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Multigenerational effects of tris(1,3-dichloro-2-propyl) phosphate on the free-living ciliate protozoa *Tetrahymena thermophila* exposed to environmentally relevant concentrations and after subsequent recovery☆

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ABSTRACT

Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) is considered a re-emerging environmental pollutant, and exposure to environmentally relevant concentrations has been shown to cause individual developmental toxicity in zebrafish and the water flea (*Daphnia magna*). However, multigenerational effects during exposure to TDCIPP and after subsequent recovery were unknown. In the present study, individuals of a model aquatic organism, the ciliated protozoan, *T. thermophila* were exposed to environmentally-relevant concentrations of TDCIPP (0, 300 or 3000 ng/L) for 60 days (e.g., theoretically 372 generations) followed by a 60-day period of recovery, during which *T. thermophila* were not exposed to TDCIPP. During exposure and after exposure, effects at the molecular, histological, individual and population levels were examined. Multigenerational exposure to 300 or 3000 ng TDCIPP/L for 60 days significantly decreased numbers of individuals, sizes of individuals, expressed as length and width of bodies, number of cilia, and depth and diameter of basal bodies of cilia, and up-regulated expressions of genes related to assembly and maintenance of cilia. Complete or partial recoveries of theoretical sizes of populations as well as sizes of individuals and expressions of genes were observed during the 60-day recovery period. Effects on number of cilia and depth and diameter of basal body of cilia were not reversible and could still be observed long after cease of TDCIPP exposure. Collectedly, and shown for the first time, multigenerational effects to *T. thermophila* were caused by exposure to environmentally relevant concentrations of TDCIPP. Also, there were multi-generational effects at the population level that were not caused by carry-over exposure to TDCIPP. The “permanent” alterations and their potential significance are discussed.

1. Introduction

Organophosphate esters (OPEs), and particularly OP triesters, are high production volume chemicals that have been in use since the 1970s (van der Veen and de Boer, 2012). OP triesters are used as flame retardants (FRs), plasticizers and as performance additives to engine oils, and found in hydraulic oils plastics, foams, textiles, floor

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polishes, waxes and furniture. In recent years production and use of some OP triesters have been increasing and coincident with the regulation and phase-out of some brominated FR substances such as polybrominated diphenyl ethers (PBDEs) (van der Veen and de Boer, 2012). Production volumes for the OPEs, tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), triphenyl phosphate (TPHP), and tris(2-chloroisopropyl) phosphate (TCIPP), in the United States increased from less than 14,000 metric tonnes per year in the mid-1980s, to 5000–25,000 metric tonnes in 2006, and to approximately 38,000 metric tonnes per year in 2012 (Schreder et al., 2016).

OPEs are not chemically bonded to the related products and thus they are more likely to be released to the environment (van der Veen and de Boer, 2012). It has been reported that OPEs are frequently measured in various environmental media and biota (Sundkvist et al., 2010; Carignan et al., 2013; Meeker et al., 2013). Recently, TDCIPP has been identified as one of the primary OPEs, and frequently detected in indoor air, dust, natural waters, and tissues of wildlife and human (van der Veen and de Boer, 2012). For example, in effluents from sewage treatment plants in Sweden, TDCIPP has been reported to occur at concentrations as great as 3 μg/L (Marklund et al., 2005). In natural waters, TDCIPP has been detected at sub-ppb (<μg/L) concentrations and the maximum concentration ever reported is 377 ng TDCIPP/L in seawaters along the coast of China near the cities of Qingdao and Xiamen (Hu et al., 2014). Furthermore, TDCIPP has also been detected in yellow perch (*Percafluviatilis*) from Djupasjön Lake in Sweden, at concentrations as great as 140 ng/g lipid weight (Sundkvist et al., 2010). In China, up to 251 ng TDCIPP/g lipid weight was detected in catfish (*Clariusfuscus*) and grass carp (*Cyprinusidellus*) from the Pearl River (Ma et al., 2013).

Published data suggest that exposure to TDCIPP causes various toxicities depending on organisms tested, such as endocrine disruption (Kojima et al., 2013; Liu et al., 2013; Zhang et al., 2014; Wang et al., 2015a), neural toxicity (Dishaw et al., 2011, 2014; Ta et al., 2014; Wang et al., 2015b, 2015c), hepatoxicology (Crump et al., 2012; Farhat et al., 2013, 2014a, 2014b; Liu et al., 2016) and developmental and reproductive toxicity (Liu et al., 2012; Liu et al., 2013; McGee et al., 2012; Farhat et al., 2013; Fu et al., 2013; Wang et al., 2015a). For example, acute exposure to TDCIPP affects embryonic development by delaying remethylation of the zygotic genome and embryonic epiboly and results in significantly greater mortality in embryos of zebrafish (McGee et al., 2012; Fu et al., 2013). Exposure to TDCIPP decreased lengths of head and bill, masses and size of gallbladder in embryos of chicken (Farhat et al., 2013, 2014a, 2014b). Using the model aquatic organism and ciliated protozoa *T. thermophila*, it has been found that acute exposure to TDCIPP decreases biomass by reducing number of cells, size of cells and quantity of cilia (Li et al., 2015b). Furthermore, two studies have reported that chronic exposure to relatively small concentrations of TDCIPP resulted in bioconcentration of the chemical, which resulted in adverse effects on reproduction and developmental of F_0 zebrafish (Wang et al., 2015b, 2015c); TDCIPP was also detected in F_1 eggs following parental exposure, causing developmental neurotoxicity in F_1 larvae of zebrafish (Wang et al., 2015b). Recently, effects of TDCIPP on development have been evaluated in zebrafish and the water flea (Crustacea; *Daphnia magna*) exposed to environmentally relevant concentrations (Li et al., 2015a; Zhu et al., 2015). Exposure of zebrafish to environmentally relevant concentrations of TDCIPP for 120 days significantly down-regulated expressions of genes included in the growth hormone/insulin-like growth factor (GH/IGF) axis, and resulted in female-biased inhibition of growth (Zhu et al., 2015). Treatment with 65 or 550 ng TDCIPP/L for 28 days resulted in lesser length of F_0 and first generation (F_1) *Daphnia magna*, and down-regulation of genes involved in synthesis of proteins and metabolism and endocytosis pathways might be responsible for the observed developmental inhibition (Li et al., 2015a).

While natural populations can be exposed to toxicants over several generations, evaluation of multigenerational effects has seldom been done. In this study, *T. thermophila* was used as a model to investigate multigenerational effects of exposure to environmentally relevant concentrations of TDCIPP at the individual and population levels. Effects during and after subsequent recovery were evaluated. *T. thermophila* was selected because it has a short generation time (around several hours depending on culture condition and seeding density), which allows several hundreds of generations to be produced within several weeks.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals and reagents were purchased from the following sources: TDCIPP was from TCI Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and a stock solution was prepared in dimethyl sulfoxide (DMSO); TRIzol reagent was from Invitrogen (NJ, USA); PrimerScript™ RT reagent Kit (Perfect Real Time) and SYBR® Green Premix Ex Taq™ ‖ (TilRNaseH plus) were from Takara (Liaoning, China); Penicillin G, streptomycin sulfate and amphotericin B were from Sigma (MO, USA); Proteosepeptone was from BactoDifco (USA); Yeast extract was from Oxioid Ltd. (Basingstoke Hampshire, England); D (+) glucose was from Biosharp (USA); Ethylendiaminetetraacetic acid monosodium ferric salt (Fe-EDTA) was from Aladdin (Shanghai, China).

2.2. Culturing of T. Thermophila and growth curves

T. Thermophila SB210 was purchased from Tetrahymena Stock Centre, Cornell University, New York, USA, and was cultured in super proteose peptone (SPP) medium (pH 7.0) at 30 °C with shaking at 135 rpm as described before (Li et al., 2015b). SPP medium included 2% proteose peptone, 0.1% yeast extract, 0.2% D (+) glucose, 0.003% Fe-EDTA, 100 units/mL penicillin G, 100 mg/L streptomycin sulfate and 0.025 mg/L amphotericin B. In order to obtain appropriate seeding density for the subsequent multigenerational experiment, growth curves were produced. Cells that grew to mid-logarithmic phase were inoculated into new SPP media in triangular flasks, and a total of six seeding densities were included. Cells were cultured at 30 °C with shaking at 135 rpm, and during culture period cell densities were determined using haemocytometer as a previous description (Li et al., 2015b).

2.3. Protocols for multigenerational experiment

Based on preliminary growth curves, a seeding density of 2×10^4 cells/mL was selected for use in the multigenerational experiment, which consisted of two phases: 60-day exposure and 60-day recovery. An exposure period of 60 days was selected since *T. thermophila* has a short generation time, which allows several hundreds of generations to be produced within the exposure and recovery periods. Briefly, cells were seeded at a density of 2×10^4 cells/mL and exposed to 0, 300 or 3000 ng/L TDCIPP for 24 h. After that, cell density in the control and exposure groups was determined, and the cells inoculated into new control media or exposure media containing the corresponding concentration of TDCIPP at a density of 2×10^4 cells/mL and the exposure was continued for another 24 h. This process was repeated until the end of exposure duration of 60-day. Both control and exposure groups received 0.1% DMSO, which did not cause multigenerational

toxicity of *T. thermophila* since, after seeding for 24 h, the densities of *T. thermophila* from growth curve with seeding density of 2×10^4 cells/mL and control group were comparative in this study. After exposure, recovery was studied by transferring cells to medium in the absence of TDCIPP. Inoculation protocol was the same as that used during the exposure to TDCIPP. Briefly, cell density in the pre-control group was determined and cells were inoculated into fresh medium without DMSO at a density of 2×10^4 cells/mL. For pre-exposure groups, the same density of cells to the pre-control group was inoculated into fresh medium without TDCIPP and DMSO, and the recovery was continued for another 24 h. This process was repeated until the end of recovery duration of 60-day. Cells were collected after 30-, 60-, 90- or 120-day exposure or recovery to examine effects on body size, cilia quantity, cilia ultrastructure and gene expression. During the period of exposure and recovery, densities of cells before inoculation were determined every 24 h for calculation of theoretical populations (Equation (1)).

$$Pn = Dn \times Vn \times \prod_{k=0}^{n-1} \frac{Dk}{2 \times 10^4} \quad (1)$$

where P is theoretical population; D is the density of *T. Thermophila*; V is the volume of medium; n is the day when theoretical population is calculated ($n \geq 1$).

2.4. Quantification of TDCIPP in culture medium or cells

TDCIPP in culture medium was extracted by use of liquid-liquid extraction (LLE). In brief, an aliquot of 0.6 mL of medium was transferred into a disposable, glass culture tube (16 × 125 mm). A volume of 20 μL of internal standard (50 ng/mL in methanol), d_{15}-TDCIPP, was spiked into the sample and mixed well by use of a vortex mixer. Then, an aliquot of 2 mL of hexane was added to the tube, and mixed with vortex for approximate 1 min. The vortexed mixture was then placed into an ultrasonic-cleaner (1.9 L, 35 kHz, 140 W from VWR, Mississauga, Canada), and ultrasonicated for 10 min at room temperature. Then, the mixture was centrifuged for 5 min to separate the two layers of aqueous medium and hexane solvent, and the upper hexane layer was carefully transferred to a new disposable culture tube. The ultrasonic extraction process (with hexane) was repeated two more times, and the collected hexane layer extracts were combined. Then, the solvent in the combined extract were evaporated under a steam of nitrogen to dryness, and 200 μL MeOH was added into the tube. The tube was mixed by vortex mixing for 10 s, and transferred into a LC vial for instrument analysis.

Details on the treatment of samples of cells and parameters for instrumental conditions can found in previously published reports (Su et al., 2014; Chu and Letcher, 2015). In brief, collected samples of cells were carefully transferred into disposable, glass culture tubes, which were further added with a volume of 4 mL of 50/50 (v/v) dichloromethane/hexane extraction solvent, 20 μL of d_{15}-TDCIPP internal standard (50 ng/mL), 0.2 g sodium chloride (NaCl) and 1.2 g anhydrous magnesium sulfate ($MgSO_4$), respectively. The mixture in tube was further vortexed for approximate 1 min, ultrasonicated in the ultrasonic-cleaner for 10 min at room temperature, and centrifuged for 5 min. The upper solvent layer was transferred to a new tube. The sample of cells was extracted following a same ultrasonic extraction process for other two more times. The collected extraction solvents were gently evaporated under a steam of nitrogen to dryness, and 1 mL MeOH was added to the tube. The re-dissolved extract was further cleaned-up with a 300 mg aliquant of PSA bonded silica, and ready for instrumental analysis. Quantification of TDCIPP in extracts was performed by use of a Waters XEVO-TQ-S ultra-high performance liquid chromatography-tandem mass spectrometer (UPLC-MS/MS) with an atmospheric pressure chemical ionization source (APCI) (Waters Limited, Milford, MA, USA) operated in positive mode, as detailed elsewhere (Su et al., 2014; Chu and Letcher, 2015).

During quantification of TDCIPP in medium or samples of cells, there were unavoidable background contamination with an average of 0.11 ± 0.02 ng TDCIPP/medium sample or 0.18 ± 0.03 ng TDCIPP/cell sample. In the present study, reported concentrations are corrected by subtraction of background concentrations. Method limits of detection (MLODs) for TDCIPP were defined as three times the standard derivation of measurements in all controls, and thus were 0.06 ng/medium sample and 0.09 ng/cell sample. Based on its internal standard, d_{15}-TDCIPP, mean recoveries of TDCIPP were 81 ± 10% for all medium samples and 87± 26% for all samples of cells.

2.5. Transmission electron microscopy (TEM) imaging

TEM imaging was performed in Wuhan Regional Centre of Life Science Instrument, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China, by use of previously described methods (Li et al., 2015b). Briefly, cells were collected and fixed in 2.5% glutaraldehyde. After that, cells were scraped, pelleted, dehydrated, infiltrated and embedded, and then ultrathin sections were obtained and stained. Hitachi HT7700 TEM was used for imaging of cilia ultrastructure.

2.6. Quantitative real-time polymerase chain reaction (RT-qPCR)

RT-qPCR was performed using previously described methods and met requirements for minimum information for publication of quantitative real-time PCR experiment (MIQE) guidelines (Bustin et al., 2009). Briefly, total RNA was isolated using TRIzol reagent following the manufacturer's instructions, and purities of RNA were determined by measuring 260/280 nm ratios and then concentrations of RNA were measured using an Epoch Microplate Spectrophotometer (Bio Tek Instruments, Inc., Winooski, VT, USA). Reverse transcription and RT-qPCR were performed using PrimerScript™ RT reagent (Perfect Real Time) and SYBR® Green Premix Ex Taq™ II (TilRNaseH plus) kits. Sequences of genes were obtained from Tetrahymena Functional Genomics Database (http://tfgd.ihb.ac.cn/) and primers were designed using Primer 3 software (http://frodo.wi.mit.edu) (see Table S1). Expressions of ten genes, which are responsible for assembly and maintenance of cilia (Seixas et al., 2003, 2010; Avasthi and Marshall, 2012; Lechtreck, 2015), were examined. Expressions of three genes (chlamydial polymorphic outer membrane protein repeat containing protein, ubiquitin carboxyl-terminal hydrolase family protein and Cysteine proteinase 3 precursor were not changed upon exposure to TDCIPP so they were used as housekeeping genes to normalize for variations among quantifications. Thermal cycling was set at 95 °C for 15 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative gene expression to control was calculated by the $2^{-\Delta\Delta CT}$ method. Three biological replicates were included in each concentration.

2.7. Statistical analyses

Statistical analyses were performed by Kyplot Demo 3.0 software (Tokyo, Japan). Normality and homogeneity assumptions were examined by the Kolmogorov-Smirnow test and Levene's tests, respectively ($P < 0.05$). One-way analysis of variance (ANOVA) followed by Tukey's multiple range test were used to determine significant differences between the control and exposure/recovery groups. Significant differences between the control and exposure/recovery groups were determined by $P < 0.05$.

3. Results

3.1. Growth curves

Six growth curves of *T. thermophila* were produced in this study, and for the two higher seeding densities (1.0×10^5 and 5×10^4 cells/mL), cell densities reached platform stage by 24 h (Fig. 1). Therefore, a seeding density of 2×10^4 cells/mL was selected for subsequent multigenerational experiments since exposure and control media were renewed every 24 h during the study. Additionally, according to the produced growth curve with seeding density of 2×10^4 cells/mL, 6.2 generations of *T. thermophila* would be produced within 24 h, which was calculated using formula of "$G = \log(2)N_{24}/N_0$, (G: generations; N_{24}: populations at 24 h; N_0: populations at 0 h)". Therefore, the 60-day exposure experiment in this study means that *T. thermophila* were exposed to TDCIPP for 372 generations.

3.2. Concentrations of TDCIPP in media and cells

Continuous monitoring of concentrations of TDCIPP was conducted for both exposure media and cells collected during exposure (Table 1). Concentrations of TDCIPP were only detectable in samples collected during exposure (i.e. 30 and 60 days) or very early period of recovery (i.e. 61 day). Specifically, in the treatment exposed to 300 ng/L, concentrations of TDCIPP were 226.60 ± 0.03 and 303.70 ± 0.03 ng/L in samples of media collected at 30 and 60 day, respectively. Concentrations of TDCIPP in cells collected at 30 and 60 day were 2.70 ± 0.67 and 4.12 ± 2.64 ng/g (wet weight of cells), respectively. TDCIPP was not detectable in any medium or cells collected at 61 or later.

In medium with a nominal concentration of 3000 ng TDCIPP/L, measured concentrations of TDCIPP were 2688.89 ± 0.03 and 2376.56 ± 0.48 ng/L in medium collected at 30 and 60 day, respectively. Amounts of TDCIPP in cells collected at 30 and 60 day were 18.20 ± 6.26 and 29.56 ± 4.13 ng/g (wet weight of cells), respectively. Small concentrations of TDCIPP were also detected in medium (70.87 ± 0.04 ng/L) or cells (1.52 ± 0.01 ng/g) collected at 61 days, one day after nominal exposure to TDCIPP was stopped, after which no TDCIPP was detected in media or cells.

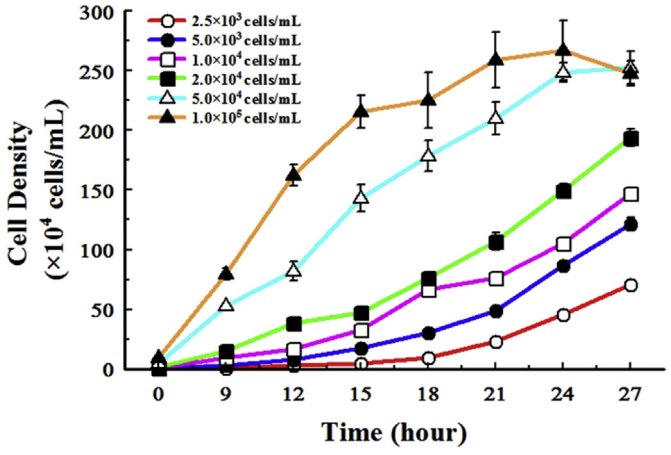

Fig. 1. Growth curves based on numbers of cells. Values are the mean ± SEM (n = 6).

3.3. Effects on theoretical populations

Exposure to 300 or 3000 ng/L TDCIPP caused a time- and dose-dependent decrease in theoretical populations compared with the control (Fig. S1A, see Supporting Information) with first significant effects observed after 5-day exposure (Fig. 2). During the recovery period, no significant effects on theoretical populations were observed due to pre-exposure to different concentrations of TDCIPP (Fig. S1B, see Supporting Information and Fig. 2).

3.4. Effects on body length and body width

Exposure to 300 or 3000 ng/L TDCIPP for 30 days significantly decreased body length of *T. thermophila* by 12.5% and 17.0%, respectively compared with the control, and a similar effect was also observed after 60-day exposure. In recovery period, a significantly decreased effect on body length was also observed due to pre-exposure to TDCIPP, but the effect was slightly less compared with that during the exposure to TDCIPP (Fig. 3A).

Body width of *T. thermophila* was significantly less by 10.6% and 14.0% after exposure to 300 or 3000 ng/L TDCIPP for 30 days, respectively compared with the control. Prolonged exposure (60 days) caused a similar effect. A slight increase in body width at 90 and 120 day was observed compared with that at 60 day, but effects on body width were still statistically significant compared with the control in recovery period (Fig. 3B).

3.5. Effects on number of cilia

Exposure to 300 or 3000 ng/L TDCIPP for 30 days significantly decreased total number of cilia of *T. thermophila* by 7.9% and 12.1%, respectively compared with the control, and exposure to the two concentrations of TDCIPP for 60 days caused a similar effect. No significant recovery effect on cilia number was observed at 90 and 120 day (Fig. 4).

3.6. Effects on depth and diameter of basal body of cilia

TEM was used to examine effects on ultrastructure of cilia during exposure to TDCIPP and the recovery period. Exposure to 3000 ng/L TDCIPP for 60 days significantly decreased depth and diameter of basal body of cilia by 7.1% and 4.5%, respectively compared with the control, and similar effects were also observed at 120-day recovery period (Fig. 5A and B). Representative images were presented in Fig. 5C and D.

Table 1
Continuous monitoring of concentrations (units: ng/L for media; ng/g for the wet weight for cells) of tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) in cell culture medium and cell samples during the exposure and recovery peroid. Values represent mean ± SEM (n = 3).

Treatments	Collected samples	Exposure peroid		Recovery peroid	
		30 day	60 day	61 day	63 day or later
300 ng/L	medium (ng/L)	226.60 ± 0.03	303.70 ± 0.03	<MLOD[a]	<MLOD
	cell (ng/g)	2.70 ± 0.38	4.12 ± 1.52	<MLOD	<MLOD
3000 ng/L	medium (ng/L)	2688.89 ± 0.16	2376.54 ± 0.48	70.87 ± 0.04	<MLOD
	cell (ng/g)	18.20 ± 3.62	29.56 ± 2.38	1.52 ± 0.01	<MLOD

[a] "<MLOD" means "lower than method limit of detection"; MLODs were 0.06 ng/mL and 0.09 ng/g for medium and cell samples, respectively.

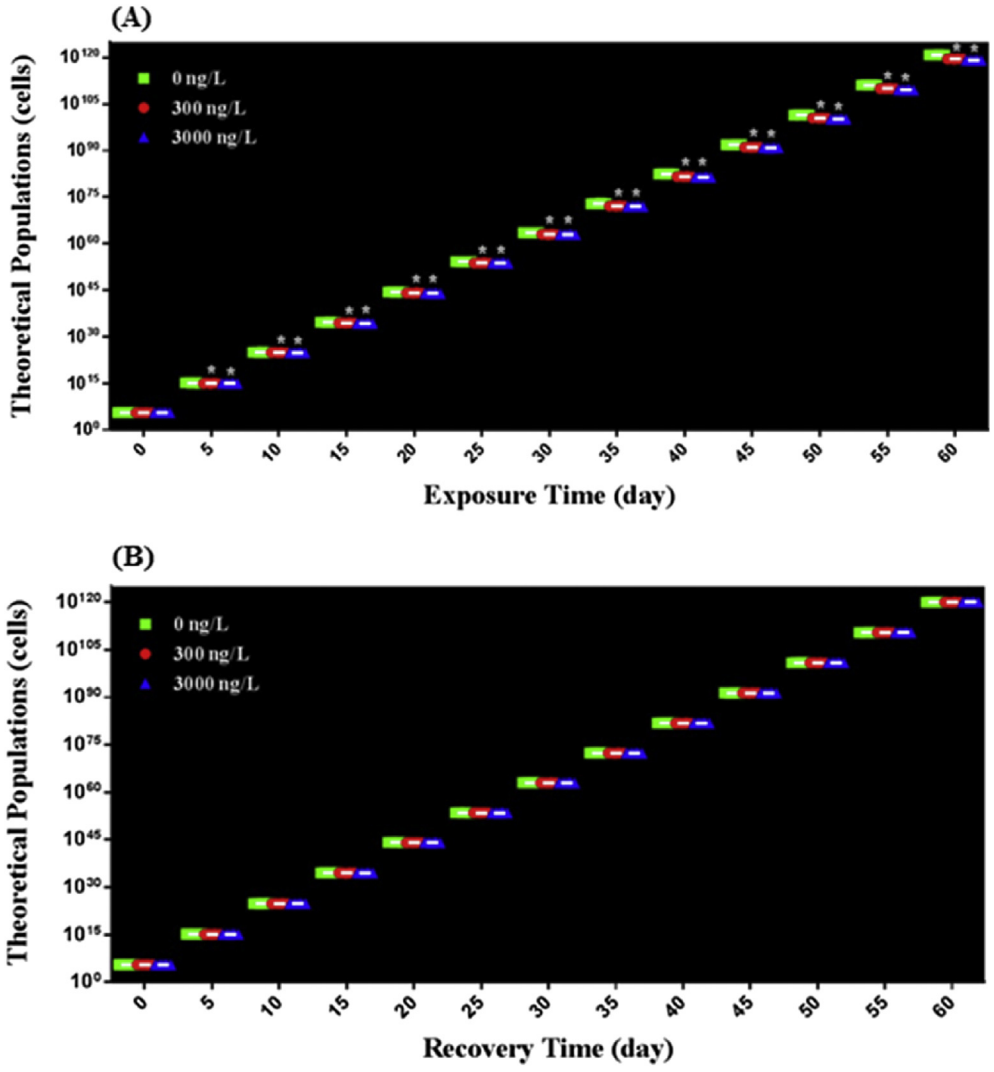

Fig. 2. Time-dependent effects on theoretical populations of *T. Thermophila* during exposure to TDCIPP (A) and after subsequent recovery (B). Values are the mean ± SEM. Significant differences from the control are indicated by $^{*}P < 0.05$. Each concentration was replicated 6 times.

3.7. Transcriptional responses of genes

Transcriptional responses of 10 genes were examined during exposure to TDCIPP and after subsequent recovery. Exposure to 3000 ng/L TDCIPP for 60 days significantly up-regulated expressions of all 10 genes tested, including three genes from intraflagellar transport protein (*ift*) family (*ift52*, *ift81* and*ift172*), and seven genes from cpn60 chaperonin protein family (*tcp-1*) (*3700.m00089*, *3706.m00106*, *3715.m00106*, *3731.m00045*, *3698.m00091*, *2.m02183* and *16.m00478*). After 60-day recovery, the expressions of three *ifts* (*ift52*, *ift81*, *ift172*) and two *tcp-1s* (*3700.m00089* and *16.m00478*) were not changed compared with the control, but a significant up-regulation in expressions of the other *tcp1* genes (*3706.m00106*, *3715.m00106*, *3731.m00045*, *3698.m00091* and *2.m02183*) was observed (Fig. 6).

4. Discussion

One of the main objectives in the present study was to investigate whether multigenerational effects persisted after the *T. thermophila* was transferred into fresh medium without TDCIPP. This makes it critical to continuously monitor concentrations of TDCIPP in exposure medium or cells to clarify whether the multigenerational effects observed in the period of recovery were caused by the remaining TDCIPP in the exposure system or cells. As expected, TDCIPP was not detectable in either exposure media or cells within two days (after 63 day or later) of being transferred to medium with a nominal concentration of 0.0 ng/L TDCIPP. Thus, it can be concluded that observed adverse effects at 63 day or later resulted from previous exposure for 60 days to TDCIPP.

Multigenerational exposure to environmentally relevant concentrations of TDCIPP significantly decreased populations of *T. thermophila*, but the population recovered once TDCIPP was no longer in the media. Previous studies demonstrated that acute exposure to relatively great concentrations of TDCIPP caused multiple toxicities (Jarema et al., 2015; Liu et al., 2016), but no information was available about multigenerational effects of environmentally relevant concentrations of TDCIPP. It has been recognized that exposure to chemicals can cause multigenerational effects through genomic and epigenetic effects. Thus, to expand on the ecological relevance of risk assessments information on multigenerational effects was deemed necessary. Although a previous study demonstrated that exposure to relatively high concentrations of TDCIPP (0.01, 0.1 or 1 μM) for 5 days significantly

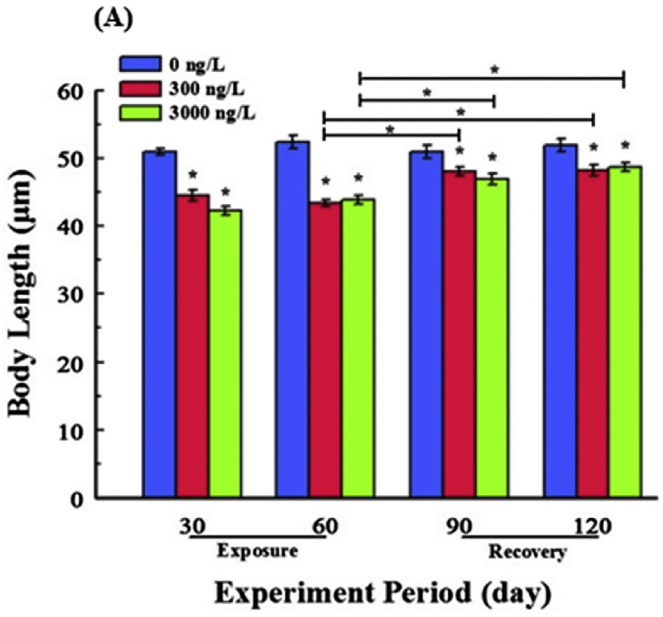

Fig. 3. Effects on body length (A) and body width (B) after TDCIPP exposure and recovery. Values are the mean ± SEM (n = 30). Significant differences from the control or between exposure and recovery are indicated by $^{*}P < 0.05$.

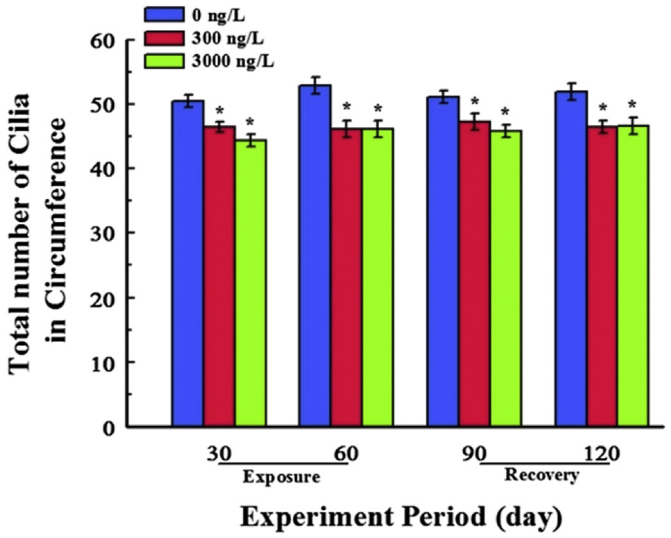

Fig. 4. Effects on total number of cilia in after exposure to TDCIPP and then the 60-day recovery. Values are the mean ± SEM (n = 30). Significant differences from the control or between exposure and recovery are indicated by $^{*}P < 0.05$.

decreased biomass of *T. thermophila*, exposure concentrations used in that study were greater than environmental concentrations (Li et al., 2015b) and thus could not provide reliable information for assessment of potential effects of TDCIPP. In the present study, a theoretical 372-generation exposure and 372-generation recovery experiment was performed using *T. thermophila* as a model, organism to study effects of multigenerational exposures to environmentally relevant concentrations of TDCIPP. While populations of *T. thermophila* were reduced due to exposure to TDCIPP, those effects were reversed once cells were inoculated into new media without TDCIPP.

Multigenerational exposure to environmentally relevant concentrations of TDCIPP resulted in smaller sizes of individual *T. thermophila*, although this effect was not reversed after the 60-day recovery period during which time there was no exposure to TDCIPP. Inhibition of growth of individuals by TDCIPP has been reported in chicken and zebrafish embryos exposed to relatively great concentrations and in *Daphnia magna* and zebrafish larvae exposed to environmentally relevant concentrations (McGee et al., 2012; Farhat et al., 2013, 2014a, 2014b; Fu et al., 2013; Li et al., 2015a; Zhu et al., 2015). Relatively great concentrations of TDCIPP (0.01, 0.1 or 1 μM) for 5 days significantly decreased body size of *T. thermophile* (Li et al., 2015b). In this study, multigenerational exposure to environmentally-relevant concentrations of TDCIPP significantly decreased body size of *T. thermophile*. Therefore, results of the present study were consistent with those of previous studies. However, results of the study, results of which are reported here, went further and reported that prolonged exposure (372 generations) of *T. thermophila* to environmentally relevant concentrations of TDCIPP caused similar developmental toxicity (reduced individual size) and the toxic effects were partially reversible during a subsequent 372 generations.

TDCIPP decreased number of cilia, changed ultrastructure of cilia, and up-regulated expressions of genes related to assembly and maintenance of cilia, and most of these effects were not reversed during the 60 day recovery during which *T. thermophila* were not exposed to TDCIPP. The present study suggested that cilia might be one of toxic targets for TDCIPP exposure in *T. thermophila* since numbers of cilia were less after exposure to the least concentration compared with other parameters tested such as relative biomass (Li et al., 2015b). Also, effects of TDCIPP on cilia evaluated after multigenerational exposure to environmentally-relevant concentrations were consistent with previous findings. Cilia are conserved eukaryotic organelles with important sensory and motile functions (Mitchell, 2007), and thus defects of cilia caused by TDCIPP might decrease capability of *T. thermophila* to sense risks and reduce its speed of escaping when facing predators. In ciliates, each cilium is stabilized by a conventional basal body, and assembly and maintenance of cilia is dependent on bidirectional trafficking of protein complexes between basal body and cilia tip (Avasthi and Marshall, 2012; Taschner et al., 2012; Lechtreck, 2015; Bayless et al., 2016). Therefore, in the present study, ultrastructure of the basal body of cilia was examined, and the decrease in depth and diameter of the basal body of cilia might be responsible for the observed reduced number of cilia since changes of basal body of

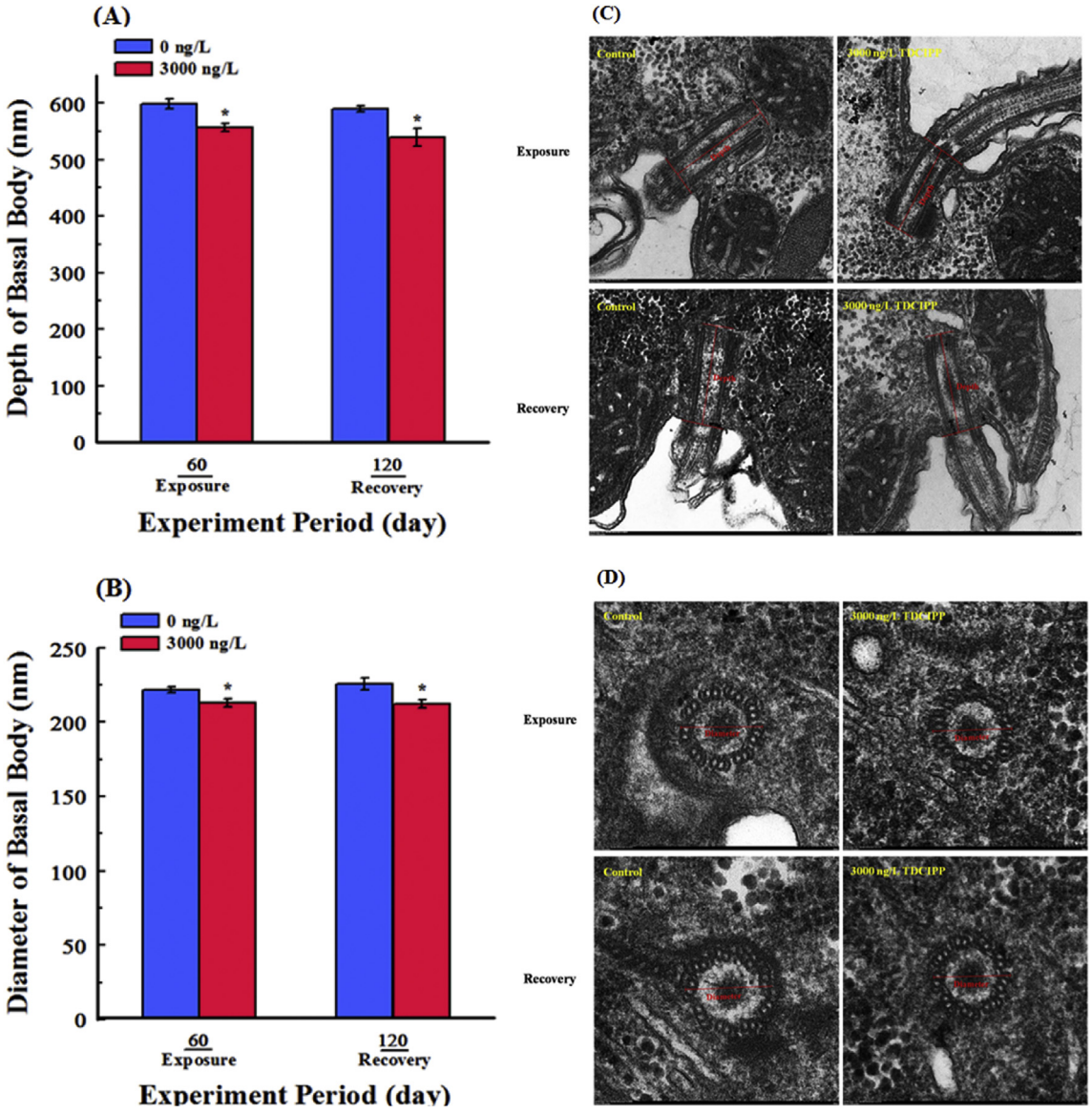

Fig. 5. Effects on depth (A) and diameter (B) of basal body after TDCIPP exposure and recovery. Values are the mean ± SEM (n = 18–54). Significant differences from the control are indicated by $^{*}P < 0.05$. Represented transmission electron microscopy images for changed ultrastructure of depth (C) (bar = 200) and diameter (D) (bar = 100) of basal body.

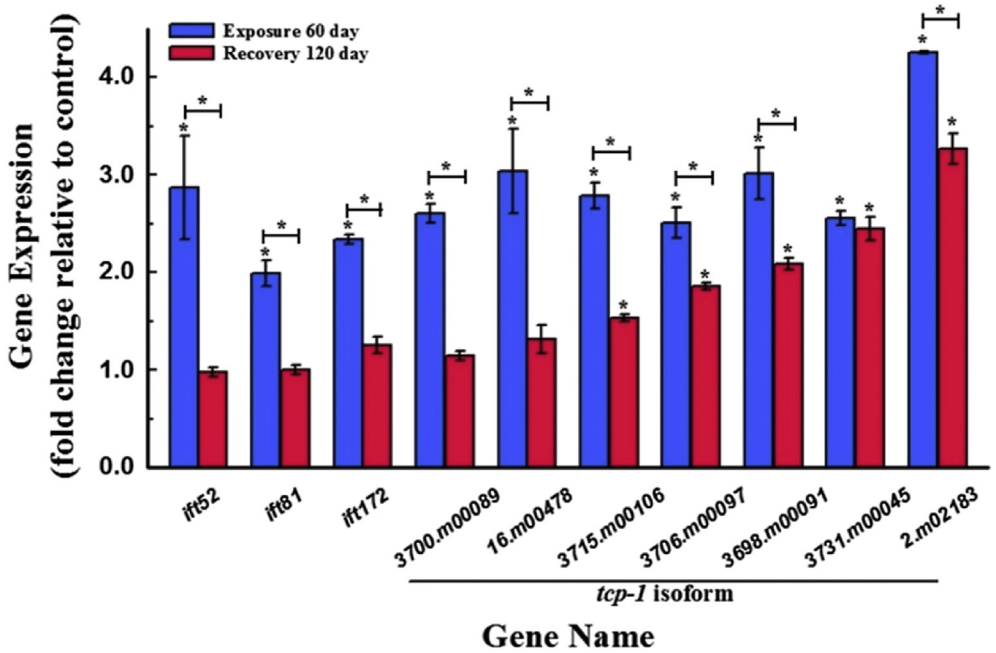

Fig. 6. Effects on the relative expression of genes after exposure to 3000 ng/L TDCIPP for 60 days and after subsequent 60-day recovery. Values are the mean ± SEM (n = 3). Significant differences from the control are indicated by $^{*}P < 0.05$.

cilia might inhibit growth and accelerate abscission of cilia. Furthermore, expressions of ten genes were also examined in this study. The ten genes are included in two functional classes and are responsible for assembly and maintenance of cilia (Seixas et al., 2003, 2010; Avasthi and Marshall, 2012; Lechtreck, 2015). Exposure to TDCIPP significantly up-regulated expressions of all ten genes, and thus the up-regulation was considered as a feedback response since number of cilia and depth and diameter of basal body of cilia were decreased. Recovery of effects has been considered as an important factor in assessments of potential long-term effects of chemicals. Similar to exposure experiments, adverse effects were also observed during the 60-day recovery period, including decreased number of cilia, changed ultrastructure of cilia and up-regulated expressions of genes, although transcriptions of partial genes tested (e.g., *ift52*, *ift81*, *ift172*, *tcp-1-3700.m00089*, and *tcp-1-16.m00478*) were unchanged compared with the control. These results suggested that TDCIPP might have the capability to cause long-term damage in lower tropic-level organisms such as in the protozoa *T. thermophila*. Consistent up-regulations of genes between exposure and recovery experiments indicated that changes in expressions of genes were not direct effects of TDCIPP which thus further supported the hypothesis that up-regulation of genes was a feedback response for the reduced number of cilia observed.

Taken together, to our knowledge we report for the first time that multigenerational exposure to environmentally relevant concentrations of TDCIPP significantly decreased the size of the population, size of individuals, expressed as length and width of bodies, number of cilia, and depth and diameter of basal body of cilia in *T. thermophila* and these results suggest that TDCIPP in natural waters might cause a significant threaten to *T. thermophila* due to multigenerational exposure. Furthermore, effects at the population level could be recovered once TDCIPP was no longer in the media. However, the “permanent” changes in number of cilia, ultrastructure of cilia, and expression of several genes related to assembly and maintenance of cilia were also observed. Here, it should be noted that *T. thermophila* has a short generation time (around several hours depending on culture condition and seeding density), and thus the multigenerational effects observed might be adaptive changes. The object of the study was to evaluate multigenerational effects of TDCIPP after exposure to environmentally relevant concentrations, and whether the multigenerational effects observed were caused through genomic or epigenetic changes remains to be determined.

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Appendix A. Supporting information

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.08.034.

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Supporting Information

Multigenerational Effects of Tris(1,3-dichloro-2-propyl) Phosphate on the Free-Living Ciliate Protozoa *Tetrahymena Thermophila* Exposed to Environmentally Relevant Concentrations and after Subsequent Recovery

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Table S1. Sequences of primers for the genes tested.

Gene ID	Gene Name	Isoform	Sense primer（5'-3'）	**Antisense primer （5'-3'）**	Product length (bp)
TTHERM_00648910	IFT52/Intraflagellar transport protein 52		TGCGGCAGAAATAAAGAAGA	TGCGATTCTTATGACCACCA	178
TTHERM_00089240	IFT172/intraflagellar transport particle protein 172		GCAGTTTGCACAGCTGAAAG	CCCATTCCTTGCCAATCTTA	199
TTHERM_01013160	IFT81/Intraflagellar transport protein 81		TTGCAGAAAAAGCTGCAAAA	CACCTTGTTGGGCAATACCT	186
TTHERM_00037060	TCP-1/cpn60 chaperonin family protein	2.m02183	CTCAAGGACGGCAAGTTTGT	TTGAGCTTGGGGATGATTTC	197
TTHERM_00134970	TCP-1/cpn60 chaperonin family protein	3698.m00091	CCTAAACCCAAAACCAAGCA	TTCATCATCGAAACCCCATT	156
TTHERM_00149340	TCP-1/cpn60 chaperonin family protein	3700.m00089	CAAATGGCTCTTGCTGTTGA	CTTCAGCGATTTCGACCTTC	169
TTHERM_00196370	TCP-1/cpn60 chaperonin family protein	16.m00478	TCCCTATGTTCTTCGCTGCT	TCTTAGGGGGACCGAAAGTT	156
TTHERM_00239290	TCP-1/cpn60 chaperonin family protein	3706.m00097	TTGCTTGGCTGTTGCTAAGA	CCACAGTTTTGAGCCAAGGT	189
TTHERM_00497960	TCP-1/cpn60 chaperonin family protein	3715.m00106	GAAGATGCATGAAGGCCAAT	TCGTGACCAGCATTATCAGC	178
TTHERM_00670500	TCP-1/cpn60 chaperonin family protein	3731.m00045	GCGAAGACCCCTCTAAGTCC	ATCAGCCCAACCAAGATCAG	200
TTHERM_00047040	Ubiquitin carboxyl-terminal hydrolase family protein		GCAAAATGGAATGGAGCATC	GCCCCATCCATCATTGATAC	176
TTHERM_00001480	Chlamydial polymorphic outer membrane protein repeat		AAACATTCCCTGATGGCTCA	AGCTCTGTAGCCATCACCTTG	183
TTHERM_00191270	Cysteine proteinase 3 precursor, putative		TTAGCTACTGCTGGCCCAAT	AATGCTCACCCCAACTGTTC	187

Figure S1

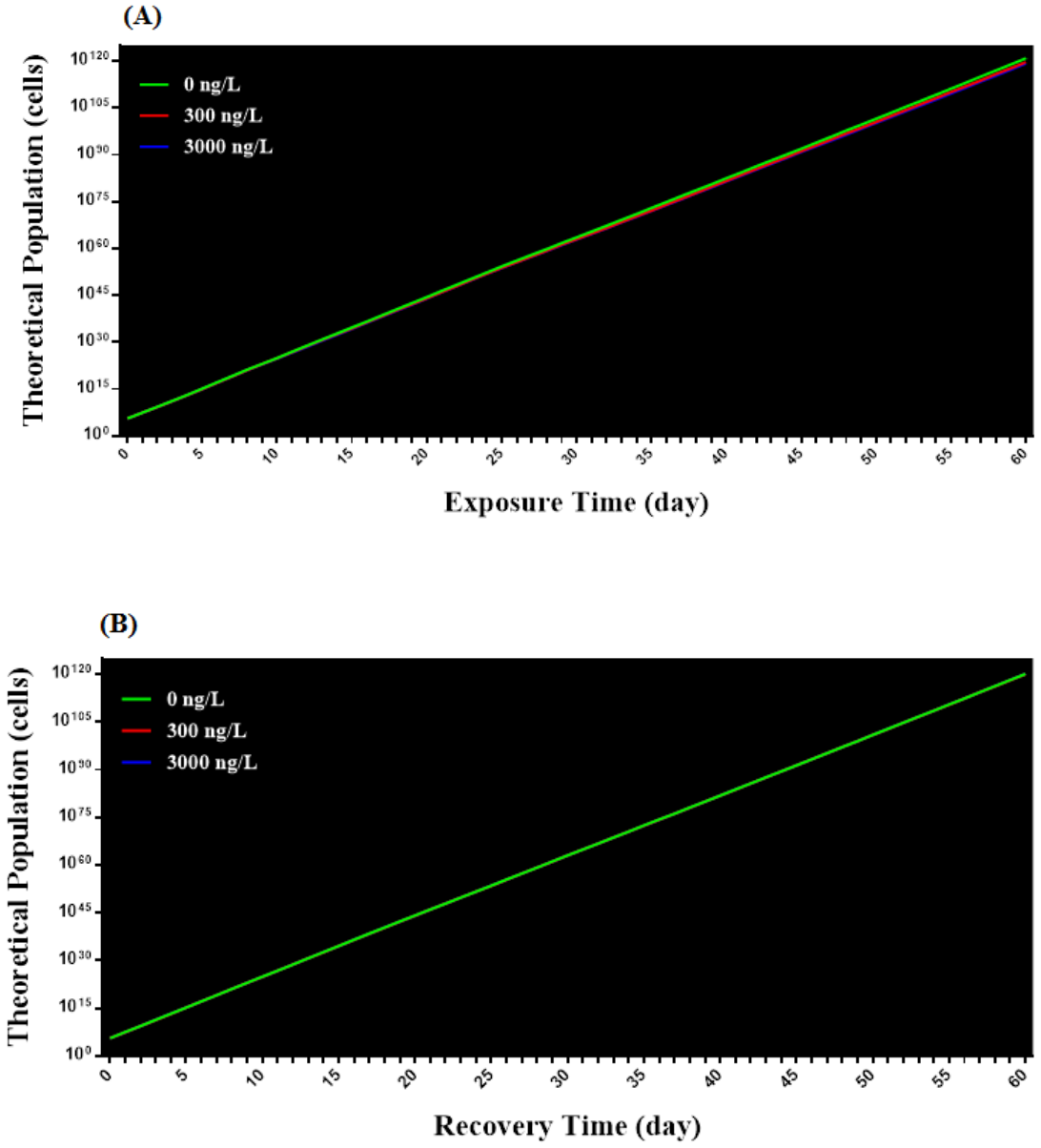

Figure S1: Time-dependent effects on theoretical populations during TDCIPP exposure (A) and after subsequent recovery (No significant effects were observed, so population curves from different concentrations were overlapped) (B). Values represent mean ± SEM. Significant differences from the control are indicated by $*P < 0.05$. Each concentration contains 6 biological replicate.