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What contributes to the sensitivity of microalgae to triclosan?

Stephanie Franz[a], Rolf Altenburger[a], Hermann Heilmeier[b], Mechthild Schmitt-Jansen[a,*]

[a] UFZ – Helmholtz Centre for Environmental Research, Department Bioanalytical Ecotoxicology, Permoserstr. 15, 04318 Leipzig, Germany
[b] TU Bergakademie Freiberg, Interdisciplinary Ecological Center, Leipziger Str. 29, 09599 Freiberg, Germany

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ABSTRACT

Differential sensitivities of microalgae to triclosan have been reported, which may have significant implications for environmental risk assessment of this widely used biocide. Therefore, the aim of this study was to derive a mechanistic understanding of varying microalgal sensitivity to this substance.

The toxicity of triclosan was evaluated using microalgal systems varying in biological complexity, exposure time and systematic position (a synchronized culture of the chlorophyte *Scenedesmus vacuolatus*, a diatom *Nitzschia palea* cultivated in suspension as well as attached to surfaces and periphyton communities). The results revealed (1) differences in sensitivity of the selected microalgal systems of three orders of magnitude and (2) highest sensitivity of the chlorophyte to triclosan in the range of environmental concentrations. To investigate algal sensitivity to triclosan in more detail, bioavailability was considered by investigating suspended and attached living algae. Differences in the generation time (in comparison to test duration) of the species were addressed by evaluating and modeling concentration–time–effect relationships. However, varying sensitivities of the selected microalgal systems remained unexplained. Comparison of species-specific toxic responses to calculated effect concentrations, derived from quantitative relationships for narcosis and uncoupling mode-of-action, leads us to the conclusion that triclosan may address multiple target sites in different microalgal species.

1. Introduction

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) is widely used as a broad-spectrum bactericide in pharmaceuticals and personal care products (PPCPs). It has been used as an ingredient in hand-disinfecting soaps, deodorants, household cleaners, dental hygiene products and textiles for over 30 years (Singer et al., 2002). The widespread use of triclosan becomes apparent in wastewater treatment plants (WWTPs). In the UK concentrations of up to 21.9 $\mu g\,L^{-1}$, or in Greece of up to 23.9 $\mu g\,L^{-1}$ were measured in the influent of WWTPs (Reiss et al., 2002; Sabaliunas et al., 2003; Stasinakis et al., 2008). Elimination processes such as biodegradation, phototransformation, sorption or sedimentation can remove up to 96% of triclosan in WWTPs (Singer et al., 2002; Reiss et al., 2002; Lindström et al., 2002; Guang-Guo and Kookana, 2007). Nevertheless, the residual amount of triclosan in wastewater effluents resulted in surface water concentrations of 1.4–90 $ng\,L^{-1}$ (Singer et al., 2002; Tixier et al., 2002; Lindström et al., 2002) or even up to 2.3 $\mu g\,L^{-1}$ in U.S. streams (Kolpin et al., 2002).

Triclosan is effective against a wide range of gram-positive and gram-negative bacteria as well as yeast and mold species at low concentrations. MIC-values (minimum inhibitory concentration) of triclosan for different bacteria stains generally range between 10 and 3000 $\mu g\,L^{-1}$ (Bhargava and Leonard, 1996). Several studies have investigated the toxicity of triclosan on higher aquatic organisms (Orvos et al., 2002; Ishibashi et al., 2004; Tatarazako et al., 2004; Canesi et al., 2007; DeLorenzo et al., 2008) and have recently been reviewed in Capdevielle et al. (2008). EC_{50} values ranged from 0.7 to 390 $\mu g\,L^{-1}$ with EC_{50} values for crustaceans (*Daphnia magna* mortality 390 $\mu g\,L^{-1}$, *Ceriodaphnia dubia* survival and reproduction 240 $\mu g\,L^{-1}$, 48 h), fish (mortality 260–370 $\mu g\,L^{-1}$, 96 h), higher plants (*Lemna gibba* growth inhibition >62.5 $\mu g\,L^{-1}$, 7 d), and five microalgal species (growth inhibition 0.7 to >66 $\mu g\,L^{-1}$, 96 h). The microalga *Selenastrum capricornutum* (EC_{50} 4.7 $\mu g\,L^{-1}$, growth inhibition) was reported to be 30-fold more sensitive to triclosan than the bacterium *Vibrio fisheri* (EC_{50} 150 $\mu g\,L^{-1}$, bioluminescence inhibition) (Tatarazako et al., 2004). In conclusion, microalgae were the most sensitive organisms to triclosan but significant differences in sensitivity in the range of two orders of magnitude were found between algae (Orvos et al., 2002), giving rise to the question for the reason of these findings.

A first explanation might be sought in the physico-chemical properties of triclosan. Triclosan has a pK_a value of 8.1, which

* Corresponding author. Tel.: +49 341 235 1523; fax: +49 341 235 2401.
E-mail address: mechthild.schmitt@ufz.de (M. Schmitt-Jansen).

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is in the range of pH values of surface waters and test medium, respectively. Therefore, the pH-regime during exposure, which may change during primary production of autotrophic organisms as a result of carbon consumption, may result in different extent of ionization of triclosan. Photodegradation during exposure of autotrophic organisms may also be a relevant process as photodegradation of triclosan in its dissociated form is reported to be very fast (Tixier et al., 2002; Lindström et al., 2002). Therefore, comparable exposure conditions are essential for comparative sensitivity studies in different phytoassays.

Significant differences in sensitivity may result from varying bioavailability of the compound, e.g. for different life forms of algae. Bioavailability may be lower in biofilm-associated cells than for suspended cells because of the presence of extracellular polymeric substances (EPS) that may reduce diffusion, as has been reported for bacterial biofilms exposed to triclosan (Tabak et al., 2007). This may be even more pronounced for periphyton as has been shown for inorganic toxicants, with a negative correlation between toxicity and algal biomass (Guasch et al., 2003).

While acute and chronic effects are differentiated in aquatic toxicity, the ratio between exposure duration and generation time often remains unclear. For organisms with generation cycles in the range of days, such as microalgae, standard exposure durations of 24–72 h could result in acute or chronic effects, respectively. As the duration of a full reproduction cycle varies significantly between different algae, the influence of exposure time in relation to generation time needs to be considered in comparative studies. Therefore, acute effects are defined as effects resulting from experiments lasting shorter than one reproduction cycle and chronic effects from experiments that last longer than one reproduction cycle.

Moreover, different modes-of-action of triclosan have been reported for bacteria. Triclosan has been described to block the lipid synthesis by inhibiting the enzyme enoyl-acyl carrier protein reductase (FabI) (McMurry et al., 1998; Levy et al., 1999) and to destabilise the cell membrane (Villalain et al., 2001), causing structural perturbations with resultant loss of permeability-barrier functions (Phan and Marquis, 2006). A specific mode-of-action in algae has not been reported, so far.

Based on the above considerations, several possible factors were identified to account for the remarkable differential sensitivities of microalgae to triclosan. The objective of this study was to link monospecies-based toxicity data to effects in systems of higher biological complexity. Additionally, the relevance of exposure time in relation to generation time was addressed by observing and modelling acute and chronic effects.

Therefore, the effect of triclosan was investigated for the unicellular chlorophyte *Scenedesmus vacuolatus*, the diatom *Nitzschia palea* growing in suspension and attached on surfaces and for periphyton communities under comparable exposure conditions. An experimental design suitable for a variety of microalgal systems was developed, to exclude the influence of physico-chemical conversion of triclosan and to enable measurements of the same observation parameter (inhibition of photosynthesis). Besides, the study aimed to clarify whether the life form, generation time or taxonomic position of microalgal species determines its sensitivity to triclosan.

2. Materials and methods

2.1. Cultivation of test organisms

A synchronized culture of the unicellular chlorophyte *S. vacuolatus* (strain 211-15 SAG, Göttingen, Germany) was grown photoautotrophically. The composition of the inorganic, sterilized and buffered test medium and the conditions of the cultivation were described by Altenburger et al. (2004). The cells were synchronized by a light/dark cycle of 14/10 h and a periodic dilution to the standard cell density of 1×10^6 cells mL^{-1} at the beginning of the light phase (Altenburger et al., 2004).

N. palea (strain unknown) was obtained from the association of the Wahnbach-Talsperrenverband (Siegburg, Germany). The suspended culture was cultivated in flasks in a climate chamber at $20 \pm 2\,^{\circ}C$ under continuous light with a light intensity of 130 μmol photons $m^{-2}\,s^{-1}$ and was grown in a sterile Bacillariophycean medium (Schlösser, 1994). This diatom species was used for experiments to compare the toxicity to suspended as well as attached living algae. For testing biofilms, the cells were transferred into 24-well plates with glass discs (diameter 1.5 cm) as a substrate. The cells were allowed to settle and to grow on discs for 1 day. Pre-studies revealed that they do not detach from the discs after this time by washing. The cultivation conditions adjusted for the suspended culture were also used for the biofilm establishment.

Periphyton was grown in 20-L aquaria. The water was taken from the Mulde River, Germany and replaced weekly to ensure a constant inoculation of algal cells and to avoid nutrient depletion. Algae colonised the glass discs (diameter 1.5 cm), which were arranged vertically in Plexiglas holders in the aquaria. The cultivation conditions are described in detail in Schmitt-Jansen and Altenburger (2005). The biofilms were grown for at least 2 weeks. Physico-chemical parameters (pH value, oxygen content, soluble ion concentration) were measured in the fresh river water and in the aquaria before renewing the water after 1 week. The pH in fresh river water was in average 7.7 and increased up to 9.5 during a week. Oxygen content ranged from 6.0 to 18.8 μg L^{-1}. The mean conductivity was 466 μS cm^{-1} and ranged from 269 to 757 μS cm^{-1}.

2.2. Standardised test design for suspended and attached microalgae

To determine acute and chronic effects of triclosan on suspended and attached microalgae, a standardised protocol was developed to avoid differences in responses caused by different testing conditions. The standard test vessels (Pyrex culture tubes, QVF Glastechnik GmbH, Wiesbaden, Germany) were altered to hold the biofilm glass discs in a vertical position, so the same test vessels could be used for experiments with algae in suspension and with biofilms. Magnetic bars were applied for stirring the medium, to homogenise algal suspension and reduce boundary layer effects. The studies were conducted at $28 \pm 0.5\,^{\circ}C$ in a water bath. The pH value during the test period was adjusted below the pK_a value of 8.1 to pH 7 or below, to minimize physico-chemical conversion of triclosan, and was stabilised over the test period using buffered media. The buffer and the pH had to be optimized for each system. Toxicity experiments were conducted using simulated sunlight provided by a halogen lamp (SOL500, Dr. Hoenle, Munich, Germany). To prevent photodegradation of triclosan, the UV-part of the spectrum (<400 nm) was filtered with an UV-filter (clear-SR, WIPA-Technik, Tettnang, Germany). For *N. palea* a different filter (Opalfilm, silver, Haverkamp, Münster, Germany) was used to eliminate UV as well as to reduce light intensity to 100 μmol photons $m^{-2}\,s^{-1}$, which was in the range of cultivation conditions and optimal light conditions for *N. palea* (von Denffer, 1949).

Triclosan (CAS RN: 3380-34-5, purity 99.8%, Calibochem, Switzerland) was dissolved in dimethylsulfoxide (DMSO, CAS RN: 67-68-5, Merck, Germany). Controls and solvent controls were prepared for each experiment. The used amount of 1‰ DMSO in the final test solution had no effect on the results (data not shown). The dilution series for triclosan were adjusted to the sensitivity of the