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Short ingestion tests as alternative proposal for conventional range finding assays with *Thamnocephalus platyurus* and *Brachionus calyciflorus*

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ABSRACT: The goal of this study was to evaluate whether short 1 h sublethal assays may predict the results of 24 h lethality assays with rotifers *Brachionus calyciflorus* and anostracan crustaceans *Thamnocephalus platyurus*. The test bionts were hatched from cysts. Inhibition of ingestion was observed after 15 min of incubation of rotifers and crustaceans with the suspension of carmine and latex beads, respectively. Nine compounds with different modes of action were used as toxicants zinc ions, sodium dodecyl sulphate, p-nitrophenol, 3, 5-dichlorophenol and pharmaceuticals propranolol, fluoxetine, abamectin, doramectin and ivermectin. The toxicity values observed in the ingestion tests were very close to the mortality values over a wide range of toxicity from a low toxic surfactant to very toxic avermectins. The ratio between the 1 h EC_{50}'s in the ingestion test and the 24 h LC_{50}'s in the lethality test was below 2 in all cases for rotifers, and 7 in 9 cases for crustaceans. The toxicity of zinc and 3,5-dichlorophenol in the Thamnotoxkit F™ was 15-fold higher and 10 fold lower than in the ingestion test, respectively. The 24 h LC_{50} values are within the range of 25-400 % of the 1 h EC_{50} values for almost all toxicants tested with the exception of p-nitrophenol for *B. calyciflorus* and zinc and 3, 5-dichlorophenol for *T. platyurus*. Short, 1 h ingestion assays Rotoxrapid and Rapidtoxkit are good predictors of the mortality over the next 24 h and can be used as a range finding tests for representatives of pharmaceuticals and surfactants.

Keywords: *Avermectins; Bioassay; Ingestion assay; Pharmaceuticals in the environment; Toxkit*

INTRODUCTION

Rotifers and crustaceans play an important role in many aquatic ecosystems as suspension feeders. With their high assimilation efficiencies, rotifers convert a considerable portion of their food into biomass (Snell and Janssen, 1995). On the other hand, they are often the first food for many larval fish. They are an important link between the nanoplankton and macrozooplankton. Rotifers, especially the genus *Brachionus,* have been used in ecological studies as early as the 1970s. In 1989, a standard toxkit protocol with *Brachionus calyciflorus* was proposed by the group of Prof. Persoone from the University of Ghent in Belgium (Snell and Persoone, 1989) and two years later, in 1991 an ASTM standard acute toxicity test was published (ASTM, 1991). Since then, rotifers in ecotoxicological studies have been used more often (Snell and Janssen, 1995; Preston *et al*., 2001; Sanchez-Fortun and Barahona, 2005;Dahms *et al*., 2011). Anostracan crustacean *Thamnocephalus platyurus* has been used in ecotoxicology for 15 years (Centeno *et al*., 1995). Its high sensitivity has been shown in many kinds of samples including pesticides (Fochtman *et al*., 2000; Bakuolia *et al*., 2008; Palma *et al*., 2008), cyanobacterial toxins (Törökné *et al*., 2000) and pharmaceuticals (Na³êcz-Jawecki and Persoone, 2006; DellaGreca *et al*., 2007; Kim *et al*., 2009).

Ingestion is an ecologically important behaviour that directly affects growth and reproduction. A feeding activity can be assessed by allowing a small population to feed on unicellular algae or special artificial food. An ingestion rate may be a good estimator of toxicity in suspension-feeding microinvertebrates. One of the main advantages of the ingestion assay is its speed (Snell, 2005). An ingestion test with rotifers was developed in 1994 (Juchelka and Snell, 1994) and refined ten years later (Snell, 2005).

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Rapidtoxkit test utilises larvae of anastracan *T. platyurus* freshly hatched from the dormant cysts. In the Rapidtoxkit assay the sublethal physiological reaction of the organisms to toxic stress is assessed by introducing a suspension of red latex beads (5 µm) in the test medium following a 1 h exposure of the organisms to the toxicant, and by observing the presence or absence of coloration of the digestive tract under a dissection microscope. The test was introduced by MicroBioTests in 2004 as a screening tool for water contamination emergencies resulting from accidental or deliberate introduction of toxicants in water supplies (Rapidtoxkit, 2004; Törökné *et al*., 2007).

Standard acute lethality assays last at least 24 h, and when testing unknown samples, they require preliminary range finding tests, which prolong the time of analysis by additional 24 h. Moreover, the new set of larvae should be hatched from the cysts, which double the cost of analysis. The goal of this paper is to evaluate whether a short 1 h sublethal assay may predict the results of the 24 h lethality assay, and whether this test may be used as a range finding test. Three groups of toxicants were chosen due to their widespread detection in environmental samples: metals (Reza and Singh, 2010), simple organics and pharmaceuticals (Giri *et al.,* 2010).

MATERIALS AND METHODS

Chemicals

Nine compounds were tested as toxicants, namely zinc ions (as $ZnSO_4$ pure p.a., POCh, Poland), sodium dodecyl sulphate (SDS specially pure, Serva), p-nitrophenol (PNP pure p.a., Sigma), 3,5-dichlorophenol (DCP 97 %, Sigma), propranolol (PL analytical standard, Sigma), fluoxetine (FLU analytical standard, Sigma), abamectin (ABM analytical standard, Sigma), doramectin (DOM analytical standard, Sigma) and ivermectin (IVM analytical standard, Sigma). The last three compounds are hardly soluble in water, thus stock solutions (1 mg/mL) were prepared in methanol, and the working solutions were prepared by dilution the stock solutions in diluent (moderately hard US EPA medium, EPA, 2002).

In the tests with rotifers and anostracans, the suspension of carmine (Sigma) and red latex beads (MicroBioTests, Belgium) respectively were used as artificial food.

Toxicity tests

Thamnotoxkit F™

The Thamnotoxkit F™ microbiotest is a 24 h acute lethal toxicity test with anostracan crustacean *Thamnocephalus platyurus*. Organisms were hatched from dormant cysts after 24-40 h incubation at 25 ºC in synthetic freshwater (moderately hard US EPA medium, EPA, 2002) under continuous illumination (3000–4000 lux). The assays were carried out in 24 well multiplates according to the standard operational procedure for this microbiotest (Thamnotoxkit F™, 1995). In each plate five concentrations of sample plus negative control were tested. Samples were prepared in 2-fold dilution series in three replicates with ten animals having been added to each well that contained 1.0 mL of tested dilution. After 24 h of incubation at 25 ºC in the dark the dead organisms were counted. On the basis of the percentage of mortality the 24 h LC_{50} and 24 h LC_{20} were calculated with a graphical interpolation method (EPA, 2002).

Rapidtoxkit - ingestion test with T. platyurus

The test was based on the Rapidtoxkit protocol (Rapidtoxkit, 2004) with modifications (Na³êcz-Jawecki and Persoone, 2006). Shortly, the test was performed in 12 well multiplates. Each well contained 5 mL of the dilution of the sample and 20 testbionts. The *T. platyurus* larvae should be older than in the mortality test (hatching should last 30-40 h). After 1h incubation in darkness at 25 ºC the suspension of red latex beads was added. The crustacea fed on artificial food for 15 min, then they were killed by one drop of Lugol solution. The number of intoxicated larvae in each concentration was counted with the use of dissection microscope (10x magnification). The organism was counted as intoxicated, when its digestive track was colourless, without any red particles (Fig. 1). On the basis of the percentage of affected crustaceans 1 h EC_{50} and 1 h EC_{20} values were calculated with graphical interpolation method (EPA, 2002).

Rotoxkit F™ with modifications

The Rotoxkit F™ microbiotest is a 24 h acute lethal toxicity test with freshwater rotifer *Brachionus calyciflorus*. Organisms were hatched from dormant cysts after 16–18 h incubation at 25 ºC in synthetic freshwater (moderately hard US EPA medium, EPA, 1993) under continuous illumination (3000–4000 lux). The assays were performed according to the standard

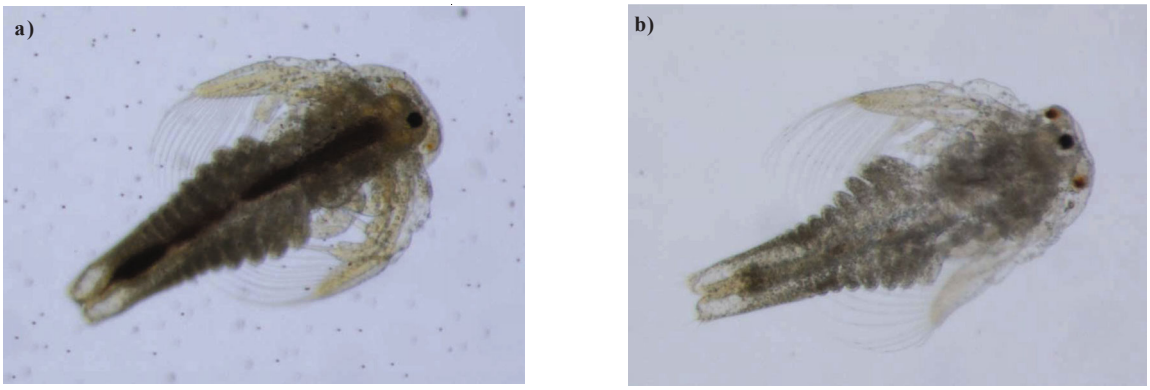

Fig. 1: Rapidtoxkit assay with *Thamnocephalus platyurus*. Larvae with artificial food (a) and affected larvae with colorless digestive track (b)

operational procedure for this microbiotest (Rotoxkit F™, 2000) with modifications. Disposable polistyrene 48-well multiplates were used as test containers. Two samples may be tested in one plate, five concentrations of each plus negative control. Samples were prepared in 2-fold dilution series in three replicates, and ten animals were added to each well containing 0.5 ml of tested dilution. After 24 h incubation at 25 ºC in the dark, the dead rotifers were counted. On the basis of the percentage of mortality, the 24 h-LC_{50} and 24 h-LC_{20} were calculated with a graphical interpolation method (EPA, 2002).

RotoxRapid - ingestion test with B. calyciflorus

The test was performed in a 48 well multiplate and the preparation of the plate was the same as in the mortality test. After 1 h incubation in darkness at 25 ºC, the suspension of carmine was added. The final suspension of carmine in the test wells was 5,000/mL. Rotifers fed on the carmine particles for 15 min, after which they were killed by one drop of Lugol solution. The number of feeding and non-feeding rotifers in each concentration was counted with the use of dissection microscope (20x magnification). On the basis of the percentage of non-feeding rotifers 1 h-EC_{50} and 1 h-EC_{20} values were calculated. There was no distinction for the intensity of the particle uptake during the calculation of the number of organisms which took coloured particles (Fig. 2).

RESULTS AND DISCUSSION

Brachionus calyciflorus

B. calyciflorus was the most sensitive to FLU with the LC_{50} below 1 mg/L (Table 1). Zinc ions, DCP and other pharmaceuticals with the LC_{50} values between 1 and 10 mg/L are classified as toxic, while SDS was harmful to the rotifer with the LC_{50} value between 10 and 100 mg/L. However, the partial mortality was caused by lower concentrations of SDS, and the threshold toxicity values (24 h-LC_{20}) for all toxic compounds were below 10 mg/L. PNP did not cause lethal effects in all concentrations up to 20 mg/L.

The toxicity values observed in the RotoxRapid were very close to the mortality values. The ratio between the 1h-EC_{50}'s in the ingestion test and the 24 h-LC_{50}'s in the lethality test was below 2 for all compounds tested (Table 1 and Fig. 3). Slightly more pronounced differences between the two assays were observed for EC_{20} and LC_{20} values. The EC_{20}/LC_{20} ratio was higher than the EC_{50}/LC_{50} ratio in the case of 5 out of 9 toxicants tested, reaching the values of 2.36 and 2.06 for PL and FLU, respectively (Table 1). Contrary to that, Zn and SDS affected rotifers' ingestion of the carmine at the levels lower than the lethal concentration. This phenomenon may be explained by the induction of the resistance to toxicants. As it is known, metalothioneins which are responsible for the resistance to metals of many animals, have not been reported in rotifers yet. The toxic stress caused by PNP after 1 h was not observed in the Rotoxkit F™ assay in all concentrations up to 20 mg/L. Thus, the toxicity endpoints cannot be calculated. The volatile organic compounds should be tested with a special procedure (Na³êcz-Jawecki and Sawicki, 1999). However, volatility can not be the only reason for the decrease of PNP toxicity, since in the case of DCP the results of both assays with rotifer are similar.

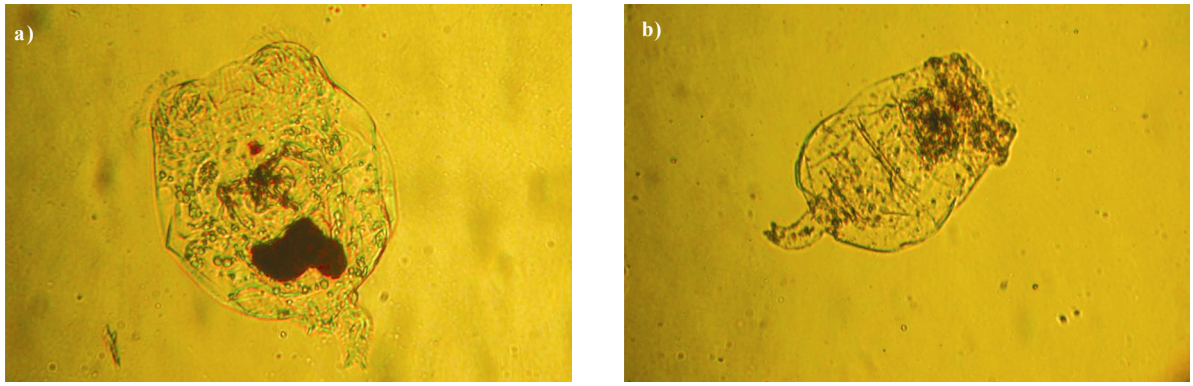

Fig. 2: Ingestion assay with *Brachionus calyciflorus*. Rotifer with artificial food (a) and affected rotifer with colorless digestive track (b)

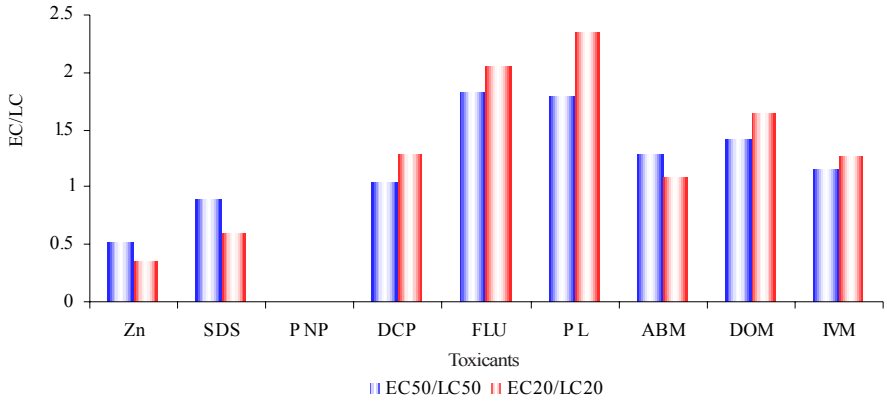

Fig. 3: The ratio of the toxicity values in the ingestion test to the mortality test (I/M) for *B. calyciflorus*

Table 1: The toxicity of 9 chemicals towards the rotifer *Brachionus calyciflorus*

Toxicant	Abbreviation	Ingestion test (mg/L)				Mortality test (mg/L)				I/M*	
		1 h-EC_{50}	SD	1 h-EC_{20}	SD	24 h-LC_{50}	SD	24 h-LC_{20}	SD	EC_{50}/LC_{50}	EC_{20}/LC_{20}
Zinc (Zn^{2+})	Zn	2.15	0.47	0.88	0.56	4.10	1.69	2.51	0.99	0.52	0.35
SDS	SDS	12.22	3.53	5.44	3.73	13.78	4.92	9.08	5.65	0.89	0.60
p-nitrophenol	PNP	12.75	0.10	8.80	0.10	> 20	-	> 20	-	-	-
3,5-dichlorophenol	DCP	5.27	2.00	4.04	1.70	5.08	1.20	3.14	1.20	1.04	1.29
Fluoxetine	FLU	0.97	0.34	0.64	0.39	0.53	0.26	0.31	0.12	1.83	2.06
Propranolol	PL	3.88	0.48	2.76	0.13	2.15	0.58	1.17	0.37	1.80	2.36
Abamectin	ABM	3.08	0.40	2.31	0.76	2.38	0.81	2.11	0.94	1.29	1.09
Doramectin	DOM	2.86	0.32	1.70	0.48	2.03	1.04	1.03	0.52	1.41	1.65
Ivermectin	IVM	1.81	0.29	1.31	0.22	1.57	0.08	1.04	0.22	1.15	1.26

* - Ratio of the toxicity value in the ingestion test to that in the mortality test

The ingestion ratio in the test with rotifers is an excellent predictor of the mortality over the next 24 h. The dilution series of a sample tested in the standard Rotoxkit F™ assay comprises five 2 fold concentrations. If the highest concentration tested in Rotoxkit F™ is equal to 400 % of 1 h EC_{50}, both toxicity values 24 h-LC_{50}, and 24 h-LC_{20}, are within the range of tested dilutions (Fig. 4). The only exception was PNP, which did not cause the mortality in all tested concentrations.

Thamnocephalus platyurus

The anostracan crustacean *T. platyurus* was extremely sensitive to antihelminthic pharmaceuticals. The 24 h LC_{50} values were 5.7, 8.9 and 13.2 µg/L for IVM, DOM and ABM, respectively (Table 2). A 1 h incubation in Rapidtoxkit at concentration of 20 µg/L of the drug was enough to observe a lethal response (data not presented). These compounds generated also very low threshold toxicity values, which were 3.4 to 4.7 fold lower than the 1 h EC_{50}. Avermectins enhance inhibitory neurotransmission by activating glutamate-gated chloride channels in neurons and myocytes. The results clearly show that bioavailability of the compounds is very high and the neurotoxic action is very fast. However, to some degree, the inhibitory effect was reversible and did not cause death of the organism. Thus, for these toxicants the 1 h EC/24 h LC ratios were below 1. Zinc ions and FLU were very toxic to the crustacean with 24 h-LC_{50} values below 1 mg/L, while SDS and PNP were considered as harmful with 24 h-LC_{50}'s of 15.1 and 27.4 mg/L, respectively.

Like in the case of the rotifer assays, the ratio between the 1 h EC_{50}'s in the ingestion test and the 24 h-LC_{50}'s in the lethality test was below 2 for almost all compounds tested. However, there were two exceptions. The toxicity of Zn drastically increased over the time of incubation. Both median and threshold toxicity values in the Thamnotoxkit F™ were 15 and 18 fold lower than in the ingestion test (Table 2 and Fig. 5). On the other hand, DCP affected the ingestion of *T. platyurus* in the concentrations 10-fold lower than the lethal levels. The reason for this recovery cannot be explained by the high volatility of the compound. PNP with the similar vapour pressure was only 2-fold more toxic in the Rapidtoxkit compared to Thamnotoxkit F™. The Rapidtoxkit assay is a good predictor of the mortality over the next 24 h. The 24 h LC_{50}'s are within the range of 25-400 % of the 1h EC_{50} values for 7 out of 9 toxicants tested (Fig. 6). Due to the high 24 h LC_{50}/LC_{20} ratio, the 24 h LC_{20} values are outside this range in 4 cases. Nowadays, it is a great challenge to create a quick and simple test method for detecting toxicity of various samples including unknown chemical substances and environmental samples. Acute toxicity tests with rotifer *B. calyciflorus* and anostracan *T. platyurus* have been used in many ecotoxicological laboratories due to their cost-effectiveness and high sensitivity to various toxicants. They seem to be useful tools of the risk assessment for pharmaceuticals and their metabolites that find their way into aquatic ecosystems because of their high sensitivity to some of these substances (Na³êcz-Jawecki and Persoone, 2006; Snell and Joaquim-Justo, 2007). *B. calyciflorus* and *T.*

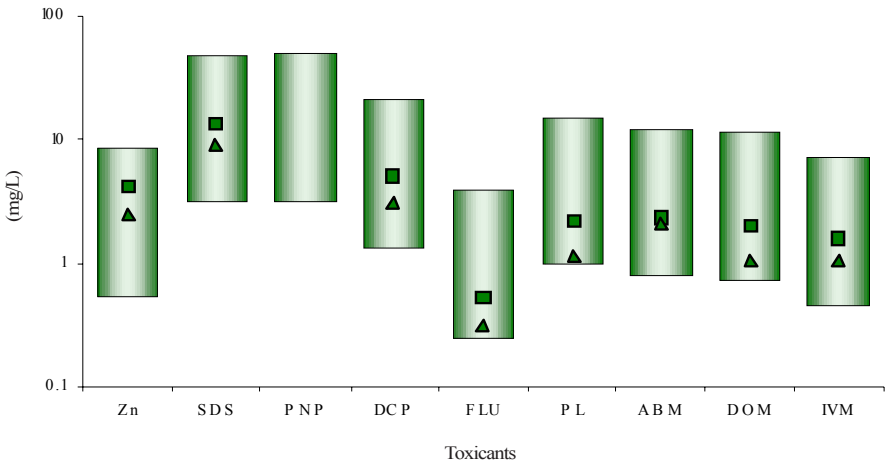

Fig. 4: Comparison of concentrations used in the ingestion test with rotifers (bars) with the 24 h LC_{50} (■) and 24 h-LC_{20} (▲) values in the Rotoxkit F™ assay. Bars comprise the concentrations of 400-25 % of the 1h-EC_{50} for each toxicant

Table 2: The toxicity of 9 chemicals towards the anostracean *Thamnocephalus platyurus*

Toxicant	Abbreviation	Ingestion test (mg/L)				Mortality test (mg/L)				I/M*	
		1 h-EC_{50}	SD	1 h-EC_{20}	SD	24 h-LC_{50}	SD	24 h-LC_{20}	SD	EC_{50}/LC_{50}	EC_{20}/LC_{20}
Zinc	Zn	5.00	1.01	2.90	0.81	0.33	0.03	0.16	0.04	15.15	18.12
SDS	SDS	21.20	8.01	12.60	5.30	15.1	4.84	7.96	2.32	1.40	1.58
p-nitrophenol	PNP	12.80	0.76	5.76	0.58	27.4	1.20	12.30	2.80	0.47	0.47
3,5-dichlorophenol	DCP	0.34	0.01	0.18	0.03	3.02	0.42	2.09	0.36	0.11	0.09
Fluoxetine	FLU	1.60	0.60	0.68	0.21	0.85	0.11	0.42	0.13	1.88	1.62
Propranolol	PL	5.50	0.55	2.83	0.73	3.24	0.09	1.68	0.18	1.70	1.68
Abamectin	ABM	0.0100	0.0005	0.0023	0.0005	0.0132	0.0015	0.0033	0.0011	0.76	0.70
Doramectin	DOM	0.0072	0.0014	0.0021	0.0005	0.0089	0.0014	0.0035	0.0012	0.81	0.60
Ivermectin	IVM	0.0052	0.0011	0.0011	0.0003	0.0057	0.0012	0.0033	0.0011	0.91	0.33

* - ratio of the toxicity value in the ingestion test to that in the mortality test

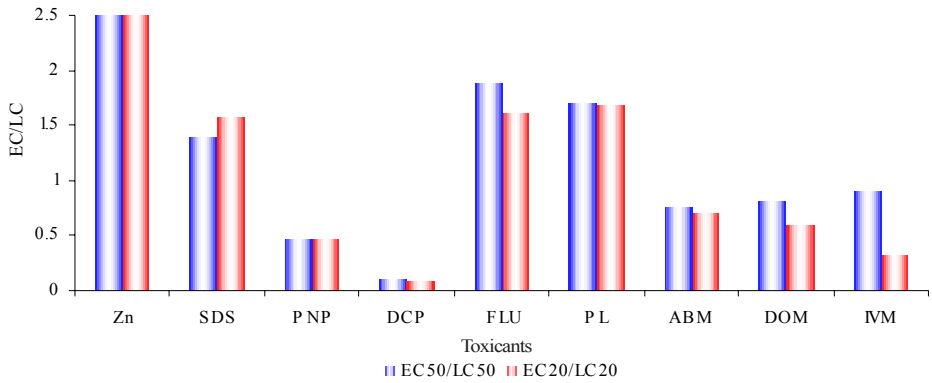

Fig. 5: The ratio of the toxicity values in the ingestion test to the mortality test for *T. platyurus*

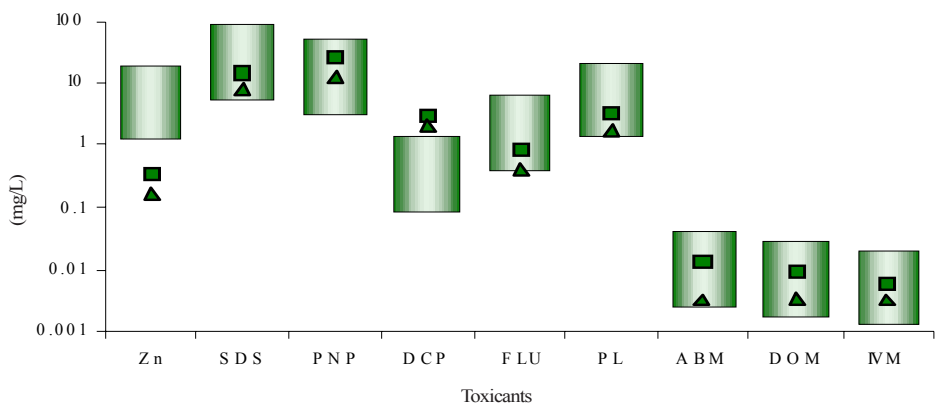

Fig. 6: Comparison of concentrations used in the Rapidtoxkit F™ (bars) with the 24h LC_{50} (■) and 24 h-LC_{20} (▲) values in the Thamnotoxkit F™ assay. Bars comprise the concentrations of 400-25 % of the 1 h EC_{50} for each toxicant

platyurus were applied by Isidori *et al*. (2005) to evaluate the ecotoxicity of naproxen and its photoproducts.

Conventional assays with animals last at least 24 h, and require preliminary, range finding tests, which prolong the time of analysis by additional 24 h. At the end of the 20th century new biotests were developed with a very short time of exposure of planktonic crustaceans. The principle of the Fluotox (Janssen and Persoone, 1993) and IQ Toxicity Test™ (Hayes *et al*., 1996) is an *in vivo* detection of enzymatic inhibition, visualised by fluorescence of the whole organism under a UV light source. For Daphnia, the 1-h enzymatic inhibition (expressed as 1 h-EC_{50}), very well correlated (r = 0.8) with the immobilization 48 h-EC_{50} for 20 compounds (Persoone, 1992).

Ingestion tests are based on a well known reaction of filter-feeding biota, i.e. stopping of ingestion of food (or artificial particles) when exposed to a toxic stress (Na³êcz-Jawecki and Persoone, 2006). The ingestion ratio has been applied as an endpoint in toxicity assays with protozoans (Juchelka and Snell, 1995), rotifers (Juchelka and Snell, 1994), crustaceans (De Coen *et.al*., 1998) and anostraceans (Rapidtoxkit, 2004). De Coen *et al*. (1998) discovered a very significant (r=0.9) correlation between the EC_{50}'s based on the ingestion rate of *D. magna* and the 24 h-immobilisation assay.

Na³êcz-Jawecki and Persoone (2006) modified the procedure of Rapidtoxkit so that the assay could be applied to test various dilutions of samples. In the case of 28 pharmaceuticals, an overall correlation coefficient of 0.96 between the two microbiotests was found, confirming the good predictive potential of the 1 h stress-based Rapidtoxkit to reveal mortality after a prolonged exposure of the crustacean test species to chemical compounds. This investigation is similar to the data collected by authors for pharmaceuticals, including both very toxic avermectins and less toxic FLU and PL. Juchelka and Snell (1994) compared the toxicity of 10 compounds using acute, chronic toxicity endpoints and ingestion rate for rotifer *B. calyciflorus*. They found out that only 4 cases the ingesition NOEC was comparable to reproduction NOEC in. As regards the other toxicants, especially metals, the ingestion test was 2.5-10 fold less sensitive. This corresponds well with he authors' findings for *T. platyurus* where ingestion was affected by 15-fold higher levels of zinc. Moreover, Juchelka and Snell (1994) suggest that the ingestion assay was more sensitive that the 24 h mortality test. However, they compared the 24 h LC_{50} values with the ingestion NOEC. The obtained data clearly shows that when comparing the same toxicity values (EC_{50} with LC_{50} and EC_{20} with LC_{20}), the results are similar. This high correlation means that the sublethal toxicity signal is a signal that the organisms will die, if further exposed to the toxicant for one day.

CONCLUSION

The toxicity values observed in the ingestion tests were very close to the mortality values over a wide range of toxicity from a low toxic surfactant (SDS) to very toxic avermectins. The 24 h-LC_{50} values are within the range of 25-400 % of the 1h-EC_{50} values for almost all toxicants tested with the exception of p-nitrophenol for *B. calyciflorus* and zinc and 3,5-dichlorophenol for *T. platyurus*. Short, 1 h ingestion assays RotoxRapid and Rapidtoxkit are good predictors of the mortality over the next 24 h and can be used as a range finding tests for representatives of pharmaceuticals and surfactants.

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