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Toxicity and genotoxicity of the quaternary ammonium compound benzalkonium chloride (BAC) using *Daphnia magna* and *Ceriodaphnia dubia* as model systems

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ABSTRACT

The toxicity and genotoxicity of the cationic surfactant benzalkonium chloride (BAC) were studied using *Daphnia magna* and *Ceriodaphnia dubia* as model systems. Acute and chronic toxicity testing were performed according to the international standard guidelines and the genotoxicity was detected through the comet assay on cells from whole organisms *in vivo* exposed. Acute effects occurred at concentrations in the order of tens of μg/L in *D. magna* and hundreds of μg/L in *C. dubia*. Chronic effects were found at one order of magnitude less than short-term effects maintaining the same difference in sensitivity between *D. magna* and *C. dubia*. BAC induced relevant DNA damage, in both cladocerans; the lowest adverse effect levels were 0.4 and 4 ng/L for *D. magna* and *C. dubia*, respectively. As these effective concentrations are far lower than BAC occurrence in surface waters (units of μg/L) a concerning environmental risk cannot be excluded. The findings of this study showed that *D. magna* and *C. dubia*, could be used as model organisms to detect acute and chronic toxicity as well as genotoxicity at the whole organism level.

1. Introduction

Benzalkonium chloride (BAC) belongs to the quaternary ammonium compounds (QACs) which are positively charged organic chemicals, primarily used as disinfectants, antimicrobials or surfactants, but also as emulsifiers and food preservatives. BAC is a mixture of alkylbenzyl dimethyl ammonium chlorides of numerous alkyl chain lengths with a positively charged tetra substituted ammonium atom. Antibacterial activity of BAC is based on its amphiphilic property having both distinct hydrophobic and hydrophilic region. The hydrophilic region interacts with the bacterial cell membrane and then the hydrophobic region penetrates the bilayer causing cell lysis (Fazlara and Ekhtelat, 2012). Quaternary ammonium compounds, and along them BAC, are broadly used in industry and household and they end up in soil and waters as waste residues preventing the biodegradation of natural environments due to their toxicity to many aquatic and terrestrial species. BAC has been detected in hospital effluents at concentrations of 6 mg/L (Sütterlin et al., 2008), in sediments at concentrations up to 206 μg/kg (Ferrer and Furlong, 2002) and, although dilution in surface waters could be supposed, toxic effects on aquatic organisms cannot be excluded. Among surfactants, cationic surfactants are known as the highest dangerous compounds for the ecosystem (Ivanković and Hrenović, 2010) and BAC is known to be toxic towards the Gram-negative bacteria *Vibrio fischeri* and *Pseudomonas putida* (Sütterlin et al., 2008) and towards algae such as *Chlorella vulgaris* (Chen et al., 2014) and *Isochrysis galbana* and *Chaetoceros gracilis* (Pérez et al., 2009). Aquatic and terrestrial invertebrates are common organisms to study BAC toxicity. Acute toxicity was found to the planarian *Dugesia japonica* in the order of mg/L (Li, 2012) even if crustaceans are among the most studied aquatic organisms for their high sensitivity to cationic surfactants (Ivanković and Hrenović, 2010). In most cases, these studies address the acute toxicity of BAC while chronic toxicity findings are rather lacking. Sorption on sediments or soil strongly affects the bioavailability and then the toxicity of BAC. Chen and collaborators (2014) found that sorbents such as humic acids, sediments and clay minerals reduced the acute toxicity of BAC in crustaceans, fish and in terrestrial animals such as *Lumbriculus variegatus*. The toxicological characterization of BAC has also concerned its possible genotoxicity, and in previous studies, BAC showed genotoxic properties in mammalian and plant cells (Deutschle et al., 2006; Ferk et al., 2007; Wu et al., 2011) while it was not mutagenic in bacterial reverse assays (Ferk et al., 2007; Laurie et al., 2013). As the

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biological activity of BAC is due to functional and morphological alterations as well as on triggering DNA damage, the concurrent evaluation of toxicity and genotoxicity of BAC on the same bio-indicators could be of great ecotoxicological relevance. In this perspective, *Daphnia magna* and *Ceriodaphnia dubia*, two freshwater crustaceans, which represent key organisms of the aquatic food chain, as they are a food source for amphibians, fish, and other aquatic species, could be used as model organisms to detect acute and chronic toxicity as well as genotoxicity at the whole organism level of benzalkonium chloride. *D. magna* and *C. dubia* are largely used in both standard acute and chronic toxicity testing and, recently, they have been used as sensitive biomarkers to detect genotoxicity (Parrella et al., 2015).

The aim of this study was to assess the overall impact of benzalkonium chloride in two freshwater crustaceans. *D. magna* and *C. dubia* were used in standard tests to detect the acute and chronic toxicity while cells from whole daphnids were *in vivo* exposed to BAC to detect genotoxicity in the single-cell gel electrophoresis assay. In order to establish possible differences between nominal and actual concentrations, BAC concentration in test solutions was also investigated.

2. Materials and methods

2.1. Chemicals

Alkyldimethylbenzylammonium chloride (BAC), linear formula $C_6H_5CH_2N(CH_3)_2RCl$ where R = C_8H_{17} to $C_{18}H_{37}$, CAS Number 63449-41-2, purity ≥ 95% and acetic acid were supplied by Sigma–Aldrich (Milano, Italy). Eosin Y was provided by Carlo Erba (Milano, Italy). Sodium acetate 3-hydrate was obtained by PanReac (Barcelona, Spain).

2.2. Test organisms

Daphnia magna Straus and *C. dubia* were coming from healthy laboratory mass cultures. *D. magna* was maintained in moderately fresh water (hardness 170 mg L^{-1}, expressed as $CaCO_3$) at 20 ± 1 °C and *C. dubia* in ISO medium (hardness 250 mg L^{-1} expressed as $CaCO_3$) at 25 ± 1 °C as reported by the respective standard guidelines (ISO 10706, 2000; ISO 20665, 2008) with a 16:8 h light:dark cycle (600 lux). Mass culture organisms were fed with a combination of 5 g/L each of yeast (*Saccharomyces cerevisiae*), alfalfa (*Medicago sativa*) and flake food, in addition to the unicellular green alga *Pseudokirchneriella subcapitata* (10^8 cells/mL). The organisms to expose both in acute and chronic tests were less than 24 h old and obtained from daphnids from the second to the fifth brood.

2.3. Determination of benzalkonium chloride concentration in test samples

The concentrations of benzalkonium chloride were determined using the spectrophotometric method according to Ma et al. (2014) procedure with slight modifications. Briefly, a suitable volume of each test solution containing benzalkonium chloride at the beginning of each acute toxicity and genotoxicity test and after 24 h and 48 h (in the dark) was transferred to a 10-mL volumetric flask, then 2 mL of eosine Y (5 mM) solution and 1.5 mL of the acetate buffer solution (pH 4.0) were added. The same procedure was repeated to prepare the reagent blank replacing benzalkonium chloride with fresh water. The test samples for chronic toxicity were added with 100 μL algae (10^8 cells/mL) and after 24 and 48 h (maximum time of exposure between two renewal of test solution for *C. dubia* and *D. magna*, respectively), were examined after filtration of the solution through 0.22 μm acetate filter. The absorption of the ion association complex solution at 550 nm was measured by a Shimadzu UV-1700 spectrophotometer in reference to a blank.

2.4. Acute and chronic toxicity tests

Acute tests were performed in two independent experiments, five test concentrations and four replicates per each concentration from 100 μg/L (dilution factor 3) for *D. magna* and seven test concentrations and three replicates from 1000 μg/L (dilution factor 3) for C. *dubia* in the dark, for 48 and 24 h, respectively without food. Preliminary range finding tests (dilution factor = 10) using five concentrations of BAC were performed in order to identify the concentration range to be used in definitive tests. Experiments were performed according to OECD 202 (2004) for *D. magna* and U.S. EPA (1993) (applied to reference toxicant) procedures for *C. dubia*.

The test endpoint for *D. magna* acute test was inhibition of mobility, and the concentration found to immobilize 50% of crustaceans in 48 h was considered as the EC50, while the test endpoint for *C. dubia* acute test was mortality, and the concentration that resulted in a 50% effect in 24 h was indicated as the LC50.

Chronic tests were performed in two independent experiments with organisms individually exposed in glass beakers, six test concentrations and ten replicates per each concentration from 6 μg/L (dilution factor 3) for *D. magna* and from 90 μg/L (dilution factor 3) for C. *dubia*, about one fifth of the respective acute EC50 values. The *C. dubia* population growth inhibition tests (ISO, 20665, 2008) were performed over 7 days at 25 °C with a 16:8 h light: dark cycle (600 lux). *D. magna* tests were performed over 21 days of exposure according to OECD 211 (2008) at 20 °C with the same light: dark cycle.

Dissolved oxygen and pH were checked at the beginning and the end of each test. For each test, data were processed only if the validity criteria were satisfied (dissolved oxygen concentration in the exposed solutions > 4 mg/L and pH within the range 7–8 according to the guidelines).

Daily, at the renewal time, the offspring produced by each parent organism, was counted and removed and the organisms were fed on 200 μL of the food combination indicated above for maintenance. For all chronic tests, a test-medium control series (negative control) was used in addition to the test series. According to the respective guidelines the mean number of living offspring per living parent in the negative control was >60 for *D. magna* and >15 for *C. dubia*. The reproduction output of the organisms exposed to the samples was compared to that of the negative control to determine the median reproduction inhibition concentration percentage (EC50).

Results were analysed using ToxRat Professional software, Version 2.10.05 (Alsdorf, Germany) to calculate, the effective percentage for each dilution. The results were then pooled by Prism5 (Graphpad Inc., CA, USA) to estimate, by non-linear regression (log agonist *vs.* normalized response-variable slope), the dilution giving 50% effect.

2.5. Comet assay

The Comet assay was carried out on cells coming from whole neonate organisms exposed to five concentrations of BAC starting from about one hundredth of the respective acute EC50 values found for *D. magna* and *C. dubia*. After 24 h, the exposed organisms were placed in 1 mL of phosphate–buffered saline (PBS) with 20 mM ethylene diamine tetra-acetic acid (EDTA) and 10% dimethyl sulfoxide (DMSO) and then disintegrated by serial pipetting. Hydrogen peroxide was used as the positive control. After centrifugation (5000 rpm), the cells were spread onto microscope slides