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Acute Toxicity Tests Using *Phyllospora comosa* (Labillardiere) C. Agardh (Phaeophyta: Fucales) and *Allorchestes compressa* Dana (Crustacea: Amphipoda)

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Despite recent developments in chronic sublethal testing, traditional acute lethal tests are still widely used as a standard ecotoxicological procedure by environmental agencies to assess the potential impact of chemicals on ecological systems. Calow (1992) has argued that ecotoxicological tests should be ecologically relevant, reliable and repeatable while admitting that these three criteria may at times be mutually exclusive. To make toxicological tests more ecologically relevant, it is important that local species (preferably from a number of trophic levels) be used. The test organisms should be commonly available and capable of providing consistent results so as to address the other concerns, i.e., reliability and repeatability.

This paper reports upon results of a series of ecotoxicological tests on two intertidal/subtidal organisms, namely *Phyllospora comosa* (Phaeophyta: Fucales) and *Allorchestes compressa* (Crustacea: Amphipoda), which are abundant on the shores of southeast Australia. The toxicants tested were tributyltin, 2,4-dichlorophenol, 2,4-dichlorophenoxyacetic acid, and formaldehyde. Previous investigations on *A. compressa* had been centered on heavy metals (e.g., Ahsanullah et al. 1988), while there has been no toxicological tests developed in Australia using marine macrophytes. Here, we compare the toxicity data of specific chemicals on the two different target organisms, and investigate the influence of different life stages (age and size) on the ecotoxicological responses. We also explore the implications of these on the search for a more relevant, reliable and repeatable ecotoxicological protocol for Australian waters.

MATERIALS AND METHODS

Healthy, mature specimens of *Phyllospora comosa* were collected at low tide from ocean beaches at Sorrento, Victoria. Plants were sexed following Burridge (1990); healthy male and female receptacles were placed separately into 15-mL sea water filled polythene vials and maintained on ice while being transported to the laboratory.

Gamete release was initiated according to the procedure of Burridge et al.

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(1993) by placing vials on ice under a light source of about 200 $\mu Em^{-2}s^{-1}$. Gamete release usually occurred within 4 to 6 hr. Female release was observed when eggs and stalks appeared on the receptacle surface; male release was observed when the water in the vial containing the receptacles turned a milky white color as a result of spermatozoid discharge (Burridge and Hallam 1993). Verification of actively swimming sperm was made using a compound light microscope prior to attempting fertilisation. Good sperm releases should result in a sperm count in the vicinity of 10^6 cells/mL. Fertilisation was achieved by mixing the entire contents of a female and male culture vial into a 400-mL glass culture chamber, containing 200-mL of millipore filtered sea water. The culture chamber was covered with parafilm, agitated and maintained at 15 °C under cool fluorescent light of 120 $\mu Em^{-2}s^{-1}$ in a light:dark regime of 12:12 hr. Twelve to fourteen hr following gamete mixing, receptacles were removed from the culture chambers and unattached zygotes could be observed scattered over the substratum surface. Non-fertilised eggs (on the surface of receptacles) maintained at 15 °C and isolated from sperm became necrotic after about 72 hr.

Stock toxicant solutions of 1000 mg/L in sea water were prepared for tributyltin (TBT: $[CH_3(CH_2)_3]_3SnCl$, 96%, Aldrich T5,020-2), 2,4-dichlorophenol (2,4DCP: 99%, Aldrich 10,595-3), 2,4-dichlorophenoxyacetic acid (2,4D: 98%, Aldrich D7,072-4) and formaldehyde (CH_2O: 37%, Aldrich 25,254-9) prior to testing and serially diluted over test concentration ranges. All stocks were prepared using fresh millipore filtered sea water. Treatment solutions were placed into 10-mL glass beakers which had been washed in phosphate free detergent, rinsed in tap water, rinsed in distilled water, washed in absolute ethanol, rinsed again in distilled water and air dried. Whatman No.1 filter paper was added to each beaker to provide a substratum for zygote attachment. Subsequent tests involved both 1-d-old zygotes and 7-d-old differentiated embryos.

Twenty five zygotes or embryos (Figures 1 and 4) were pipetted into 10-mL beakers containing treatment solutions (temperature: 15 °C; pH: 7 $\pm$ 1; DO: 7 $\pm$ 1 mg/L). Four replicates of 25 zygotes or embryos were established for each treatment, sample size having been determined from power analysis (Zar 1984) (power = 0.86). Each beaker was covered with parafilm and then placed back into incubation. Percentage mortality of 1-d-old zygotes and 7-d-old embryos was recorded at 24, 48, 72 and 96 hr using a dissecting microscope. Zygotes surviving to 96 hr exhibited cell wall development and presence of extra cellular adhesive (Figure 3). Mortality was assumed only for zygotes or embryos that were obviously necrotic (Figures 2 and 5). Controls exhibited normal germination (Burridge and Hallam 1993).

Stocks of laboratory-cultured *Allorchestes compressa* were obtained from Swan Bay of Queenscliff region in Victoria. The amphipods were maintained on the seagrass (*Heterozostera* sp.) in sea water (temperature: 20 $\pm$ 1°C; salinity: 34 $\pm$ 2‰; pH: 8 $\pm$ 1) in aerated 40-L glass tanks. The animals

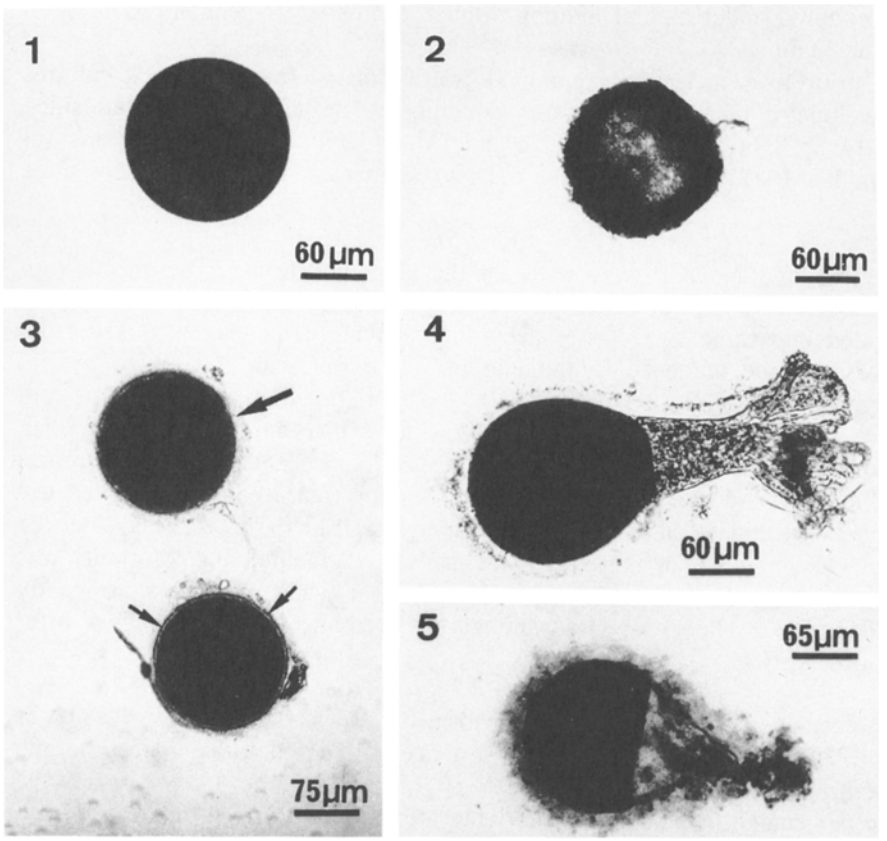

Figure 1. A 12-hr old zygote. Figure 2. A necrotic zygote cultured in 10^{-6} mg/L TBT at 72 hr. Figure 3. Surviving zygotes cultured in 100 mg/L 2,4D showing a developed cell wall (small arrows) and extracellular adhesive (large arrow). Figure 4. A 7-d-old embryo (control). Figure 5. A necrotic 7-d-old embryo in 10 mg/L TBT.

Table 1. NOEC/LOEC for the 96-hr mortality tests conducted on *Phyllospora comosa* and *Allorchestes compressa*. All concentrations are expressed in mg/L.

SPECIES	TBT	2,4DCP	2,4D	CH_2O
P. comosa (1-d-old)	$<10^{-6}/10^{-6}$	$<10^{-6}/10^{-6}$	10/100	<0.1/0.1
P. comosa (7-d-old)	$<10^{-6}/10^{-6}$	$10^{-4}/10^{-3}$	100/1000	1/10
A. compressa (small)	0.025/0.05	0.075/0.10	/	/
A. compressa (medium)	0.05/0.075	0.05/0.075	/	/
A. compressa (large)	0.0125/0.025	0.075/0.10	/	/

were cultured under natural lighting through a number of generations prior to experimentation.
3 wk prior to each testing, amphipods were isolated from the stock cultures and acclimated under the laboratory conditions. The animals were maintained on a 14 hr light: 10 hr dark cycle and fed *ad libitum* with the seagrass (cf Ahsanullah 1982). Food was changed and sea water replenished every three days.

Only TBT and 2,4DCP were used for the amphipod tests. Experiments with each toxicant were preceded by a range-finding test. Amphipods were separated into three size classes according to their body lengths (taken from the base of the antennae to the end of the telson with the animal gently straightened): 7 $\pm$ 1 mm (small), 10 $\pm$ 1 mm (medium) and 12 $\pm$ 1 mm (large). Ten amphipods of each size class were placed in a glass petri dish containing 20-mL of a test solution of a specific concentration. Test animals were not fed and there were three replicates for each treatment. All the test chambers were then placed under a 14 hr light: 10 hr dark cycle and kept for 96 hr. Observations for mortality were made at 12 hr intervals. Mortality was assumed when there was no sign of movement when animals were gently prodded with a glass rod. Dead animals were enumerated and removed after each observation.

All percentage data were arcsine transformed prior to the analyses of variance (Zar 1984). Two-factor analyses of variance were used to evaluate time and concentration effects on the mortality of *P. comosa*. For *A. compressa*, the mean percentage of amphipods surviving after 96 hr was determined for each treatment. Two-factor analyses of variance were used to compare the percentage mortality data among animals in the three size classes, exposed to different toxicant concentrations. Pair-wise comparisons of the 96-hr mortality data between the control and individual treatment groups were performed using Dunnett's tests (Zar 1984) to determine the Lowest Observable Effect Concentration (LOEC) and the No Observable Effect Concentration (NOEC) for each toxicant and target organism.

RESULTS AND DISCUSSION

For *Phyllospora comosa*, exposure of 1-d-old zygotes to 2,4D (Figure 6a) resulted in a very rapid onset of mortality at 1000 mg/L, all zygotes having died within 24 hr of exposure. Dilutions of 0.01, 0.1, 1.0 and 10.0 mg/L produced mortality between 2.0 and 16.0 %, while 100 mg/L dilutions resulted in 50.0% mortality. Controls exhibited a mortality of approximately 2% at 96 hr. Tests using 2,4DCP conducted over 96 hr resulted in 100% mortality of the 1-d-old zygotes at 72 hr for concentrations above 100 μg/L (Figure 6b). At 24 hr dilutions between 100 μg/L and 0.1 mg/L resulted in low (1-3%) mortality, while 1.0 mg/L produced 11.0% mortality. All 1-d-old zygotes exposed to TBT concentrations of 0.1 and 1.0 mg/L died within 24 hr, while controls at 96 hr exhibited mortality of about 10% (Figure 6c). The

zygotes experienced 100% mortality within 24 hr when exposed to 100 mg/L of formaldehyde and at 10 mg/L after 96 hr (Figure 6d). Dilutions of 0.1 and 1 mg/L produced 40-50% mortality at 96 hr. Two-factor analyses of variance for the tests on *P. comosa* confirmed that both increased concentration and duration of exposure had a significant effect on mortality. All results were significant to $P < 0.001$ for both concentration and time. In all cases there were also significant interactions between time and concentration.

For *Allorchestes compressa*, data on the effect of TBT indicated that there were significant differences in the percentage mortality after 96 hr when the animals were exposed to different concentrations ($F_{8,54} = 328.52$, $P < 0.001$), and when animals of different size classes were used ($F_{2,54} = 521.67$, $P < 0.001$) (Figure 7). Similarly for 2,4DCP, there were significant differences in the percentage mortality in animals of different sizes ($F_{2,66} = 132.48$, $P < 0.001$), and exposed to different concentrations ($F_{10,66} = 174.10$, $P < 0.001$) (Figure 7). There were significant interactions between size and concentration for both toxicants.

The LOECs and NOECs for both *Phyllospora comosa* and *Allorchestes compressa* are summarized in Table 1. Based on the *P. comosa* data, TBT appeared to be most toxic, followed by 2,4DCP, formaldehyde and then 2,4D. Further, the 1-d-old zygotes were more sensitive than the 7-d-old embryo for all toxicants. For *A. compressa*, the NOEC and LOEC values for TBT are much higher than those previously reported (Bryan et al. 1989; Alzieu 1991; Gibbs et al. 1991; Dowson et al. 1993; Lapota et al. 1993). The apparent ability of small amphipods to tolerate higher concentrations of TBT and 2,4DCP than large individuals is counterintuitive as larger individuals are often expected to be more resistant to environmental stressors. It is possible that the mode of action of these toxicants may be different for plants and animals. This aspect clearly deserves further investigation.

Our results indicate that *P. comosa* zygotes are extremely sensitive to low TBT and 2,4DCP concentrations. The results of mortality testing using TBT are consistent with studies on other species including *Crassostrea gigas* (Alzieu 1991; Gibbs et al. 1991), *Nucella lapillus* (Dowson et al. 1993), *Mytilus edulis* (Lapota et al. 1993) and *Ilyanassa obsoleta* (Bryan et al. 1989) where sensitivity has also been recorded at concentrations in the 10^{-6}-10^{-5} mg/L range. Formaldehyde has been shown to have 96-hr LC_{50} values generally in the 10.0 to 1000.0 mg/L range and toxicity thresholds in the low mg/L range (Verschueren 1983). Results on *P. comosa* are consistent with these values although 100% mortality for concentrations of 10 mg/L and higher indicate a greater sensitivity for this organism than work previously published. For 2,4D, toxicity thresholds of a similar order of magnitude to *P. comosa* (> 10 mg/L) have been identified for bacteria (*Pseudomonas putida* at 6.0 mg/L), microalgae (*Microcystis aeruginosa* at 2.0 mg/L and *Scenedesmus quadricauda* at 3.6 mg/L) and protozoans (*Entosiphon sulcatum* at 0.5 mg/L and *Uronema parduzci* at 1.6 mg/L) using the cell multiplication

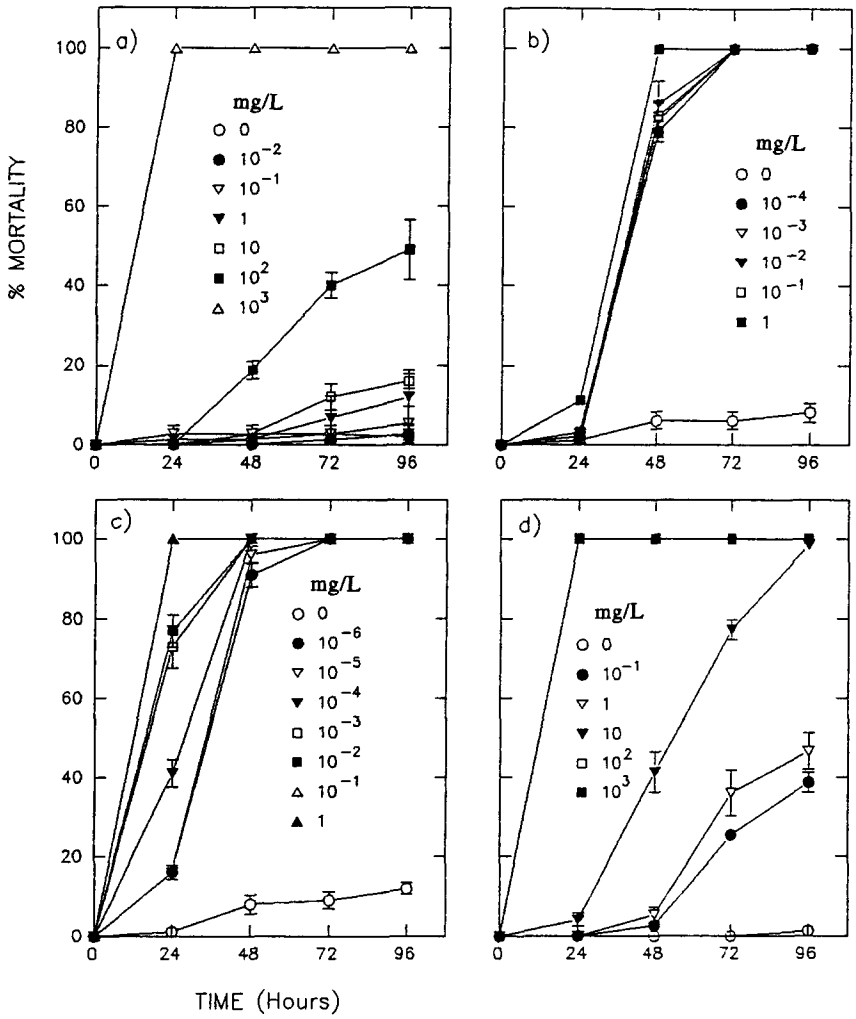

Figure 6. 96-hr cumulative mortality on 1-d-old *P. comosa* zygotes in a) 2,4-dichlorophenoxyacetic acid, b) 2,4-dichlorophenol, c) tributyltin, and d) formaldehyde.

inhibition test (Verschueren 1983).

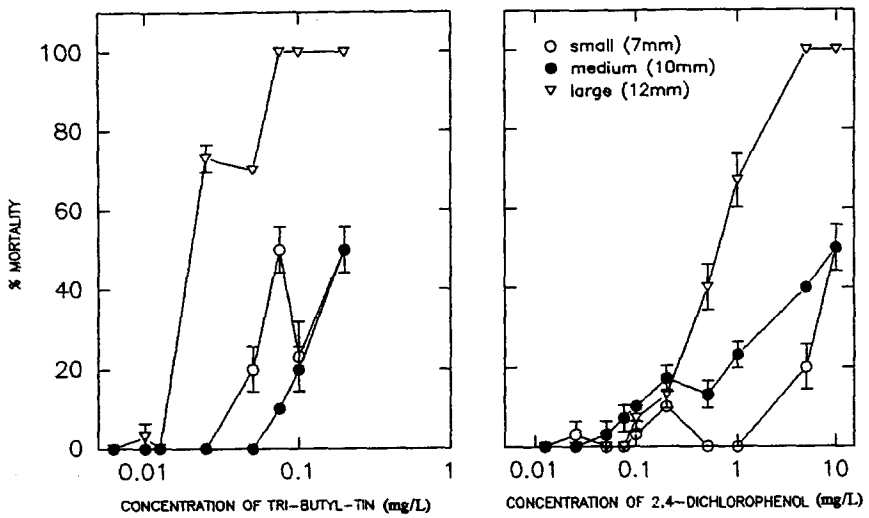

Figure 7. 96-hr mortality test on *A. compressa* in tributyltin and 2,4-dichlorophenol (logarithmic scale).

Both *P. comosa* and *A. compressa* are potentially useful as target organisms in acute toxicity testing. However, results of this study show that the response of the two species to applied toxicants may differ by several orders of magnitude and, that size and/or age of the organism(s) tested will have a significant influence on the test results. Prudent environmental protection/regulation should utilise the most sensitive life stage of the most susceptible organism from each trophic level. Findings here suggest that there is a need to obtain toxicity data from a wider range of organisms, preferably at least one from each trophic level in the system investigated. It is also possible that developments in sublethal chronic tests will help to alleviate some of these problems, and this will be the subject of a later paper.

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