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Ecotoxicological potential of the biocides terbutryn, octhilinone and methylisothiazolinone: Underestimated risk from biocidal pathways?

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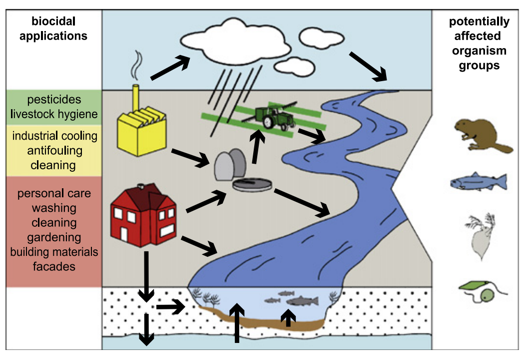
HIGHLIGHTS

- Toxicity of terbutryn, octhilinone and methylisothiazolinone was determined for *Daphnia magna*
- In-vitro tests with rainbow trout liver cells (RTL-W1) and ovarian cells of hamsters (CHO-9) showed low toxicity
- Based on published data, PNEC values were calculated for TB (0.003 μg/l), OIT (0.05 μg/l) and MI (0.5 μg/l)

GRAPHICAL ABSTRACT



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ABSTRACT

The use of biocides by industry, agriculture and households increased throughout the last two decades. Many new applications with known substances enriched the variety of biocidal pollution sources for the aquatic environment. While agriculture was the major source for a long time, leaching from building facades and preservation of personal care and cleaning products was identified as new sources in the last few years. With the different usage forms of biocidal products the complexity of legislative regulation increased as well. The requirements for risk assessment differ from one law to another and the potential risk of substances under different regulations might be underestimated. Still EC_{50} and predicted no-effect concentration (PNEC) values gained from testing with different species are the core of environmental risk assessment, but ecotoxicological data is limited or lacking for many biocides. In this study the biocides widely used in facade coatings and household products terbutryn, octhilinone and methylisothiazolinone were tested with the *Daphnia magna* acute immobilisation assay, the neutral red uptake assay and the ethoxyresorufin-O-deethylase (EROD) assay, performed with rainbow trout liver (RTL-W1) cells. Further, the MTT assay with the ovarian cell line CHO-9 from Chinese hamster was used as mammalian model. Octhilinone induced the strongest effects with EC_{50} values of 156 μg/l in the *D. magna* assay, while terbutryn showed the weakest effects with 8390 μg/l and methylisothiazolinone 513 μg/l respectively. All other assays showed higher EC_{50} values and thus only weak effects. EROD assays did not show any effects. With additional literature and database records PNEC values were calculated: terbutryn reached 0.003 μg/l, octhilinone 0.05 μg/l and methylisothiazolinone 0.5 μg/l. Potential ecotoxicological risks of these biocides are discussed, considering environmental concentrations.

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1. Introduction

Despite the occurrence of emerging contaminants such as pharmaceuticals, biocides (or pesticides when used in agriculture) belong to the major pollutants and potential threats for the aquatic environment, with increasing concentrations being detected in rivers, lakes and coastal waters (Malaj et al., 2014; Kaonga et al., 2016). While agriculture was supposed to be the major source of pesticide/biocide pollution throughout recent years, different studies revealed the significance of other sources of biocidal active substances especially in surface water: These substances may enter the environment due to an improper use of pesticides by private households (Blanchoud et al., 2004 and 2007), the use of home and personal care products (Zhang et al., 2015) as wells as their use as material protection products in a variety of applications. One of the best documented cases of the latter is tributyltin used as antifouling agent in boat paintings, leading to strong unintended ecotoxicological effects on aquatic life (Alzieu, 2000).

Biocides used for material protection can also be found in most building and construction materials (Wangler et al., 2012). Therefore, this application gained attention in recent years as a new source of biocidal pollution in watercourses (Burkhardt et al., 2012; Bollmann et al., 2014a). Different biocidal active substances are used in renders, plasters, wood or paintings to protect the facades and roofs of buildings from unwanted microbial, fungal and algal growth. Several studies showed that these biocides are leached from building architecture by rainfall events and can reach surface waters by rainwater runoff (Bester et al., 2014). For example, Wittmer et al. (2011) could demonstrate that urban biocide loads in a Swiss river catchment were in the same range as the most widely-used agricultural pesticides despite of substantially lower amounts of urban biocides used. Leaching from biocide-treated materials depends on several factors with intensity of precipitation being among the most important parameters (Jungnickel et al., 2008; Wittmer et al., 2011). As intensification of heavy precipitation events are expected due to climate change (Min et al., 2011), the impact of biocide leaching from facades on aquatic habitats might even rise in future. However, adverse effects of these substances on non-target organisms cannot be excluded at the amounts released currently (Burkhardt et al., 2009).

Approaches to estimate the overall amount of biocides released to the environment are missing, mainly due to inefficient regulation, in some cases leading to neglecting the toxicological and ecotoxicological potential of the substances used. Wieck et al. (2016) showed that 65% of the biocides used in household and personal care products are not covered by the EU biocidal regulation (European Parliament and Council, 2012).

Besides others, terbutryn (TB) as triazine herbicide/algaecide as well as octhilinone (OIT) and methylisothiazolinone (MI) as isothiazolinone bactericides/fungicides belong to the most frequently used biocides in construction materials as in-can or film preservatives (Schoknecht et al., 2012; Bollmann et al., 2014a). Terbutryn was widely used as agricultural pesticide until it was banned within the EU in 2002. Other usages are still allowed although terbutryn is recognized meanwhile as a threat to aquatic life and belongs to the priority substances since 2013 (European Parliament and Council, 2013) according to the Water Framework Directive (WFD; European Parliament and Council, 2000). Due to this status many data are available, including ecotoxicological results and environmental quality standards (EQS). Octhilinone and methylisothiazolinone are not regulated by the WFD. Although many studies are available on the leaching of biocides from building surfaces (Burkhardt et al., 2012), ecotoxicological data are rather scarce. Risk assessment for these substances is therefore often performed with estimations, extrapolations or models. Accordingly, there is a strong need for ecotoxicological data to allow for a thorough risk assessment of these substances. For most ecotoxicological risk assessment approaches a predicted environmental concentration (PEC) of a substance is related to a predicted no-effect concentration (PNEC) of the same. In order to derive the PNEC, at least data from tests with algae, fish and *Daphnia magna* are requested. PNEC values gained from chronic instead of acute tests are preferred, but due to the complexity of chronic testing, acute data is often used, usually with a safety or assessment factor. While algae and *D. magna* are accepted and frequently used organisms in standardized tests, the use of vertebrates such as fish as well as mammals for ecotoxicological testing is subject of ethical discussions (Lillicrap et al., 2016; Scholz et al., 2013) and should be avoided whenever possible. Cell-based tests can contribute in solving this problem, though the results are not always completely comparable to the ones from whole organism tests (Schirmer, 2006).

As leaching of biocides from building materials was demonstrated (Burkhardt et al., 2012; Wangler et al., 2012; Bollmann et al., 2014a; Schoknecht et al., 2016), we are interested in possible effects of these substances in the aquatic environment. The use of different cellular and organismic test systems will help to fill the gap of ecotoxicological data to allow for a thorough environmental risk assessment. In the current study we used the highly standardized *Daphnia magna* test as a measure for the acute toxicity for aquatic invertebrates. Additionally, we used three established cell-based toxicity assays with different endpoints to check if they are alternatives for whole animal testing. Cytotoxicity to vertebrate cells was tested with the neutral red uptake assay. The activity of the cytochrome P4501A system was studied as one important cellular detoxification mechanism using the ethoxyresorufin-O-deethylase (EROD) assay. Both tests were performed with rainbow trout liver cells. Finally, cell viability of mammalian cells was tested with the MTT assay. By comparing our results with literature and database entries, we were able to calculate PNEC values from the results of the most sensitive species. Using the PNEC values and literature data on environmental concentrations, the potential risk for the aquatic environment could be estimated.

2. Materials and methods

2.1. Biocide stock solutions

Standards of terbutryn, octhilinone and methylisothiazolinone were purchased from Labor Dr. Ehrenstorfer GmbH (Augsburg, Germany). Table 1 is summarizing the names and numbers of the three substances. Stock solutions of the biocides were prepared with standard fresh water (ISO/DIS, 2010) for the acute toxicity test and dimethyl sulfoxide (DMSO; purity > 99.8%; Carl Roth, Karlsruhe, Germany) for neutral red uptake, EROD and MTT assay, respectively. The stock solutions were stored at 4 °C in dark and used for the experiments within few days after preparation by mixing with standard fresh water, Leibowitz medium (Biowest, Nuaillé, France) or phosphate buffered saline (PBS, Biowest, Nuaillé, France) according to the specific test requirements.

2.2. Acute toxicity assay with *Daphnia magna*

The acute toxicity for fresh water invertebrates was tested using the DaphToxKit F™ magna (MicroBioTests, Gent, Belgium) following the OECD Guideline 202 (OECD, 2004) and DIN EN ISO 6341 (ISO/DIS, 2010). Maintenance of animals and dilution of the biocide solutions were performed with standard fresh water, prepared with deionized water by Milli-Q-Academics (Merck-Millipore, Germany) and salt solutions from DaphToxKit F™ magna (MicroBioTests, Gent, Belgium) with the concentrations: $NaHCO_3$ (67.75 mg/l), $CaCl_2 \times 2\ H_2O$ (294 mg/l), $MgSO_4 \times 7\ H_2O$ (123.25 mg/l) and KCl (5.75 mg/l). Standard fresh water was stored at 4 °C in the dark and brought to a temperature of 20 ± 1 °C as well as pre-aerated with an aquarium pump before use.

Neonates of *Daphnia magna* aged <24 h were exposed to the biocides in 24-well plates (MicroBioTests, Gent, Belgium) without feeding prior to exposure. Range-finding tests determined the 0–100% response range for each biocide. Different concentrations of the biocide solutions and one control with pure standard fresh water were tested on each