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Published in:
Environmental Toxicology and Chemistry

Link to article, DOI:
[10.1002/etc.3649](https://doi.org/10.1002/etc.3649)

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):
Ribbenstedt, A., Mustajärvi, L., Breitholtz, M., Gorokhova, E., Mayer, P., & Sobek, A. (2017). Passive dosing of triclosan in multi-generation tests with copepods - Stable exposure concentrations and effects at the low μg l-1 range. *Environmental Toxicology and Chemistry*, 36(5), 1254-1260. DOI: 10.1002/etc.3649

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PASSIVE DOSING OF TRICLOSAN IN MULTIGENERATION TESTS WITH COPEPODS – STABLE EXPOSURE CONCENTRATIONS AND EFFECTS AT THE LOW $\mu\text{g/L}$ RANGEANTON RIBBENSTEDT,[†] LUKAS MUSTAJÄRVI,[†] MAGNUS BREITHOLTZ,[†] ELENA GOROKHOVA,[†] PHILIPP MAYER,[‡]
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(Submitted 30 August 2016; Returned for Revision 22 September 2016; Accepted 10 October 2016)

Abstract: Ecotoxicity testing is a crucial component of chemical risk assessment. Still, due to methodological difficulties related to controlling exposure concentrations over time, data on long-term effects of organic chemicals at low concentrations are limited. The aim of the present study was, therefore, to test the applicability of passive dosing to maintain stable concentrations of the organochlorine bactericide triclosan in the water phase during a 6-wk multigeneration population development test with the harpacticoid copepod *Nitocra spinipes*. Triclosan was loaded into silicone (1000 mg), which was used as passive dosing phase in the exposure vials. The distribution ratio for triclosan between silicone and water ($D_{\text{silicone-water}}$) was 10466 ± 1927 . A population development test was conducted at 3 concentration levels of triclosan that were measured to be 3 $\mu\text{g/L}$ to 5 $\mu\text{g/L}$, 7 $\mu\text{g/L}$ to 11 $\mu\text{g/L}$ and 16 $\mu\text{g/L}$ to 26 $\mu\text{g/L}$. The results demonstrate that passive dosing is applicable for long-term ecotoxicity testing of organic chemicals, including during significant growth of the test organism population. Shifts in the demographic structure of the population during exposure suggest the most severe effects were exerted on juvenile development. Progressively lower development index values in the populations exposed to increasing triclosan concentrations suggest developmental retardation. The results further stress the need for chronic exposure during ecotoxicity testing in chemical risk assessment because even the most sensitive endpoint was not significant until after 7 d of exposure. *Environ Toxicol Chem* 2017;36:1254–1260. © 2016 The Authors. Environmental Toxicology and Chemistry published by Wiley Periodicals, Inc. on behalf of SETAC.

Keywords: Passive dosing Triclosan Chronic toxicity Silicone Exposure

INTRODUCTION

Reliable ecotoxicological data are crucial for sound risk assessments of chemicals. In environmental risk assessment, criteria for protection goals are generally focused on populations, communities, and ecosystems [1,2]. Yet, many existing ecotoxicological test protocols used for chemical assessment are based on short-term toxicity tests [3]. Adverse effects, which are detectable by long-term exposure to chemicals at low concentrations, are thereby rarely assessed. One important reason behind the limited data on long-term effects is methodological difficulties related to controlling exposure concentrations over time [4,5].

Maintaining stable exposure concentrations during ecotoxicological testing is essential during chronic tests. Traditionally in ecotoxicity testing of organic chemicals, the test substance is added to the test medium in a solvent solution. This procedure works well for polar, nonvolatile, chemicals and chemicals that do not significantly degrade or transform during the test period. However, many contaminants of environmental concern are hydrophobic or semihydrophobic. These chemicals partition to organic matter in the test medium or to the walls of the test vial and, depending on their properties, may also volatilize, leading to significant decrease in exposure concentrations. Passive

dosing offers a method to overcome these problems [6]. In passive dosing experiments, a polymer (e.g., silicone) is loaded with chemicals and brought into contact with a medium (e.g., water). Once equilibrium is established between the polymer and the water phase, the chemical concentration in the medium is equal to the concentration in the polymer divided by the polymer–medium partition coefficient. A major advantage of using passive dosing in ecotoxicological testing is that the polymer can compensate for losses of the test substance due to sorption, volatilization, and degradation. Another advantage of using passive dosing is that solvent spiking of the chemicals is avoided, which otherwise may cause problems with solvent toxicity [7].

The applicability of passive dosing in experimental ecotoxicology has been demonstrated in a large number of studies [6,8–13]. Silicone polymers are favorable to use in passive dosing because of the high diffusion coefficients of organic chemicals in silicone compared with other materials [14]. Different passive dosing approaches include casting silicone in vials [6], using o-rings [12], rods [11], or sheets [15]; and span from low-volume tests with cells in microwell plates [12] to 2-L tests with aquatic invertebrates and fish [16]. Passive dosing has also been applied in ecotoxicological tests with terrestrial organisms [17]. Less studied is the applicability of passive dosing in long-term tests for investigations of chronic toxicity, although a few recent studies demonstrate its potential [18,19]. In long-term incubations, problems arise with increasing levels of organic matter in the test medium as test organisms grow and produce metabolites during the test. In addition, organisms need to be fed and provided with clean water, which further complicates the possibility of maintaining stable exposure concentrations of the test substance.

This article includes online-only Supplemental Data.

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Published online 12 October 2016 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/etc.3649

The aim of the present study was to test the applicability of passive dosing to maintain stable concentrations of the bacteriocide triclosan in the water phase during a 6-wk population development test with *Nitocra spinipes*, a benthic harpacticoid copepod with well-documented biology and wide global distribution [20–22]. In ecotoxicology, it has been extensively used to study the effects of a wide range of environmental contaminants using physiological, developmental, and reproductive endpoints, as well as responses in population growth and genetics [23–26].

The chlorophenol triclosan is a broad spectrum antibacterial agent used in personal care, veterinary, and industrial products. It is not sufficiently removed during the wastewater treatment process and, as a result, is commonly detected in aquatic ecosystems in ng/L to low µg/L concentrations [27]. Moreover, there is strong evidence that aquatic species such as algae, invertebrates, and certain fish taxa are very sensitive to this chemical [28]. Recently, concerns were raised regarding microbiome-mediated effects of antibacterial substances in various animals, including arthropods that are particularly dependent on their microflora for feeding and growth [29]. These findings emphasize the relevance of chronic tests on triclosan toxicity for a sound risk assessment.

MATERIALS AND METHODS

Chemicals and materials

Silicone (1-2577 Conformal Coating, Dow Corning) was purchased through Elfa Distrelec AB. Methanol and n-hexane were acquired from Merck. Milli-Q grade water was generated using a Milli-Q water purification system (Merck Millipore). Triclosan (Irgasan DP300) was a gift from Ciba-Geigy, now Novartis, and ¹³C₆-Triclosan was acquired from ALSACHIM.

General test design

To test the chronic toxicity of triclosan, we used a multigeneration population development test with the crustacean *N. spinipes*. Based on the generation time of 15 d to 20 d for *N. spinipes* under the present experimental conditions [23], the population development test covers 2 to 3 generation cycles [30]. Specific details are provided in the section *Exposure test*. The test starts with a mixed population, whose development is followed by weekly observations, and ends after 5 wk to 6 wk when a steady state is reached. The organisms are fed with phytoplankton and the test medium is renewed regularly. To enable a stable exposure concentration of triclosan during these steps, 2 sets of exposure vials were used (Figure 1). One set was equilibrating with water while the other was in use. Each week when the organisms were counted, they were moved to the other set of vials. Thus, during the population development test, each set of vials was actively used in total for 3 wk and kept equilibrated, without animals and feed, for 3 wk.

Preparing silicone passive dosing phase in exposure vials

To prepare passive dosing vials, either 500 mg or 1000 mg of silicone were added to preweighed glass vials (10 mL or 24 mL), where it polymerized into a silicone elastomer. At purchase, the silicone was dissolved in approximately 30% solvent, which was accounted for when silicone was added to the vials. The final amounts of silicone after evaporation of solvent were 533 ± 19 mg and 1037 ± 17 mg, respectively. Vials were left for 48h in a fume hood before they were heated to 110 °C for 48h in an oven. After cooling to room temperature, the vials were rinsed 3 times with 5 mL of ethanol during a 48-h period. Then 5 mL of Milli-Q water were added to the vials and left for 24 h, after which the vials were dried through heating to 110 °C for approximately 2 h. All vials were then weighed, capped, and stored in darkness at room temperature until use.

Loading of triclosan into silicone

Loading of triclosan into the passive dosing silicone followed the procedure described by Birch et al. [13] with minor modifications. First, 50 µL methanol solution with triclosan were added to 10-mL vials. Then, 50 µL Milli-Q water were added repeatedly (4 times), after which 100 µL, 200 µL, and 300 µL Milli-Q water were added at 10 min intervals until the silicone was covered by water. To load the 24-mL vials, a larger volume of water was required to cover the silicone surface. Therefore all volumes of water and methanol were doubled and 600 µL water was added as a last step. The loaded vials were shaken for 17 h at 150 rpm. Then, 5 mL Milli-Q water were added to all vials, with subsequent shaking for 24 h at 170 rpm. After that, water was removed from the vials, and the silicon surface was wiped with lint-free tissue. All water equilibration steps of water with silicone during the exposure study were carried out by adding 10 mL of GF/C filtered seawater to the vials followed by shaking for 16 h at 150 rpm.

Determination of distribution ratio between silicone and water

Triclosan is a weak acid with a pKa of 8.1. Only the neutral form at the present pH in the exposure vials will distribute to silicone. Therefore the term distribution ratio ($D_{\text{silicone-water}}$) is used instead of partition coefficient to describe triclosan's partitioning between silicone and water. Silicone in 3 groups of vials was loaded with 3 different amounts of triclosan (in triplicates); approximately 700 µg, 2000 µg, and 6000 µg. Concentrations of triclosan in silicone (C_{silicone} µg/kg) and water (C_w ; µg/L) were determined after equilibration (see section *Determination of triclosan concentration in water and silicone* for description of the analytical work) and $D_{\text{silicone-water}}$ (kg/L) was calculated as the ratio between the 2

$$D_{\text{silicone-water}} = \frac{C_{\text{silicone}}}{C_w} \quad (1)$$

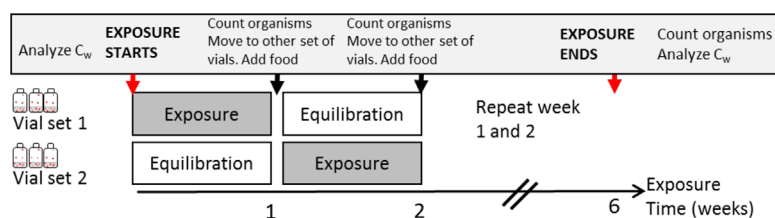


Figure 1. General work flow of the present exposure study to test the effects of triclosan on population development of *Nitocra spinipes* using passive dosing to maintain stable exposure concentrations.

Control of exposure concentration of triclosan

The stability of triclosan concentrations in the water phase, (i.e., the exposure concentration), was evaluated in several ways. Vials with triclosan-loaded silicone were equilibrated with water and left for 5 wk, (i.e., the approximate duration of the population development test). Thereafter, the triclosan concentration in the water phase was analyzed. This test demonstrates the stability of the system without any organisms added. A second 2-wk test was performed with 3 concentrations of triclosan in triplicate and organisms to test water concentration stability in the exposure scenario, (i.e., with periodical exchange of water) and increasing total biomass of copepods as a result of organism and population growth (i.e., increasing organic carbon content available for sorption). Two sets of vials were used, and each set of vials was extracted 3 times by equilibrating 3 sets of 10 mL Milli-Q water. This test was first performed with 500 mg silicone and then repeated with 1000 mg (at 1 triclosan concentration). Finally, triclosan concentration in the water phase was analyzed in both sets of vials, before and after the population development test.

The first 10 mL of water that were equilibrated with silicone after loading had approximately a 3 times higher triclosan concentration than the second and third volumes of water extracted (Supplemental Data, Table S1). This was tested using both 500 mg and 1000 mg of silicone casted in the vials. The ratio between concentrations in the second and first extraction was 2.9 and 3.3, respectively. We do not have an explanation for the approximately 3 times higher triclosan concentration in the first compared with the second extraction. To ensure a stable exposure concentration, after the loading of triclosan into the silicone, all vials were extracted with 10 mL of water, which was discarded, and then equilibrated a second time with 10 mL of water, which were used for the first week of exposure (Figure 1).

Determination of triclosan concentration in water and silicone

To analyze triclosan in the water phase, the water and animals were removed, the silicone surface was cleaned with a lint-free tissue and 10 mL of Milli-Q water was added and allowed to equilibrate with the silicone. Thereafter the water was analyzed. This procedure was used to capture the freely dissolved and thus effective concentrations of triclosan in the water phase, which does not include the fraction sorbed to organisms and other organic matter in the water phase. Subsamples of 1 mL, 2 mL, or 4 mL of water were used for chemical analysis, depending on the expected triclosan concentration. An internal standard (^{13}C -labeled triclosan) was added to the samples. Extraction was carried out by adding 2 mL of n-hexane, vortexing for 30 s followed by 3 min centrifugation (1500 rpm). The extraction was repeated and the extracts of each vial were pooled; the volume was adjusted to 300 μL through heating and applying a soft stream of nitrogen. The extract was transferred to 1.5-mL GC-vials with micro-inlets and weighed before the addition of a volumetric standard.

The silicone phase was extracted according to Smith et al. [6]. Briefly, methanol (5 mL for 500 mg, 10 mL for 1000 mg) was added to the vial and left to equilibrate for 24 h. The extraction step was repeated and the 2 methanol extracts for each vial were pooled. Two thoroughly weight-monitored dilutions based on the theoretical amount loaded into the silicon were carried out before addition of the internal standard.

All samples were run on a Thermo 1310 gas chromatograph coupled to a mass spectrometer (with a PTV-injector coupled to an ISQ LT; Thermo Fisher scientific) run in SIM

mode. A Thermo TG-5SILMS column (30 m \times 0.25 m inner diameter, 0.25 μm) was used and the sample injection volume was 1 μL . Selected ions were m/z 218, m/z 288, and m/z 290 for ^{12}C -triclosan and m/z 294 and m/z 296 for ^{13}C -triclosan. The column heat ramp was from 45 $^{\circ}\text{C}$ to 280 $^{\circ}\text{C}$ with a 15 $^{\circ}\text{C}$ increase per minute. The signal integration and quantification of the results were based on a 6-point calibration curve ($R^2 > 0.992$) using the software Xcalibur Ver 2.2 (Thermo Fisher scientific). The mean recovery of the triclosan analyses in water was $72 \pm 21\%$. No triclosan was detected in any of the blank or control samples.

Exposure study

The treatments included control and 3 target triclosan concentrations (15 $\mu\text{g/L}$, 30 $\mu\text{g/L}$, 60 $\mu\text{g/L}$) each in triplicate. The test concentrations were selected based on a pilot study on larval development (see Supplemental Data). The exposure vials (pre-equilibrated vials with GF/C-filtered natural brackish water, salinity 6 ‰, from Brunnsviken, Baltic Sea) were prepared with 3 individuals of each of 3 life stages of *N. spinipes*: nauplii (larvae), copepodites (juveniles), and adults (males and ovigerous females carrying egg sack). The test vials were kept in darkness at $22.5 \pm 1^{\circ}\text{C}$, in a MIR-154-PE cooled incubator (Panasonic) throughout the exposure period (pH 7.8). To avoid buildup of organic matter, the water phase was completely renewed once every week. At the same time, the number of animals in each life stage was recorded. Copepods were transferred to Petri dishes, counted using a Leica MZ6 light microscope (Leica Microsystems), and transferred to equilibrated vials from a new set. Feeding was carried out after each transfer (as well as at the start of the test) by adding *Rhodomonas salina* corresponding to a final concentration of 5×10^7 cells/mL. Algal concentrations were measured using Z2TM Coulter Counter (Beckman Coulter). After the sixth week of exposure the animals were counted for the last time and incubations were terminated.

Analysis of ecotoxicological data and statistics

Abundance of the age classes (nauplii, copepodites, and adults) and total population size (individual/replicate) were used as response variables when evaluating effects of time and triclosan concentration. Using the abundance of each age class, we also calculated a development index (DI), which incorporates both survival and metamorphosis success in copepods:

$$DI = \frac{\sum k_i \times n_i}{NS} \quad (2)$$

where k_i is the assigned stage value (in the present study: 1 for nauplii, 2 for copepodites, and 3 for adults), n_i is the number of copepods at that stage, and NS stands for the total number of individuals [31]. This index reflects the demographic structure of the population and has been found useful for evaluating effects of toxic food on copepods [32]. To further characterize the demographic structure, we also calculated the sex ratio in each population as percentage of females in the well-established populations (i.e., after 28 d of incubation; this period was sufficient to avoid stochastic fluctuations related to low population size).

Using a restricted maximum likelihood unequal variance mixed model with random intercepts to decompose the variances and derive parameter estimates, we estimated the variance in the abundance of juvenile age classes (nauplii and copepodites), adults (females and males), and total population

size due to 1 random effect—experimental container (“tank”), and 2 fixed effects—time (weekly observations over 6 wk) and triclosan concentration (measured values 4 µg/L, 9 µg/L, and 21 µg/L corresponding to target concentrations 15 µg/L, 30 µg/L, and 60 µg/L; Table 1). The random effect was always retained in the model while evaluating the fixed effects, because the containers were part of the experimental design as a measure to capture environmental variation due to intrapopulation stochastic effects, rather than an explicit focus of the study. Therefore, testing whether the containers were significantly different from each other within a treatment was irrelevant in this case. To evaluate fixed effects and their interactions with sex ratio, a generalized linear model (GLM) was used, because “tank” effect was never significant for this variable. All abundance endpoints fulfilled assumptions of normality of variances after Box-Cox transformation; no transformation was necessary for development index and sex ratio values. The analyses were performed in S-PLUS 8.0 (TIBCO Software). Descriptive statistics are given as means and standard deviations (SDs) unless otherwise stated; significance level was set at $\alpha = 0.05$.

Mass balance calculation

A mass balance calculation of triclosan in the exposure unit was performed to confirm that the silicone contained enough triclosan to compensate for losses due to sorption to organic carbon. A dry weight of 5 µg per individual and an organic

carbon content of 50% was used [33–35]. The organic carbon content per vial used for the mass balance was based on 500 individuals, to simulate conditions with high organic carbon content [30]. The fraction of triclosan in the organic carbon phase (f_{oc}) was calculated as

$$f_{oc} = \frac{C_{oc} \times M_{oc}}{C_{oc} \times M_{oc} + C_{silicone} \times M_{silicone} + C_w \times V_w} \quad (3)$$

where C_{oc} is the concentration of triclosan in organic carbon (µg/kg), M_{oc} is the amount of organic carbon in the system (kg), $C_{silicone}$ is the concentration of triclosan in silicone (µg/kg), C_w is the concentration of triclosan in the water phase (µg/L), and V_w is the volume of water (L). Replacing C_{oc} by $D_{oc} \times C_w$ (where D_{oc} is the organic carbon to water distribution ratio) and $C_{silicone}$ by $D_{silicone-water} \times C_w$, and rearranging gives

$$f_{oc} = \frac{D_{oc} \times M_{oc}}{D_{oc} \times M_{oc} + D_{silicone-water} \times M_{silicone} + 1 \times V_w} \quad (4)$$

The D_{oc} was calculated from K_{oc} [36] and $D_{silicone-water}$ was from the present study.

RESULTS AND DISCUSSION

Loading efficiency and $D_{silicone-water}$

Loading efficiency of triclosan on the silicone phase casted in the test vials was $82 \pm 6\%$ (average \pm SD). The log $D_{silicone-water}$ for triclosan was 4.0 ($D_{silicone-water}$ 10466 \pm 1927). We are not aware of any previously published distribution ratios for triclosan between silicone and water that can be used for comparison.

Stability of exposure concentration

The initial study with vials casted with silicone and loaded with triclosan demonstrated stable concentrations in the water phase during 35 d (Supplemental Data, Figure S1; slope of the regression not significantly different from 0). The next step was to test if the silicone phase may compensate for losses due to sorption of triclosan to organic matter. After the first extraction, the exposure concentrations were confirmed for 2 more water exchanges in each vial set (Supplemental Data, Table S4). Concentrations were kept stable during the 2 extractions in vial set 2 (t -test; $p > 0.05$), whereas they decreased in vial set 1 (t -test; $p < 0.05$). These tests were performed with 500 mg silicone as passive dosing phase. To maintain a stable exposure concentration over 3 extractions, the amount of silicone was increased to 1000 mg. When the test was repeated using 1000 mg, the triclosan concentration remained stable between extraction number 2 and 3 (t -test; $p > 0.05$; Supplemental Data, Table S1). Based on this, each set of vials was used for 3 wk, allowing for a total exposure time of 6 wk. During the population development test, triclosan concentrations were analyzed in the first 10 mL of water equilibrated with silicone, which were not used for exposure, and after the termination of the exposure. As expected based on the initial findings, concentrations at the end of the experiment were approximately 3 times lower than in the first extraction (factor 2.6–3.1 in vial set 1 and factor 3.5–4.7 in vial set 2; Table 1). The mass balance calculation demonstrated that with a population of 500 individuals in each vial, the fraction of triclosan sorbed to organic matter (f_{oc}) is approximately 0.1%. This is in line with what is normally observed in passive dosing studies [13].

Table 1. Exposure concentrations (µg/L) of triclosan in the population development test^a

	Before exposure conc µg/L	End of exposure conc µg/L
Set 1	13	4.7
	17	4.6
	14	5.0
Average \pm SD	15 \pm 2	4.8 \pm 0.2
RSD (%)	16	4
	32	11
	29	12
	34	10
Average \pm SD	32 \pm 3	11 \pm 1
RSD (%)	9	7
	76	27
	79	27
	55	25
Average \pm SD	70 \pm 13	26 \pm 1
RSD (%)	19	5
Set 2	15	3.1
	16	3.3
	15	3.5
Average \pm SD	16 \pm 1	3.3 \pm 0.2
RSD (%)	5	6
	33	6.2
	32	7.0
	7	7.1
Average \pm SD	24 \pm 15	6.7 \pm 1
RSD (%)	62	8
	62	17
	78	16
	66	16
Average \pm SD	69 \pm 8	16 \pm 1
RSD (%)	12	4

^aConcentrations are displayed for the first 10 mL of water equilibrated with silicone (pre-exposure; water discarded and not used for exposure), and after termination of the exposure. conc = concentration; SD = standard deviation; RSD = relative standard deviation.

Exposure concentrations

The relative standard deviation of triclosan concentrations among the triplicates varied between 9% and 19% for vial set 1 and between 5% and 62% for vial set 2 before the start of the experiment (water not used), and between 4% and 8% for both sets of vials at the end of the experiment (Table 1). The low relative SD values (<19%) for all but 1 measured concentration both before and after the 6-wk exposure demonstrate reproducibility of exposure concentrations. The tested exposure concentrations were 3 µg/L to 5 µg/L, 7 µg/L to 11 µg/L, and 16 µg/L to 26 µg/L (Table 1). These concentrations were approximately 3 times lower than the target concentrations, due to the decrease with a factor of 3 from the first to the second extraction (as described in the section *Control of exposure concentration of triclosan*, Supplemental Data, Table S1).

Effects of triclosan on population development

In all treatments, copepod abundance increased over the exposure period, with the increase being more pronounced for juveniles than for adults (Figure 2 and Supplemental Data, Figure S2). In line with this increase, the effect of time was significant in all models (Table 2); moreover, the significant time × concentration interaction effect in all models implies that the relationship between abundance and time varied with exposure concentration. Because the main effect concentration was significant for copepodites only indicating that during this part of development the negative effect on survival was concentration-dependent on each observation occasion (Table 2). As alternative explanations, retarded naupliar development and/or enhanced maturation rate can be put forward, because both processes would decrease the copepodite abundance. However, they would also result in a synchronous accumulation of nauplii or adults, respectively. Hence, negative correlations between copepodites and these age classes should have occurred; this, however, was not the case (Supplemental Data, Figure S2; Spearman ρ : copepodites vs nauplii 0.27, copepodites vs adults 0.35; $p < 0.05$).

Analysis of the demographic structure using development index dynamics also suggests the most severe effects being exerted on the juvenile development during the first 4 wk of the exposure (Figure 3). Indeed, the progressively lower development index values in the populations exposed to increasing triclosan concentrations imply developmental retardation (Figure 3A). The shift in the development index values toward younger life stages cannot be attributed to enhanced birth rates because nauplii abundance was relatively unaffected during this period and at lower test concentrations (Figure 3 and Supplemental Data, Figure S1). The most influential parameter for development index dynamics was the decline in the percentage of copepodites, with an approximately 2-fold difference between the average values in the control (~80%) and the highest triclosan concentration (~40%). Together with the restricted maximum likelihood unequal variance models, the development index dynamics indicate that copepodites were more susceptible to triclosan than nauplii, whereas effects on the adults were not possible to separate, because they were intrinsically linked to the survival and metamorphosis of the copepodites. Moreover, no significant differences were found for sex ratio (GLM; concentration × time × sex interaction effect, Wald statistic = 0.112, $p > 0.7$) suggesting that the concentration effects were not sex specific but related to a common biological mechanism.

What can this mechanism be? We suggest that triclosan may have detrimental effects on the microbiota of copepods, which

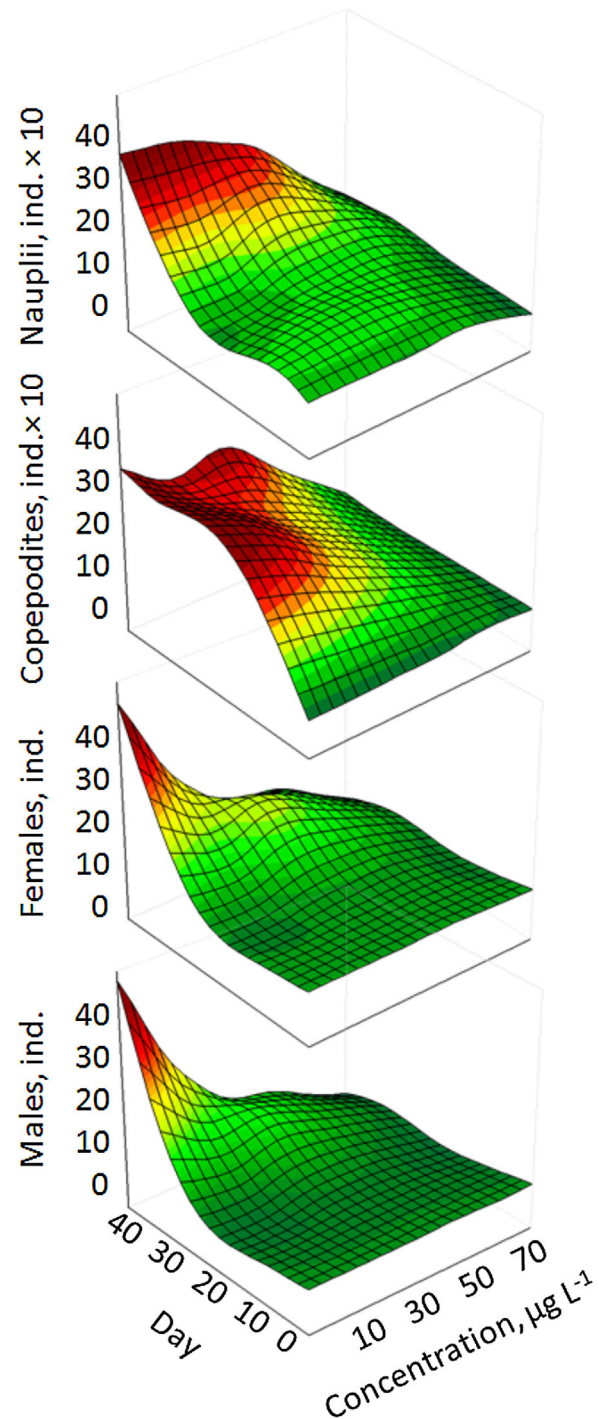


Figure 2. Changes in abundance of nauplii, copepodites, adult females, and adult males (top to bottom) of *Nitocra spinipes* exposed to triclosan (0–21 µg/L) over 42 d in the test system. Distance-weighted least squares method was used to fit the data. Note that reverse direction on the Day-axis is used to show that abundance of all categories increases with time and decreases with triclosan concentration.

results in disrupted host-microbiome functioning, decreased food assimilation, and growth. By this mechanism, nauplii would most probably be less affected than copepodites because they rely much less on the external feeding [37] compared with the latter, which gradually acquire gut microflora to facilitate digestion [38]. Exposure to antibiotics has been shown to decrease both diversity of bacterial communities associated with copepodites and development rate in *N. spinipes* [29]. In

Table 2. Restricted maximum likelihood output for the models testing effects of time elapsed from the start of the experiment (day) and measured triclosan concentration ($\mu\text{g/L}$; Table 1) on the abundance of specific age classes as well as total population size of *Nitocra spinipes*^a

Dependent variable	Predictors	Numerator <i>df</i>	Denominator <i>df</i>	<i>F</i> ratio	<i>p</i> -value
Nauplii	Concentration	3	8	1.639	0.256
	Time	6	48	23.62	<0.000*
	Concentration \times time	18	48	2.828	0.002*
Copepodites	Concentration	3	8	48.27	<0.000*
	Time	6	48	25.61	<0.000*
	Concentration \times time	18	48	5.285	<0.000*
Females	Concentration	3	8	2.704	0.116
	Time	6	48	16.14	<0.000*
	Concentration \times time	18	48	3.147	0.001*
Males	Concentration	3	8	3.111	0.089
	Time	6	48	9.625	<0.000*
	Concentration \times time	18	48	2.539	0.006*
Total	Concentration	3	8	12.40	0.003*
	Time	6	48	24.17	<0.000*
	Concentration \times time	18	48	2.294	0.012*

^a*Nitocra spinipes* were exposed to triclosan (0–21 $\mu\text{g/L}$) over 42 d in the test system. Specific age classes included juveniles (nauplii and copepodites) and adults (females and males). Population size expresses as individual replicate⁻¹. Tank was a random effect in each model; see Supplemental Data, Figure S2 for the data structure.

*Significant *p*-values.

df = degree of freedom.

turn, the antibiotic-induced decline in gut microflora has been shown to compromise feeding digestion and assimilation in similarly sized cladoceran *Daphnia magna* [39]. Because triclosan is an antibacterial agent, this mode of action would also explain the severe effects on the growth and development in microflora-dependent copepodites and less pronounced effects on nauplii.

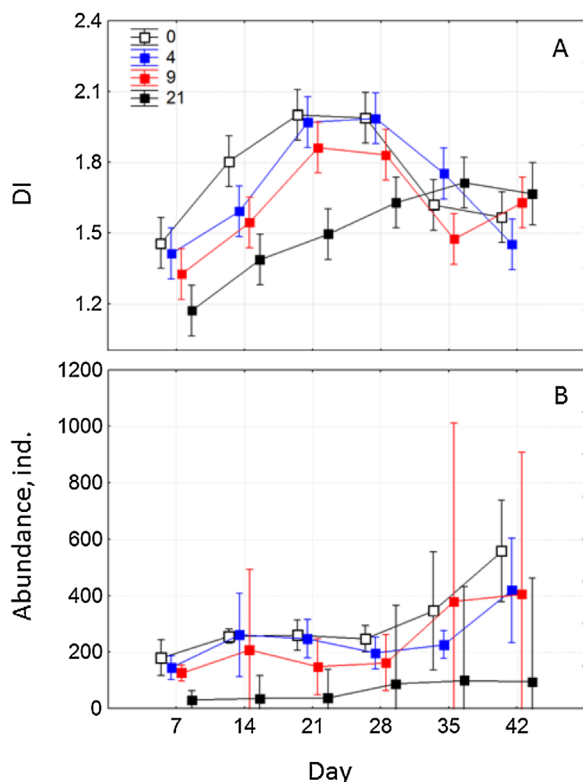


Figure 3. Population structure expressed as development index, (DI), (A) and total population abundance (B) of *Nitocra spinipes* exposed to triclosan (0–21 $\mu\text{g/L}$, indicated by color code as shown in the legend) over 42 d in the test system. Data are mean with 95% confidence interval. First day is omitted from the analysis because all values were set by experimenter and identical among the treatments.

It is also important to point out that for the exposure period of 2 wk to 4 wk, development index was the most sensitive endpoint due to the lowest between-replicate variability and no significant “tank” effect (Figure 3A) as compared with the total abundance data (Figure 3B). Beyond this period, development index decreased in all treatments, except the highest triclosan concentration, because of a large influx of nauplii produced by the cohort that was born in the first week of exposure and reached maturation in 3 wk to 4 wk from the birth (Figure 3, Supplemental Data, Figure S2). Together with stochastic population effects propagating in each container over time, this also resulted in the increased variances, both for population abundance and development index values. Therefore, in this test system, the optimal exposure period should not exceed 4 wk.

Even for this most sensitive endpoint, the concentration effect was not significant until after 7 d of the exposure in 21 $\mu\text{g/L}$ and after 14 d in 4 $\mu\text{g/L}$ to 9 $\mu\text{g/L}$ triclosan as suggested by nonoverlapping confidence intervals for mean values (Figure 3A). We are not aware of any published data on triclosan toxicity for *N. spinipes* or any other harpacticoids. However, published median effective concentration values for the crustacean *Daphnia* are in the range of 200 $\mu\text{g/L}$ to 400 $\mu\text{g/L}$; these were obtained in 24 h acute toxicity to 8 d subchronic toxicity tests [40–42]. The copepod populations in the present study were close to extinction in 2 out of 3 experimental replicates exposed to 21 $\mu\text{g/L}$ at the end of the experimental period, whereas development inhibition was observed at as low as 4 $\mu\text{g/L}$ after a period that was sufficient for completing juvenile development [23,24]. Therefore, for test organisms with complex life cycles, like copepods, it is important to include different life stages in toxicity testing and design long-term tests ensuring stable exposure concentrations and allowing detection of stage-specific chronic effects.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3649.

Acknowledgment—The present study was funded by basic faculty funding at the Department of Environmental Science and Analytical Chemistry. A. Sobek was funded by the Swedish Research Council for the Environment (Formas Project Grant #2012-1211).

Data availability—Data are available on request to the authors (anna.sobek@aces.su.se).

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