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Monitoring genotoxicity in freshwater microcrustaceans: A new application of the micronucleus assay

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ABSTRACT

We have applied the micronucleus (MN) assay to the measurement of genotoxicity in microcrustaceans. Daphnids (*Daphnia magna*) and Copepods (*Acanthocyclops robustus*) were collected *in situ* and acclimated in the lab for 24 h. The MN assay was successful with the Daphnids but not with the Copepods. Adult Daphnids were exposed to sublethal concentrations of metals (Cu, Zn, Cd) or insecticide (deltamethrin) for 2 and 7 d. Dose-dependent induction of MN was observed after 2 d exposure, with 2-fold induction at the highest doses for each chemical tested. The advantages and ecological relevance of using Daphnids in genotoxicity assessment are highlighted. The Daphnid assay may be a reliable test for aquatic genotoxicity hazard/risk assessment and a useful alternative to studies of amphibians.

1. Introduction

The effects of anthropogenic substances (including micropollutants, such as endocrine-disrupting chemicals, nanoparticles, pesticides, pharmaceuticals, and cosmetics) on aquatic systems are a growing concern [1]. There is an urgent for research and for the development of regulatory frameworks, in order to protect human and wildlife health. Genotoxic effects may lead to tumor induction or impairment of reproduction [2].

The micronucleus (MN) assay is a sensitive biomarker of chromosome damage or mitotic spindle dysfunction [3,4] and can detect both aneugenic and clastogenic effects [5,6]. The MN test consists of detecting structural and numerical chromosome aberrations; it was first established for *Vicia faba* root tips [7] and later applied to mammalian bone-marrow cells [8] and humans [9,10].

Jaylet et al. [11,12] developed the MN test with newt larvae (pleurodele amphibians: *Pleurodeles watl*) exposed to potentially toxic compounds for 16 d. Other studies applied this technique [3,4,13–15] and led to the establishment of international standards for the MN assay with two amphibian larvae, *Pleurodeles watl* and *Xenopus laevis* [16]. These systems are used for assessment of the genotoxicity of pure chemicals, mixtures, natural waters, effluents, leachates, and eluates [17–20]. The MN test has also been applied to various freshwater and marine vertebrate and invertebrate species (fish, amphibians, mollusks, echinoderms); thus, this test has shown high sensitivity and reliability for *in vivo* or *in situ* experiments [21–28]. Genotoxic responses vary among animal species. For example, among amphibians, pleurodele showed a different response compared to xenopus [15,29]. In mammals, the same chemical may induce a different magnitude of genotoxic response between rats and mice [30,31]. Pesticides may induce MN significantly in fish but not in mice [32]. These results show that we cannot rely on a single species to assess the response to genotoxicants.

The goal of this study was to apply the MN assay to two zooplanktonic species, a Cladoceran and a Copepod, and to study the sensitivity of these microcrustaceans to different contaminants: Cu, Zn, Cd, and deltamethrin. The small size of microcrustaceans may make them more sensitive to contaminants than larger species. Zooplankton are considered ecologically more relevant than animals higher in the aquatic food chain. The sensitivity of Cladocerans and Copepods to contaminants has been widely reported and they are used for ecotoxicity testing in routine hazard assessment (ISO standards based on reproduction, motility, larval development, and mortality) [33–38].

Abbreviations: ANOVA, analysis of variance; *D. magna*, *Daphnia magna*; EC_{50}, half maximal effective concentration; LC_{50}, lethal concentration 50%; MN, micronucleus; ROS, reactive oxygen species.

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2. Materials and methods

2.1. Sampling site

Cladocerans were collected from a rainwater-fed pond located in a poorly urbanized area, near the small city of Zarmdine in Tunisia. Access to the collection site is difficult, as it is located in the bottom of an ancient clay pit and surrounded by dense vegetation (mainly reeds and rushes), reducing anthropic impact. Copepods were collected from the Bir Mcherga dam near the city of Zaghouan whose water is used for irrigation (Fig. 1).

2.2. Physico-chemical data and collection of microcrustaceans

The physico-chemical parameters (temperature, salinity, dissolved O_2 and pH) were measured *in situ* using a multi-parameter probe (CONSORT C534). Cladocerans were collected by hand from the pond bank in March 2012, with a 105 μm mesh net. Copepods were collected in February 2012, by dragging a 65 μm mesh plankton net. Samples were held in clean glass jars and kept in an icebox during their transfer to the laboratory. (Some specimens were fixed in formalin for taxonomic identification [39,40].) Animals were acclimated in the laboratory for 24 h prior to experiments.

2.3. Exposure of Daphnia magna to contaminants

2.3.1. Contaminants

Cu, Zn, Cd and the insecticide deltamethrin were examined. Metal salts ($CuCl_2$, $ZnCl_2$, $CdCl_2$) were obtained from Merck (99.9% pure). Deltamethrin, a class II pyrethroid insecticide commonly used in agriculture, was purchased from Fluka (99.9% pure) and was dissolved in ethanol (70%). Sublethal concentrations of contaminants in experimental media were obtained by adding appropriate volumes of stock solutions to 0.45 μm-filtered freshwater. Only nominal contaminant concentrations are reported.

2.3.2. Exposure procedure for D. magna

Control pools of 23 specimens of *D. magna* were put in 0.45 μm-filtered fresh water (salinity 3.3 psu). As a control, animals were exposed to a volume of fresh water added to an equal volume of ethanol (70%) from the deltamethrin stock solution corresponding to the highest deltamethrin-treated group. Pools of 23 specimens of *D. magna* were exposed separately to six different sublethal concentrations (1/2, 1/5, 1/15 $LC_{50,\ 48h}$ for 2 d and 1/20, 1/50, 1/100 $LC_{50,\ 48h}$ for 7 d) of Cu, Zn, Cd, and deltamethrin. $LC_{50,48h}$ values are 18.9, 4029, 17 and 0.45 μg/L for Cu, Zn, Cd, and deltamethrin, respectively [41–43]. Metal and deltamethrin concentrations in experimental media were prepared by adding appropriate volumes of stock solutions to 0.45 μm-filtered fresh water. Vessels were pre-contaminated for 24 h before testing, to stabilize contaminant concentrations during exposures. The experiments were carried out as semi-static tests, with daily renewal of test solutions, at 22 °C +/− 2 °C with a light/dark regime of 12:12 h.

Whenever found, dead animals were removed daily. Animals were fed for 1 h every 24 h before the renewal of solutions. The experiments were conducted in triplicate. At the end of the experiments, animals were prepared for the MN assay. For Copepods, we did not run exposure tests, since the MN assay applied to control Copepods was unsuccessful.

2.4. Micronucleus assay

2.4.1. Sample treatment

Because of the small size of zooplanktonic species, MN assays were performed using a pool of ten Daphnids (50 for copepods) to obtain sufficient cells. Compared to other invertebrate cells (e.g., mollusks), microcrustacean cells are very small and appeared to stick together. Moreover, the chitinous cuticle presents an additional difficulty for cell separation. As for Daphnid specimens, both valves were delicately removed with dissection pliers. A protease was used to separate cells, in order to facilitate their observation and scoring. Whole organisms were crushed with the edge of a Pasteur pipette and incubated in a solution (50 μl) of Dispase I (Neutral protease, Sigma, France), 0.1 mg/ml for 20 min, in a water bath at 37 °C. The cellular suspension obtained was centrifuged for 5 min at room temperature at 3000 H g. After discarding the supernatant, the pellets were spread on microscope slides, air dried, fixed in methanol: acetic acid (3:1) for 1 min, and stained with 0.4% Giemsa (Sigma, France) prepared in phosphate buffer (0.025 M, pH 6.88) for 10 min. One slide was prepared from each sample of ten Daphnids (50 for Copepods) and three slides were prepared for each experimental condition.

Table 1
Ranges of temperature, pH, salinity and dissolved oxygen in Zarmdine pond and Bir Mcherga dam.

Parameter	Zramdine pond	Bir Mcherga dam
Temperature (°C)	17.9–19.6	10.3
pH	7.9–8.2	8.7
Salinity (psu)	2.48–3.32	0.8
Dissolved oxygen (mg/L)	5.36–6.62	7.2

2.4.2. Slide analysis

Slides were examined under 100× magnification using an optical microscope (Carl Zeiss, Germany) with a video recorder (Panasonic, Model NU-VZ 10 EN) connected to a TV screen to facilitate MN counting.

2.4.3. Scoring criteria

One thousand cells with preserved cytoplasm were scored per slide to determine MN frequency, according to the following criteria. MN were defined as round structures, smaller than 1/3 of the main nucleus diameter; MN had to be on the same optical plane as the main nucleus but possess boundaries distinguishable from it [44].

2.4.4. Statistical analysis

For each exposure condition, MN frequency was determined by reading three slides; the reported MN frequency is the mean of these three counts. All results were expressed as mean ± standard deviation. In all experiments, slides were coded to avoid any counting bias. Differences in MN frequencies among sites were compared by one-way ANOVA and Tukey test for multiple comparisons. Significance of differences was set at $P \leq 0.05$.

3. Results

3.1. Physico-chemical parameters

Data are presented in Table 1. Temperature, salinity, dissolved oxygen and pH ranges were within the expected normal values.

3.2. Taxonomic identification of Cladocerans and Copepods

Two Cladoceran species, *Dahnia magna* (Daphnidae family) and *Alona rectangula* (Chydoridae family), were found in the Zarmdine pond collection site. We used only *D. magna* for experiments as they are bigger and considered a model crustacean organism. With respect to Copepods from the Bir Mcherga dam, the cyclopoid *Acanthocyclops robustus* was dominant.