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Ana M. Sardo - Ulisses M. Azeiteiro - Lourdes Pereira - Fernando Morgado - Amadeu M.V.M.Soares

Angerstr. 12
85354 Freising - Germany
Phone: ++49 – (0) 8161-48420
Fax: ++49 - (0) 8161-484248
Email: parlar@psp-parlar.de
www.psp-parlar.de

HISTOLOGICAL EVALUATION OF THE EXPOSURE TO 3,4-DICHLOROANILINE IN THE ESTUARINE MYSID *Mesopodopsis slabberi,* UNDER EXPERIMENTAL CONDITIONS

Ana M. Sardo[1,2], Ulisses M. Azeiteiro[3], Lourdes Pereira[1], Fernando Morgado[1] and Amadeu M.V.M.Soares[1]

[1] Departamento de Biologia, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[2] Departamento de Zoologia, Faculdade de Ciências e Tecnologia da Universidade de Coimbra, 3004-517 Coimbra, Portugal
[3] Departamento de Ciências Exactas e Tecnológicas, Universidade Aberta; IMAR - Universidade de Coimbra, 3004-517 Coimbra, Portugal

SUMMARY

This work presents an experimental approach to test *Mesopodopsis slabberi* as a potential indicator of pollution. The toxic effects of 3,4-dichloroaniline (DCA) on the histology of this estuarine mysid were studied. After an acclimation period of two days, the mysids were exposed to different sublethal 3,4-DCA concentrations (0.10, 0.30, 0.50, 0.90, 1.00, 1.10, 1.20, 1.30 and 1.40 mg/L), for a period of 48 h. Each concentration had seven replicates, and one control. After the exposure period, organisms were sacrificed and submitted to a standard histological procedure with some modifications. Histological effects were analyzed in several tissues and damages were found in organisms exposed to concentrations higher then 0.30 mg/L. Muscular tissue, cuticular lens and gonads were clearly affected by 3,4-DCA, presenting accumulations of this toxic substance and lesions on the structures.

KEYWORDS: *Mesopodopsis slabberi*, 3,4-DCA, toxicology, experimental, histological effects.

INTRODUCTION

Most basic knowledge about toxic actions on ecosystems results from the study of toxic effects on the individuals of different species, thus, on the elementary components. A toxicant may act on the physiological and biochemical processes and, subsequently, may affect individual life-history parameters in a variety of ways, depending on life-parameter and species [1]. Public concern on the toxicity and persistence of chemicals released into the environment has led to increased interest in the environmental fate of man-made pollutants [2]. Small adult and larval crustaceans are important components of a number of food webs. Therefore, it is important to determine the sensitivity of these crustaceans to environmental contaminants and as well as to select the most sensitive species as test organisms to help establishing the permissible levels of contamination [3].

3,4-Dichloroaniline (3,4-DCA) is a hydrolysis product of several herbicides and has been detected in natural aquatic environments at substantial concentrations during the last decade. This metabolite often gets into the environment from agricultural activities, either subsequent to the application or to the industrial production processes of chemicals [1].

Mesopodopsis slabberi (van Beneden 1861) is an euryhaline and suprabenthic mysid that presents a wide geographic distribution [4], and is one of the most important mysids` species in coastal temperate Atlantic shallow waters [5]. There is an increasing need to develop tests, which measure sublethal responses to toxicants at environmentally realistic concentrations and may be used to predict environmental impact [6]. Even though *M. slabberi* is an important estuarine species, there is a lack of data concerning possible effects of pollution and pesticides on this organism. In the present work, the toxic effect of 3,4-DCA on the histological structure of *M. slabberi* was studied, using sublethal concentrations, with the purpose of contributing with new data about its toxicity and the potential for experimental ecotoxicology. Histology was selected as method, since it allows the evaluation of internal morphology, observing for bioaccumulation and, also damage.

MATERIALS AND METHODS

Sampling. Mysids were collected at Ria de Aveiro (NW Portugal), where depths vary from 5 to 7 m, with a salinity of 35 PSU, about 2 hours after high tide. All samples were collected during the day, when supra-benthic mysids are known to be near the bottom [7]. Capture was

made using a supra-benthic sledge with a collecting cup, both with 500 µm-mesh net, operated from a vessel, with 3-4 min drags. The content of the collecting cup was placed in 20 L-buckets, in order to keep the organisms alive until reaching the laboratory.

Acclimation. In laboratory, the organisms were separated from the samples, using only adult specimens, from the same class size (adults, about 11 mm). A total of 80 organisms was placed in 5 glass bottles (16 in each) with 500 mL of artificial seawater (prepared by dilution of marine salt SERA PREMIUM® in distilled water), with 25 PSU salinity (± 1 PSU) [4], and permanent and moderate aeration in a room with constant temperature (20 ± 1ºC), and without sediment. Light was provided by florescence lamps, controlled automatically, with a 14:10 h light:dark photoperiod. Acclimation took place for 48 h, in which mortality, temperature, pH, salinity and O_2 level were monitored, and 250 mL of water renewed (semi-static system) daily. A very narrow mesh net was carefully placed inside the bottles to prevent the removal of specimens, while 250 mL of water was removed. The fresh water was dripped in the bottles, with the aid of a glass rod. Organisms were fed daily with nearly hatched *Artemia* nauplii, *ad libitum*, because cannibalism has been observed when enough food is not provided [8]. Excessive food that accumulated in the bottom of the bottles was removed.

3,4-DCA exposure regime. Mysids were exposed, for a period of 48 h, to five 3,4-DCA (98% $Cl_2C_6H_3NH_2$) concentrations: 0.10, 0.30, 0.50, 0.70 and 0.90 mg/L (all prepared from a stock solution of 100 mg/L), and one control was used. All solutions were prepared with artificial seawater (prepared by dilution of marine salt SERA PREMIUM® in distilled water) with salinity 25 ± 1 PSU. Organisms were placed in bottles with 100 mL 3,4-DCA solution, one per bottle in a non-feeding regime. Seven replicates per concentration were used, totaling 42 organisms. Exposure was carried out at 20 ± 1 ºC with ambient laboratory lighting programmed for a 14:10 h light:dark photoperiod. During the exposure period, moderate aeration was kept. Salinity, pH, O_2 level, water temperature and mortality were monitored at 0, 24 and 48 h of exposure. After the histological analysis of exposed and unexposed organisms, another group of concentrations was tested (1.00, 1.10, 1.20, 1.30 and 1.40 mg/L), following the protocol described before.

Histological process. Mysids were submitted to standard histological procedures. The process suffered little modifications, due to size, fragility of the structures and chitinous coating of the species [9]. The procedure consisted of fixation in Bouin's solution for 24 hours, 70% ethanol for 24 hours, dehydration, paraffin embedding, sectioning (8 µm), staining using Haematoxyline-Eosin and mounting (with Eukitt™). The slides obtained after this process were observed in a light microscope, searching for damages caused by lead exposure. Photographs were taken with a NIKON microscope and a NIKON 'FX-35 DX machine, controlled by a NIKON AFX-DX.

Statistical Analysis. For statistical analyses, the overall effects of the days and concentrations on the mortality, through a 2-way analysis of variance (ANOVA) [10], using STATISTICA 6.0 Software Package, were investigated.

RESULTS

Throughout the acclimation period, important variations in registered physicochemical parameters were not observed and the mortality was low (1.7%). These factors suggest that a good water quality was maintained, a critical factor in mysids` acclimation [11]. During the exposure to 3,4-DCA, no significant variations were observed in the physicochemical parameters measured and mortality was also low (Tables 1 and 2). The obtained standard

TABLE 1 – Physicochemical parameters and mortality during 3,4-DCA exposure (0.10 to 0.90 mg/L)

Hours	Concentration (mg/L)	Salinity (PSU)	Temperature (ºC)	pH	DO_2 (%)	Mortality (# organisms)
0	Control	24.73±0.018	19.97±0.113	8.110±0.0053	83.06±0.892	0
	0.10	24.69±0.014	19.66±0.048	8.107±0.0029	85.89±0.844	0
	0.30	24.61±0.014	19.81±0.086	8.090±0.0062	83.73±0.947	0
	0.50	24.61±0.014	19.44±0.078	8.083±0.0036	90.69±0.624	0
	0.70	24.54±0.020	19.74±0.069	8.090±0.0049	84.90±0.285	0
	0.90	24.50±0.000	19.66±0.081	8.083±0.0052	84.94±0.932	0
24	Control	24.77±0.018	19.79±0.055	8.130±0.0000	82.87±0.813	0
	0.10	24.70±0.000	19.63±0.036	8.104±0.0020	83.71±0.722	0
	0.30	24.69±0.014	19.79±0.040	8.103±0.0057	85.47±0.773	3
	0.50	24.71±0.014	19.77±0.057	8.101±0.0055	86.99±0.878	0
	0.70	24.55±0.014	19.72±0.047	8.090±0.0053	84.38±0.636	1
	0.90	24.63±0.029	19.87±0.018	8.093±0.0046	85.87±0.865	3
48	Control	24.79±0.014	19.80±0.038	8.149±0.0014	83.37±0.545	1
	0.10	24.73±0.018	19.76±0.030	8.106±0.0030	85.09±0.528	1
	0.30	24.70±0.000	19.76±0.030	8.100±0.0031	84.73±0.992	3
	0.50	24.70±0.022	19.74±0.072	8.101±0.0046	86.63±0.817	1
	0.70	24.73±0.018	19.81±0.040	8.109±0.0026	84.66±0.898	1
	0.90	24.74±0.030	19.90±0.022	8.101±0.0036	85.50±0.721	3

Note: means ± standard error

TABLE 2 – Physicochemical parameters and mortality during 3,4-DCA exposure (1.00 to 1.40 mg/L).

Hours	Concentration (mg/L)	Salinity (PSU)	Temperature (ºC)	pH	DO_2 (%)	Mortality (# organisms)
0	Control	24.81±0.014	20.33±0.057	8.027±0.0018	84.97±0.547	0
	1.00	24.50±0.000	20.47±0.057	8.030±0.0022	84.64±0.618	0
	1.10	23.94±0.557	20.44±0.057	8.031±0.0014	84.73±0.596	0
	1.20	24.51±0.014	20.44±0.057	8.023±0.0018	84.63±0.538	0
	1.30	24.49±0.014	20.27±0.047	8.023±0.0018	84.59±0.642	0
	1.40	24.56±0.020	20.34±0.053	8.021±0.0026	85.13±0.638	0
24	Control	25.40±0.022	17.37±0.047	8.113±0.0018	83.84±0.745	1
	1.00	24.89±0.014	19.83±0.036	8.110±0.0000	83.11±0.527	1
	1.10	24.91±0.014	19.97±0.036	8.110±0.0000	84.30±0.536	0
	1.20	24.83±0.018	19.83±0.029	8.110±0.0000	83.11±0.423	1
	1.30	24.67±0.029	19.84±0.030	8.110±0.0000	84.17±0.442	1
	1.40	25.70±0.000	19.87±0.036	8.110±0.0000	83.47±0.477	3
48	Control	25.47±0.018	17.77±0.018	8.130±0.000	83.50±0.739	1
	1.00	25.10±0.000	19.90±0.049	8.130±0.0000	83.56±0.466	2
	1.10	25.06±0.020	20.01±0.040	8.130±0.0000	84.09±0.487	1
	1.20	25.10±0.000	19.83±0.047	8.130±0.0000	83.87±0.670	1
	1.30	24.90±0.000	19.89±0.051	8.130±0.0000	84.14±0.638	2
	1.40	25.77±0.018	19.96±0.030	8.130±0.0000	83.09±0.563	3

Note: means ± standard error

errors for the physicochemical parameters measured during exposure period, were very low. Among the different tested concentrations and between replicates, pH was the more constant parameter, presenting the lowest standard errors as expected, since no food was administered. Oxygen level showed some high standard errors, which can be explained by the constant aeration that did not let the probe stabilize correctly. By the analysis of means and standard errors, it can be stated that it was possible to maintain water with the same physicochemical parameters in the replicates at different concentrations.

All tested concentrations proved to be sublethal, and highest mortality rates were observed at 0.30, 0.90 and 1.40 mg/L. After statistical analysis (Tables 3 and 4), it can be stated that neither the factor day neither the factor concentration significantly affected the mortality ($p > 0.05$). Only the mortalities on concentrations between 0.10 and 0.90 mg/L were significantly different ($p < 0.05$).

TABLE 3 - Two-way ANOVA for mortality at 3,4-DCA concentrations between 0.10 and 0.90 mg/L.

	F	p
Day	$F_{1,72} = 0.69$	$p > 0.05$
Concentrations	$F_{5,72} = 2.84$	$p < 0.05$

TABLE 4 - Two-way ANOVA for mortality at 3,4-DCA concentrations between 1.00 and 1.40 mg/L.

	F	p
Day	$F_{1,72} = 1.20$	$p > 0.05$
Concentrations	$F_{5,72} = 1.38$	$p > 0.05$

All tested concentrations produced changes in the histological structure of *M. slabberi*, except that of 0.10 mg/L. Mysids exposed to 3,4-DCA concentrations in the range of 0.30 to 1.40 mg/L presented a number of histological effects. Hence, bioaccumulations and some tissue damages were found in the organisms exposed to that range of concentrations. More affected structures were gonads, mus-

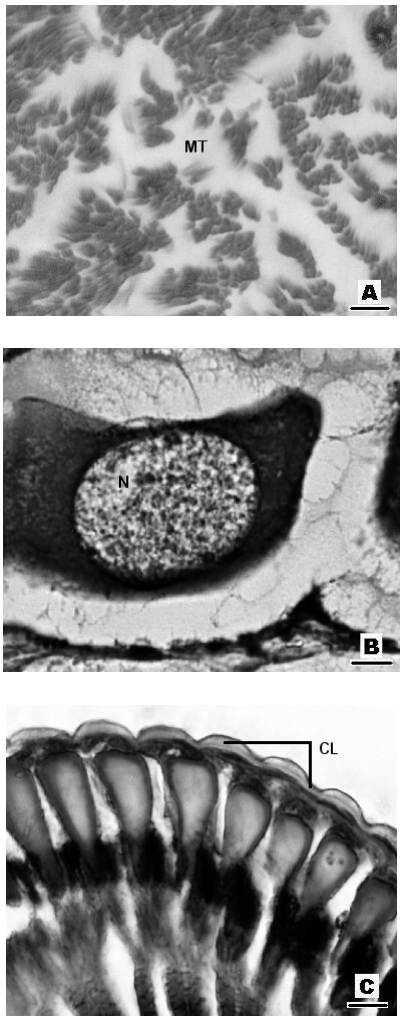

FIGURE 1 - *M. slabberi* longitudinal sections. A - Haematoxylin-Eosin staining showing a control muscular tissue (MT) (40x; scale bar = 10 µm). B - Haematoxylin-Eosin staining showing a control gonad (N = nucleus) (40x; scale bar = 10 µm). C - Haematoxylin-Eosin staining showing a part of a control eye, presenting the cuticular lens (CL) (40x; scale bar = 10 µm).

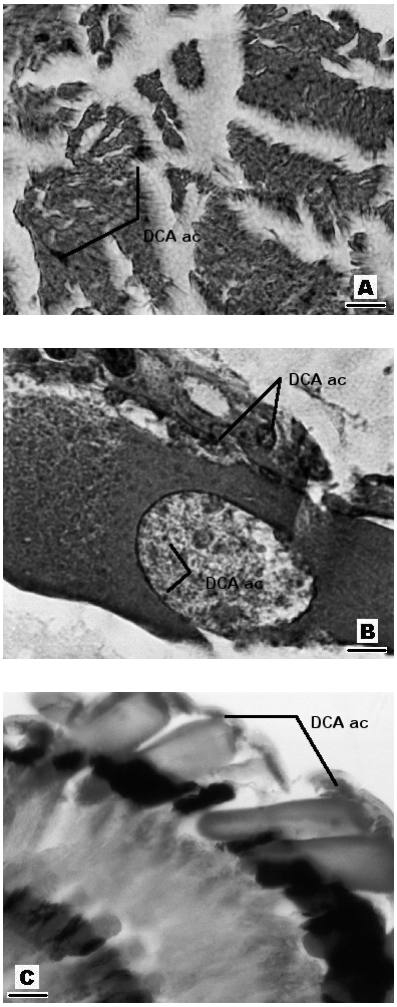

FIGURE 2 - *M. slabberi* longitudinal sections. A – Haematoxylin-Eosin staining showing the muscular tissue (MT) after exposure to 0.90 mg/L of 3,4-DCA. Note the accumulations resulting from the toxic exposure (DCA ac) (40x; scale bar = 10 µm). B - Haematoxylin-Eosin staining showing a gonad with accumulations (DAC ac) after an exposure to 1.00 mg/L of 3,4-DCA (40x; scale bar = 10 µm). C – Haematoxylin-Eosin staining showing the cuticular lens with 3,4-DCA accumulations (DCA ac), from an organism exposed to 0.50 mg/L (40x; scale bar = 10 µm).

cular tissue and the eye. Exposed gonads (Fig. 2-B) showed accumulations in nucleus and cytoplasm that seem to be the result of contact with the toxic substance. 3,4-DCA accumulations were also found in the eye, particularly in the cuticular lens (Fig. 2-C). Another histological difference observed was the presence, in great number, of accumulations in muscular tissues (Fig. 2-A). These accumulations presented no regular distribution in the tissues, or a much defined form. Muscular tissue was extremely affected by the exposure to 3,4-DCA, not only by accumulation, but also a high level of disruption of normal structure.

All the described histological damages were observed in organisms exposed to concentrations from 0.30 to 1.40 mg/L of 3,4-DCA, and histologically there were no differences between them. No histological damages were observed in the hepatopancreas, digestive cavity, intestine, heart, pericardial cavity and gills of the specimens submitted to the tested concentrations.

DISCUSSION AND CONCLUSIONS

Several recent studies tested the effects of 3,4-DCA on reproductive activity, feeding rate and mortality, both on freshwater and estuarine organisms. According to Adema and Vink [12], 3,4-DCA showed sublethal effects in the range of 0.01 to 1.0 mg/L, in a semi-static aquatic toxicity testing. Effects of 3,4-DCA on reproduction, the most sensitive of the biological responses measured, of the planktonic crustacean *Daphnia magna* occurred at concentration of $\leq$ 0.02 mg/L [13], but when the toxic effects are studied on mortality of *D. magna*, the value obtained was 0.20 mg/L [14]. For *Brachionus calyciflorus*, another freshwater organism, the LC_{50} was found to be 61.5 mg/L [14], and effects on the feeding rate were observed at 41.2 mg/l of 3,4-DCA [15].

There are much less references for estuarine species. *Gammarus pulex* LOEC was found to be 8.7 mg/L [16], and NOEC of *Pristina longiseta*, an oligochaeta, 0.8 mg/L [17]. The sublethal effects of 3,4-DCA, observed in this work for the studied species *M. slabberi*, were in the range of 0.30-1.40 mg/L.

The results indicated that histological structures of this mysid are affected at toxic concentrations of 3,4-DCA. Regarding the toxic effects, the results point to some histological damages, especially on *M. slabberi's* eye, gonads and muscular tissue, and especially the presence of 3,4-DCA accumulations, causing rather oval to spherical forms, which are visible in some essential structures. The destruction caused on the structure and organization of the muscular tissue and on gonads was also considerable.

Histological method revealed to be appropriate for this work, since it allowed the evaluation of tissues damages and other alterations, caused by 3,4-DCA. *M. slabberi* histological changes are sensitive indicators of sublethal concentrations of 3,4-DCA, accumulating in muscles, gonads and eyes.

Furthermore, the results underline the importance of toxicity studies with this mysid that has an important position in the estuarine food chain [18-20]. Since *M. slabberi* organisms undertake vertical migrations between sediment and water column [21], this species should play an important role in the process of energy and chemical compounds` transfer throughout that food chain [20]. This study may provide a baseline for future works to evaluate histological damages caused by toxic materials on mysids. The authors believe that standardized mysids` exposure toxicity tests can have an important future in the first level of hazard assessment schemes.

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CORRESPONDING AUTHOR

Ana Margarida Sardo
Department of Biology
University of Aveiro
Campus Universitário de Santiago
3810-193 Aveiro - PORTUGAL

Phone: +351 234370786
Fax: +351 234426408
e-mail: amsardo@bio.ua.pt