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Assessment of chronic effects of tebuconazole on survival, reproduction and growth of *Daphnia magna* after different exposure times

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ABSTRACT

The effect of the fungicide tebuconazole (0.41, 0.52, 0.71 and 1.14 mg/L) on survival, reproduction and growth of *Daphnia magna* organisms was monitored using 14 and 21 days exposure tests. A third experiment was performed by exposing *D. magna* to the fungicide for 14 days followed by 7 days of recovery (14+7). In order to test fungicide effects on *D. magna*, parameters as survival, mean whole body length, mean total number of neonates per female, mean number of broods per female, mean brood size per female, time to first brood/reproduction and intrinsic rate of natural increase (r) were used. Reproduction was seriously affected by tebuconazole. All tebuconazole concentrations tested affected the number of broods per female and day to first brood. At 14-days test, number of neonates per female and body size decreased by concentrations of tebuconazole higher than 0.52 mg/L, whereas at 21-days test both parameters were affected at all the concentrations tested. Survival of the daphnids after 14 days fungicide exposure did not exhibited differences among experimental and control groups. In this experiment r value was reduced (in a 22%) when animals were exposed to concentrations of 0.71 mg/L and 1.14 mg/L. Survival of daphnids exposed during 21 days to 1.14 mg/L declined, and the intrinsic rate of natural increase (r) decreased in a 30 % for tebuconazole concentrations higher than 0.41 mg/L. Longevity of daphnids pre-exposed to tebuconazole for 14 days and 7 days in clean water did not show differences from control values and all of them survived the 21 days of the test. However, after 7 days in fungicide free medium animals were unable to restore control values for reproductive parameters and length. The maximum acceptable toxicant concentration (MATC) was calculated using the r values as parameter of evaluation. MATC estimations were 0.61 mg/L and 0.46 mg/L for 14 and 21 days, respectively. Results showed that the number of neonates per female was the highest sensitive parameter to the effects of tebuconazole on *D. magna*. On the other hand, a recovery period of 7 days in a free toxicant medium would not be longer enough to reestablish normal reproduction parameters in pre-exposed tebuconazole daphnids.

1. Introduction

The use of chronic toxicity data is essential for the ecotoxicological assessment of chemicals. Chronic tests are used to assess long-term responses to contaminants. Most of them require life-cycles, and are costly and labor-intensive. Life-table analysis provides important insights into the mechanisms of population-level consequences of toxicant exposure which includes survival and reproduction effects. The intrinsic rate of natural increase (r), is a superior laboratory toxicological endpoint compared to the acute mortality because it combines reproduction and mortality chronic effects into one meaningful measure (Van Leeuwen et al., 1985; Stark et al., 1997). Several authors indicate that for some chemicals as 3,4 DCA, metals as Cd or chiral pesticides (as representative of some major groups of pollutants) chronic toxicity for *Daphnia magna* may be measured in a shorter period than the traditional 21 days of exposure with the aim to study the possibility derive new tests designs which would have higher ecological relevance (Guillermino et al., 1999; Barata and Baird, 2000; Chen and Liu, 2008). Van Leeuwen et al. (1985) evaluated r changes in *D. magna* along time when exposed to Cd and found that r values for 14 and 21 days, joint to the r calculated for the entire life table.

A delay in reproduction, caused by pollutants, is an important

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endpoint and should be included in reproduction test with daphnids. Age-specific survival and fecundity rates derived from life table experiments and subsequent calculation of *r* values are considered to be a sound basis for the description of the exponential growth under toxicant stress (Van Leeuwen et al., 1985).

Although, the use of tebuconazole and azole fungicides is forbidden in EU since 2009, it is currently used as fungicide in paddy fields and soybeans culture in other countries. Soybeans are intensively grown over large swaths of land in Midwestern US where tebuconazole is currently applied against *Phakopsora pachyrrhizi* (Ochoa-Acuña et al., 2009). Caldas et al. (2010) have revealed significant concentrations of this fungicide not only in superficial waters but also in groundwater. Tebuconazole concentrations ranged from 0.89 μg/L to 0.03 μg/L were found in groundwater in France (Baugros et al., 2008). Although the environmental levels of this pesticide are very low to induce the direct disappearance of aquatic invertebrates and other organisms, to know its sublethal effects on populations of aquatic organisms such as cladocerans, which are ecologically important components of zooplankton in freshwater ecosystems throughout the world is very important. The tebuconazole concentrations used in the present study are similar to those reported in the Albufera Natural Park (Valencia. Spain) after spray operations (Andreu et al., 2008).

The continuous changing conditions in natural systems make necessary recovery abilities after a stress situation (as chemical exposure). So, species able to recuperate their physiology in a shorter period of time and will be able to survive and reproduce before another adverse period takes place (Villarroel et al., 2000). Population recovery after toxic stress in temporary ponds may be associated with adaptational properties that have previously altered reproduction and growth (Lahr, 1997). Therefore, it would be interesting to investigate the dynamic of a population during the stress situation and recovery period in toxicant free medium after pre-exposure.

Previous research performed in our own laboratory (Sancho et al., 2010) indicated that tebuconazole had a rapid disruptive effect on fish reproduction, but no information is available in *D. magna* organisms.On the other hand, tebuconazole reduces the feeding rate of food in *D. magna* as well as the caloric content in concentrations higher than 0.52 mg/L, as previously determined by Sancho et al. (2009). Therefore, constant exposure of these organisms to tebuconazole will reduce their ability to obtain adequate nutrition for growth, reproduction and metabolic maintenance.

The objective of this study was to provide information on the chronic toxicity of this tebuconazole in survival, growth and reproduction of *D. magna* under different exposure period. Comparisons were made between the results of chronic bioassays with *D. magna* after 14 and 21 days of exposure in order to select the more predictive-informative test fortebuconazole. A daphnid population pre-exposed during 14 days to tebuconazole, was allowed to recover for 7 days in free–toxicant medium (14+7) to detect chronic cumulative effects and to evaluate the patterns of recovery by comparing the results with the other two populations of *D. magna* exposed for 14 and 21 days respectively.

2. Materials and methods

2.1. Test organisms

D. magna test organisms were obtained from a controlled Daphnia culture (clon K6, Muyssen and Janssen, 2001) was obtained several years ago from the Laboratory for Biological Research in Aquatic Pollution (Gante, Belgium). The stock animals were maintained in our laboratory following the recommendations of the EPA United (1996) and OECD (2012): total hardness, 181.8 ± 18.8 mg/L as $CaCO_3$; pH 7.9 ± 0.2; alkalinity, 4.1 mmol/L, 16 h:08 h light:dark photoperiod and at a density of less than 40 animals per liter. Stock animals were maintained in an acclimatized temperature room at 22 ± 1 °C. The daphnids used for the experiments were less than 24 h old and they were derived from a healthy parthenogenetic stock culture (no signs of stress such as high mortality, presence of males or ephipia, delay in the production of the first brood were detected in the stock culture). The reproductive output was ≥60 living offspring per adult female. The medium was renewed three times a week. Offspring were separated from the cultures 1 day before the experiment and the test animals used were always juveniles (≤24 h old from a third brood offspring). The daphnids were fed daily with the algae *Nannochloris oculata*. The algae were cultured in BBM nutrient medium (Bischoff and Bold, 1983) under constant illumination. The temperature was kept at 22 ± 1 °C. The log phase algae were harvested, centrifuged, rinsed and re-suspended. Algae density was estimated by counting with a haemocytometer. *N. oculata* density chosen was 5×10^5 cells/mL (Ferrando et al., 1995).

2.2. Test chemical

Tebuconazole (chemical name IUPAC: (*RS*)-1-*p*-chlorophenyl)-4,4-dimethyl-3-3(1*H*-1,2,4-triazol-1-ylmethyl)pentan-3-ol) technical grade fungicide used in the experiments was 96.5% pure (Bayer CropScience Limited, 2005, Germany). Stock solutions were prepared by dissolving the toxicant in acetone (1 μL/L) (Pesticide Residue Analysis Grade) and then diluting by water into selected concentrations prior to the initiation of testing. Solid-phase extraction (SPE) followed by Gas-Chromatography/Mass-Spectrophotometry techniques (GC–MS/MS) were used to confirm the presence of tebuconazole in the water at the desired concentration over the entire exposure periods (Andreu et al., 2008). Residues of tebuconazole in the water were measured using a gas chromatograph 6890N (Agilent technologies, USA) equipped with a mass-selective detector 5975 (Agilent Technologies) and autosampler 7683 Series (Agilent Technologies, USA). Separations were carried out on capillary column HP-5MS (30 m × 0.25 mm × 0.25 μm, Agilent Technologies, USA). All GC–MS chromatographic data were processed using ChemStation Software (Hewlett-Packard, USA) equipped with a NIST 2005 Mass spectral library. Helium (purity 99.9999%) was used as carrier and collision gas; the injector and detector operating temperatures were 280 and 180 °C, respectively. The column oven temperature was 280 °C. The detection time was 11.1 min. MS detector was equipped with a quadrupole analyzer operating in electron ionization mode (EI); ion source temperature was 230 °C and MS Quad temperature was 150 °C. In these conditions tebuconazole recoveries from water were not less than 90% (Andreu et al., 2008). Therefore, actual tebuconazole concentrations were not significantly different from the nominal values. Actual concentrations were in good agreement with the nominal ones. Reported concentrations in the present study are nominal values.

2.3. Chronic toxicity test

Preliminary acute toxicity test were conducted in our laboratory in order to calculate tebuconazole LC_{50} data (Sancho et al., 2009). Based on these results, daphnids were exposed for both 14 and 21 days chronic tests to the following sublethal tebuconazole concentrations of 0.41 (1/140 LC_{50}-24 h), 0.52 (1/110 LC_{50}-24 h), 0.71 (1/80 LC_{50}-24 h) and 1.14 (1/50 LC_{50}-24 h) mg/L plus the blank control and the acetone control (1 μL/L) containing the same concentration of acetone as in the toxicant concentrations tested, so the amount of solvent used remained constant. *N. oculata* in a