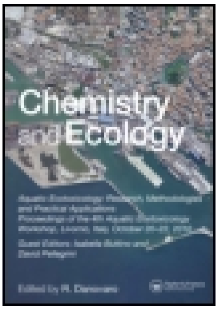

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Toxicity assessment of copper, pentachlorophenol and phenanthrene by lethal and sublethal endpoints on nauplii of *Tigriopus fulvus*

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This article evaluates the sensitivity of two endpoints in ecotoxicological tests using nauplii of *Tigriopus fulvus*: the classical index of mortality and the variation in the number of moults. The experiment was conducted by exposing the nauplii to three different types of chemical compounds: copper (heavy metal), pentachlorophenol (pesticide) and phenanthrene (polycyclic aromatic hydrocarbon). For each substance, 50% effect concentration, no observed effect concentration and lowest observed effect concentration were evaluated for both endpoints. The system showed good sensitivity for pentachlorophenol and copper, although no relevant effects were found for phenanthrene. The endpoint 'number of moults' during larval development showed higher sensitivity than the mortality endpoint.

Keywords: *Tigriopus fulvus*; moulting; copper; pentachlorophenol; phenanthrene

1. Introduction

In ecotoxicology, the endpoints can be classified into three categories: vital functions (mortality, reproduction, hatching, exuviation and growth inhibition), behavioural (swimming speed, phototactic responses, feeding rate) and biochemical (inhibition of bioluminescence, induction of activities of different enzymes, including cytochrome P_{450}, ethoxyresorufin-O-deethylase activity, acetylcholinesterase, metallothioneins, DNA changes and ratio DNA/RNA, immune dysfunctions) [1]. Of these categories, the vital functions are more commonly used as endpoints in standard protocols [2–4].

Physiological responses and cellular development indicators, such as the number of moults, are rarely considered in environmental monitoring [5].

Tigriopus fulvus (Fischer, 1860) is a meiobenthic, euryaline (2–125 psu) and eurythermal (0–35 °C) copepod species, widely distributed in the Mediterranean Sea and easy to identify [6].

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Marine copepods of the genus *Tigriopus*, which have a wide geographical distribution, short life-cycle and distinctive developmental stages, have been recommended as a promising candidate for ecotoxicological studies [7] and were shortlisted in an OECD document [8].

Copper (Cu) is an essential micronutrient vital to processes such as cellular respiration, free radical defence and cellular iron metabolism, but at elevated levels Cu is toxic to organisms, especially aquatic invertebrates [9].

Undoubtedly, contamination of Cu in saltwater environments is generally higher in harbours and estuaries [10]. Cu-based antifouling coatings on ship hulls have been a major source of Cu contamination in coastal marine environments [11].

Pentachlorophenol (PCP), an uncoupler of mitochondrial oxidative phosphorylation, acts by destroying the electrochemical potential across the inner membrane of mitochondria [12,13].

Since the 1980s, concern about the toxicity of PCP and its potential adverse effects on human beings and the environment has led to regulatory action to limit its use. In the EU, use of PCP, its salts and esters, is currently limited to two industrial applications: wood preservation (91/173 EEC, which includes sapstain control) and the impregnation of heavy duty textiles. A reduction in the use of organochlorurate pesticides induced an increase in the consumption of organophosphate and carbamates; these compounds are more dangerous to the environment than organochlorurates, but they are faster to degrade [14].

Phenanthrene (PH) is a polycyclic aromatic hydrocarbon (PAH), one of a large group of widespread organic compounds of high environmental concern. Even though PAHs occur naturally, the highest concentrations are mainly due to human activities that lead to a continuous increase in PAH levels in estuarine and marine waters [15,16]. Direct discharges into the marine environment from point sources such as wastewater treatment plants range from $<1\,\mu g \cdot L^{-1}$ to $>625\,\mu g \cdot L^{-1}$, and PAH concentrations in industrial effluents range from undetectable to $4.4\,mg \cdot L^{-1}$ [17].

The aim of this study was to evaluate the effects on mortality and moulting in nauplii of *T. fulvus* exposed to Cu, PCP and PH. The study was part of research conducted to standardise protocols for ecotoxicological assessment of the marine environment.

2. Materials and methods

2.1. *Experimental animals*

Adults of the harpacticoid copepod *Tigriopus fulvus* from natural populations collected from the coast of the Tyrrhenian Sea (Livorno, Italy) were used in the trials and were provided by the ISPRA Institute of Rome. They were transported to the laboratory in artificial seawater (Instant Ocean® 38‰ salinity) at room temperature. Species identification was confirmed by morphological analysis, as reported previously [18]. The copepods were kept in a thermostatic chamber at 20 ± 1 °C with a 16:8 h L/D photoperiod and fed with the microalgae *Tetraselmis suecica* and *Isochrysis galbana* at 1.5×10^8 and 3.0×10^8 cells·L^{-1}, respectively.

2.2. *Synchronous nauplii production*

Ovigerous females of *T. fulvus* were selected and isolated from the population to synchronise nauplii production. The selected females were fed with live algae *T. suecica* and *I. galbana* at 5×10^8 and 6×10^8 cell·L^{-1}, respectively.

After 72 h, ovigerous females were filtered from their newborn nauplii and copepodites and after 24 h, nauplii (from NI to NII stage) were separated from adults by filtration (90 μm mesh size) and used to conduct the tests. Newborn nauplii at stages NI and NII are simple to collect from ovigerous female and are homogeneous in stage development.

2.3. *Experimental solutions*

Artificial seawater (ASW), made following ASTM specifications [19], was used in the tests. A Cu stock solution of $1\,\mathrm{g{\cdot}L^{-1}}$ final concentration using $CuCl_2$ salt was made in ASW and nominal Cu concentrations used for the toxicity tests were 0, 15, 30, 60, 120, 250, 500 and $1000\,\mu\mathrm{g{\cdot}L^{-1}}$.

Pentachlorophenol, an environmentally persistent fungicide, has a $\log K_{\mathrm{ow}}$ (Octanol-water partition coefficient) of 5.05 [20] and a water solubility of $10\text{–}20\,\mathrm{mg{\cdot}L^{-1}}$ [21].

Starting with a stock solution of $10\,\mathrm{mg{\cdot}L^{-1}}$ PCP in ASW, the following test solutions were made: 0.5, 1, 2, 4, 8, 15, 30, 60, 125, 250, 500 and $1000\,\mu\mathrm{g{\cdot}L^{-1}}$.

The reported solubility limit of phenanthrene is $7.24\,\mu\mathrm{mol{\cdot}L^{-1}}$ (in freshwater at 25 °C), so, to enhance water solubility, a PH stock solution was made by dissolving it in dimethylsulfoxide (DMSO) [22].

Table 1 shows the final percentage (v/v) of DMSO in the nominal PH concentrations used in tests. Experimental concentrations were chosen on the basis of range-finding trials and on data from the literature. For each chemical solution, no aggregation was observed at the highest concentrations tested.

Copper ($CuCl_2$, purity $>99.995\%$), pentachlorophenol (C_6Cl_5OH, purity $>99.50\%$), phenanthrene ($C_{14}H_{10}$, purity $>99.5\%$) and all salts for ASW were purchased from Sigma Aldrich (Milan, Italy).

2.4. *Toxicity test*

Tests were carried out according to the protocol developed by Faraponova et al. [23], collecting synchronised nauplii at stage NI–NII (24 h old).

The tests were conducted in 12-well plates, each well containing 10 mL of test solution and 10 nauplii. ASW without chemical substances was used as control. The test was performed in three replicates per session. Four sessions of test were performed for each chemical substance.

After 96 h of incubation at 20 °C (static state 96 h test), the survival rate in each well was evaluated (endpoint mortality); this is the longest exposure time for the acute toxicity test that does not require feeding or solution substitution [24].

To evaluate the number of moults, a drop of crystal violet solution was placed in each well, making identification of the exoskeletons under the dissecting microscope easy. Because we always found a reduction in the number of moults relative to the increased concentrations of each toxic substance, the results were expressed as percentage moult reduction compared with the control, dividing the number of exoskeletons found in the samples treated by the number of those found in controls.

Table 1. Phenanthrene concentrations and respective dimethylsulfoxide (DMSO) percentages in final volumes.

	Phenanthrene concentration ($\mu\mathrm{g{\cdot}L^{-1}}$)					
	0.5	5	50	100	200	500
DMSO (% v/v)	0.015	0.03	0.06	0.12	0.25	0.5

2.5. *Statistical evaluation*

For each substance, the 50% lethal concentration at the endpoint mortality (LC_{50}) and the 50% effect concentration at the endpoint of moult reduction (EC_{50m}) were calculated at the 95% confidence limits by means of Probit [25]. Analysis of variance (ANOVA) was applied, using raw data, to test for significant differences among treatments (significance level was always set at $p < 0.05$); the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) were then determined by Dunnett's procedure [26]. Each experiment was conducted five times each with three replicates ($n = 15$).

3. Results

LC_{50}, NOEC and LOEC values obtained for the endpoint mortality of each tested substance are given in Table 2. Among the substances tested, Cu emerged as the most toxic ($LC_{50} = 50 \pm 7\,\mu g{\cdot}L^{-1}$).

The EC_{50m}, NOEC and LOEC values for the endpoint 'moult number reduction' of each tested substance are presented in Table 3.

After exposure to both Cu and PCP, the EC_{50m} values obtained for the endpoint in moult reduction (29 ± 6 and $63 \pm 25\,\mu g{\cdot}L^{-1}$, respectively) were significantly ($p < 0.05$) lower than the LC_{50} values recorded for the mortality endpoint (50 ± 7 and $134 \pm 36\,\mu g{\cdot}L^{-1}$, respectively). For PH, LC_{50}, EC_{50m}, NOEC and LOEC values could not be calculated because of the very low sensitivity to PH shown by *T. fulvus*.

3.1. *Copper*

The results obtained by exposing the nauplii to Cu are shown in Figures 1 and 2.

The effects of Cu on survival (Figure 1) were not significant at concentrations $<30\,\mu g{\cdot}L^{-1}$ ($p < 0.05$), but increased at higher concentrations, reaching 100% mortality at $250\,\mu g{\cdot}L^{-1}$, yielding a LC_{50} of $50 \pm 7\,\mu g{\cdot}L^{-1}$ (Table 2).

Table 2. Copper, pentachlorophenol and phenanthrene toxic effects related to the endpoint mortality of *Tigriopus fulvus*: NOEC, LOEC and LC_{50}.

Toxic substance	LC_{50} ($\mu g{\cdot}L^{-1} \pm$ CI)	NOEC ($\mu g{\cdot}L^{-1}$)	LOEC ($\mu g{\cdot}L^{-1}$)
$CuCl_2$ ($\mu g{\cdot}L^{-1}$)	50 ± 7	15	30
Pentachlorophenol ($\mu g{\cdot}L^{-1}$)	134 ± 36	30	60
Phenathrene ($\mu g{\cdot}L^{-1}$)	NC	NC	NC

Note: NOEC, no observed effect concentration; LOEC, lowest observed effect concentration; LC_{50}, 50% lethal concentration; NC, not calculated; CI, 95% confidence interval.

Table 3. Copper, pentachlorophenol and phenanthrene toxic effects related to the endpoint moult reduction in *Tigriopus fulvus*: NOEC, LOEC and EC_{50}.

Toxic substace	EC_{50} ($\mu g{\cdot}L^{-1} \pm$ CI)	NOEC ($\mu g{\cdot}L^{-1}$)	LOEC ($\mu g{\cdot}L^{-1}$)
$CuCl_2$ ($\mu g{\cdot}L^{-1}$)	29 ± 6	ND	15
Pentachlorophenol ($\mu g{\cdot}L^{-1}$)	63 ± 25	4	8
Phenathrene ($\mu g{\cdot}L^{-1}$)	NC	NC	NC

Note: NOEC, no observed effect concentration; LOEC, lowest observed effect concentration; EC_{50}, 50% effect concentration; NC, not calculated; ND, not detectable; CI, 95% confidence interval.

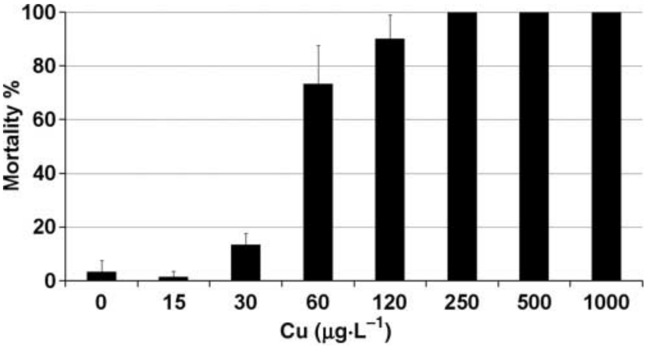

Figure 1. Mortality (%) of nauplii exposed for 96 h to increasing copper concentrations (mean ± SD; $n = 15$).

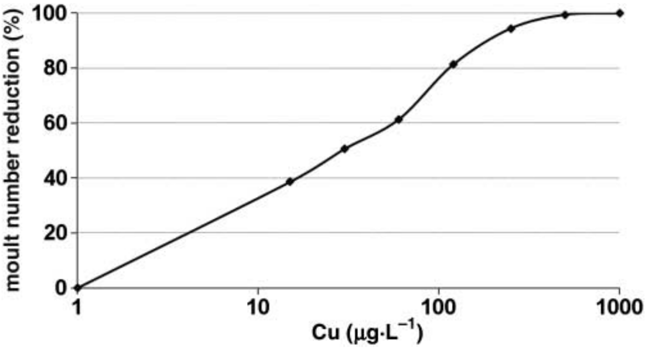

Figure 2. Reduction in number of moults (%) after 96 h exposure to copper ($n = 15$).

In Figure 2, a significant effect of Cu on the number of moults is apparent at 15 $\mu g \cdot L^{-1}$ Cu and the EC_{50m} obtained (29 ± 6 $\mu g \cdot L^{-1}$) was significantly lower than the LC_{50}.

3.2. *Pentachlorophenol*

The results obtained exposing the nauplii to PCP are shown in Figures 3 and 4. PCP has significant effects on mortality (Figure 3), starting at concentrations of 60 $\mu g \cdot L^{-1}$ (LOEC; $p < 0.05$), and reaching 100% mortality at 500 $\mu g \cdot L^{-1}$ and an LC_{50} of 134 ± 36 $\mu g \cdot L^{-1}$ (Table 2).

Significant effects on the number of moults (Figure 4) become apparent at a concentration of 8 $\mu g \cdot L^{-1}$ and the EC_{50m} obtained was 63 ± 25 $\mu g \cdot L^{-1}$, significantly lower than the LC_{50} value ($p < 0.05$).

3.3. *Phenanthrene*

DMSO had no significant effect compared with the controls in terms of mortality rate up to a concentration of 0.25%; this was the percentage of DMSO in the phenathrene at a concentration of 200 $\mu g \cdot L^{-1}$.

In terms of the number of moults, DMSO had no significant effect compared with controls up to a concentration of 0.03%, at which point it was not possible to establish the effect of PH on moult reduction.

Mortality of nauplii exposed to PH had no significant effect compared with controls up to a concentration of 200 $\mu g \cdot L^{-1}$ (data not shown).

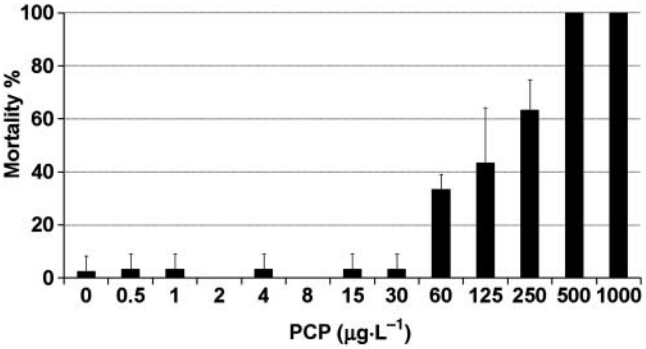

Figure 3. Mortality (%) of nauplii exposed for 96 h to increasing pentachlorophenol concentrations (mean ± SD; $n = 15$).

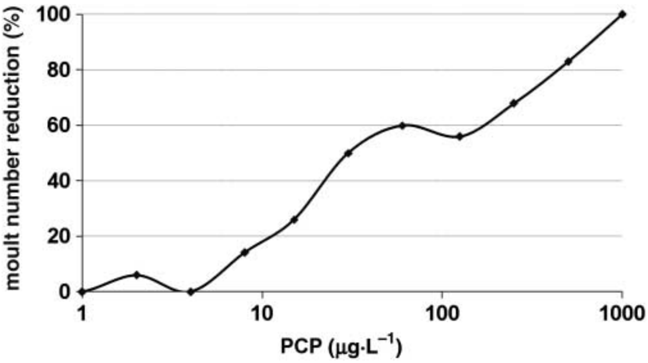

Figure 4. Reduction in number of moults (%) after 96 h exposure to pentachlorophenol ($n = 15$).

4. Discussion

The use of marine copepods of the genus *Tigriopus* as test organisms in ecotoxicological research is not new. Barnett and Kontogiannis [27] and O'Brian et al. [28] evaluated the effects of crude oil and some heavy metals on age-specific survival of the North American copepod, *T. californicus*.

Survival of early life stages has been described as the most sensitive life-history trait of copepods in many studies. For example, in experiments with meiobenthic and splashpool copepods exposed to metals, pesticides and polychlorinated biphenyls, it was reported that juveniles (nauplii) were two to four times more sensitive than adults [28–32].

In this study, Cu and PCP were very toxic for the early naupliar stages (NI–NII) of *T. fulvus*, in terms of their effect on both survival and the reduction in the number of moults. PH exposure, by contrast, showed no effect in terms of mortality or reduction in moult number.

The rank of toxicity, taking into account LOEC values, is Cu $>$ PCP for the endpoint mortality and PCP $>$ Cu for the endpoint moult reduction.

The LC_{50} value for Cu in this study was similar to values found in other test organisms (Table 4). Values reported in the literature are for different exposure periods which makes comparisons difficult. In general, however, copper LC_{50} should decrease with exposure time. Thus LC_{50} after 24 h would be expected to be higher than LC_{50} after 48 or 96 h. What this suggests is a higher tolerance for one species when the 96-h LC_{50} value is equal to or greater than the 48 or 24 h LC_{50} value for a second species [28].

Table 4. LC_{50} ($\mu g \cdot L^{-1}$) values to copper salt of some marine copepods and cladocerans.

Species	LC_{50} ($\mu g \cdot L^{-1}$)	Reference
Acartia tonsa	21 (at 72 h)	Sonsowski et al. [33]
Tigriopus japonicus	813 (at 96 h)	Kwok et al. [9]
Eurytemora affinis	58 (at 96 h)	Hall et al. [34]
Chydorus sphaericus	191 (at 96 h)	Dekker et al. [35]
Daphnia magna	60 (at 48 h)	Biesinger and Christensen [36]
Acartia clausi	60 (at 48 h)	Verriopoulos [37]
Tigriopus fulvus	10–50 (at 96 h)	Faraponova et al. [23]

Following Cu exposure, no significant differences between the endpoints of 'moult reduction' and mortality were found, despite literature reports of other crustacean species showing an increase in moult frequency after Cu exposure [38]. This is probably due to the difference in the respiratory protein present in Branchiopoda, Ostracoda, Copepoda, rhizocephalan Cirripedia (haemoglobin), whereas haemocyanin has been described in Malacostraca [39]. Haemocyanin is a copper-containing and multi-subunit protein; multiple members of the haemocyanin gene family play vital roles during moulting and reproduction in crustaceans and insects [40].

Exposure to PCP showed a very high sensitivity for EC_{50m} with respect to the LC_{50} value. For PCP, the endpoint in 'moult reduction' seems to be very sensitive to this class of chemical compounds. Scow [41] provided evidence of PCP sensitivity in these organisms: *Crassostrea gigas* (shell deposition) showed an EC_{50} of 48 $\mu g \cdot L^{-1}$ and *Palaemon elegans* an LC_{50} of 84 $\mu g \cdot L^{-1}$. In freshwater organisms, Repetto et al. [42] reported an EC_{50} for *Daphnia magna* of 400 $\mu g \cdot L^{-1}$, while Johnson and Finley [43] found an LC_{50} of 50 $\mu g \cdot L^{-1}$ for *Oncorhynchus mykiss*. Parks and LeBlanc [44] reported that, in all life stages of *D. magna*, 0.062–0.5 $mg \cdot L^{-1}$ PCP had significant effects on mortality, fecundity and reduction in the elimination of testosterone metabolites, giving clear evidence of endocrine disruption caused by PCP. Conkling and Rao [45] reviewed results for several species of intermoult adult crustaceans and found median LC_{50} ranged from 1.8 to 53 $mg \cdot L^{-1}$. Larvae were found to be more sensitive, with the LC_{50} values ranged between 84 and 649 $\mu g \cdot L^{-1}$. It was also found that moulting crustaceans were more sensitive than intermolt individuals because the uptake of PCP into the tissue after moulting was rapid.

Regarding PH, the concentrations tested here did not significantly affect mortality rate or moult reduction, although in other toxicity studies on the freshwater crustacean, *D. magna*, PH caused a decrease in growth rate by affecting the moulting process [46]. Evans and Nipper [47] showed that the copepod *Schizopera knabeni* manifest a toxic effect of PH in terms of the number of nauplii produced per female only at the highest concentration of PH (13.47 $\mu mol \cdot L^{-1}$) in solution. This was five times the highest concentration tested here.

Finally, considering the dose/effect curves obtained, it is clear that *T. fulvus* nauplii exposed to PCP and Cu showed very high sensitivity, more pronounced in moult number reduction than in mortality rate.

Further experiments are needed to optimise the ecotoxicity protocol, to make it a better predictor of chemical contamination in the environment.

Given the results obtained, *T. fulvus* remains a good candidate for a model organism in ecotoxicological research, thanks to its ease of management and its sensitivity to chemical pollutants.

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