* was again the least sensitive organism. In addition, from the results of this study we can conclude that the thiophanate methyl 70% WG reached its maximum toxic effect for *Artemia fransiscana* and *Daphnia magna* with prolonged exposure time since the calculated LC_{50} values were much lower at 48 h than the respective 24 h of incubation (Table 4). Also, in the case of photobacterium *Vibrio fischeri* the thiophanate methyl 70% WG formulation reached its maximum toxic effect at 15 min of incubation. On the contrary, the algae population recovered at 48 and 72 h of exposure where lower toxicity was observed.

The herbicide pendimethalin 60% WG exhibited higher toxicity on the unicellular marine algae *Nannochloropsis occulata* at 24 h of exposure while the population recovered at 48 and 72 h of exposure (Table 4). This is attributed to photodegradation of pentimethalin under the experimental conditions of the specific assay (Weed Science Society of America 1994). The freshwater crustacean *Daphnia magna* was much more sensitive than the marine crustacean *Artemia fransiscana*. For both organisms, higher toxicity was observed after prolonged exposure (48 h). The rotifer (*Brachionus plicatilis*) was almost as sensitive as *Daphnia magna* and also in this case the photobacterium *Vibrio fisheri* was the least sensitive organism to pendimethalin 60% WG formulation.

Finally, the herbicide S-metolachlor 31.2% + terbuthilazine 18.8% SC exhibited the maximum toxicity on the marine alga *Nannochloropsis occulata* at 24 and 48 h of exposure and a slight recovery at 72 h (Table 4). In this case, as in the case of pendimethalin 60% WG, the crustacean *Daphnia magna* was more sensitive than the crustacean *Artemia fransiscana* and the rotifer *Brachionus plicatilis.* The toxicity for crustacean and rotifers was

Table 3 Toxicity of the tested formulations on the marine rotifer *Brachionus plicatilis*, the marine crustacean *Atremia fransiscana* and the marine crustacean *Daphnia magna*

Formulation	Time of exposure (h)	Regression equation	Coefficient correlation	LC_{50} (mg/L)
Brachionus plicatilis				
Thiophanate methyl 70% WG	24	$Y = 17.27X - 11.08$	0.9686	34
Thiram 80% WG	24	$Y = 14.81X + 94.42$	0.9735	0.05
Pendimethalin 60% WG	24	$Y = 17.3X - 34.43$	0.9054	132
S-metolachlor 31.2% + terbuthilazine 18.8% SC	24	$Y = 19.19X - 27.79$	0.9135	58
Atremia fransiscana				
Thiophanate methyl 70% WG	24	$Y = 0.1775X - 0.2513$	0.9657	69
	48	$Y = 0.1547X + 0.0469$	0.9323	19
Thiram 80% WG	24	$Y = 0.1548X + 0.8105$	0.9335	0.13
	48	$Y = 0.0866X + 0.83$	0.96	0.02
Pendimethalin 60% WG	24	$Y = 0.1948X - 0.6404$	0.9	350
	48	$Y = 0.2339X - 0.5123$	0.9335	76
S-metolachlor 31.2% + terbuthilazine 18.8% SC	24	$Y = 0.2264X - 0.6598$	0.9874	168
	48	$Y = 0.2276X - 0.2658$	0.9494	29
Daphnia magna				
Thiophanate methyl 70% WG	24	$Y = 12.24X + 11.37$	0.9152	23.5
	48	$Y = 16.62X + 34.21$	0.9451	2.6
Thiram 80% WG	24	$Y = 15.61X + 104.01$	0.9292	0.03
	48	$Y = 16.66X + 123.04$	0.9062	0.01
Pendimethalin 60% WG	24	$Y = 13.35X - 12.99$	0.9627	112
	48	$Y = 15.61X - 12.01$	0.9474	53
S-metolachlor 31.2% + terbuthilazine 18.8% SC	24	$Y = 21.27X - 13.66$	0.9068	20
	48	$Y = 19.78X + 5.44$	0.906	9.5

Y = percentage inhibition, X = natural logarithm of formulation concentration

Table 4 Comparative toxicity data (EC_{50} and LC_{50} values in mg/L) of the different tested formulations on five aquatic organisms

	Thiophanate methyl 70% WG	Thiram 80% WG	Pendimethalin 60% WG	S-metolachlor 31.2% + terbuthilazine 18.8% SC
V. fischeri 5/15 min	267/117	0.45/0.32	215/225	130/96
A. fransiscana 24/48 h	69/19	0.13/0.02	350/76	168/29
B. plicatilis 24 h	34	0.05	132	58
D. magna 24/48 h	23.5/2.6	0.03/0.01	112/53	20/9.5
N. occulata 24/48/72 h	5/11.3/93	0.04/0.04/0.04	16.3/>100/>100	7.4/6.7/10.5

higher when the duration of exposure was prolonged. Finally, the photobacterium *Vibrio fisheri* was again the least sensitive organism to the tested formulation.

In summary, the above results indicate that there are significant differences in sensitivity among species when they are exposed to the same formulations. The unicellular marine green alga *Nannochloropsis occulata* was the most sensitive from the tested organisms, especially for the herbicide formulations, as expected. However, the organism recovered with prolonged exposure for almost all tested formulations. The most resistant organism was the marine photobacterium *Vibrio fischeri.* Concerning the more complex life forms the marine species *Artemia fransiscana* and *Brachionus plicatilis* seemed to be more tolerant to the toxic substances than the freshwater crustacean *Daphnia magna,* which appears to be the most sensitive aquatic organism. This is inline with the ranking of freshwater species sensitivity presented in EFSA–Q-2005-042 opinion. However we cannot exclude the possibility that the marine species can be more sensitive than freshwater organisms when other endpoints are monitored or other chemical substances with specific modes of actions are tested.

Conclusion

The above comparative toxicity data indicate that the marine crustacean species are effectively protected when data on *Daphnia magna* are used as surrogate for acute toxicity risk assessment while the comparative sensitivity of the unicellular green algae may vary considerably, depending on the mode of action of the specific formulation.

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