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TDCIPP exposure affects *Artemia franciscana* growth and osmoregulation

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Abstract

Environmental monitoring has demonstrated widespread occurrence of the flame-retardant tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), raising concerns about the impact on aquatic life. Using ^{1}H NMR and GC-MS metabolomics and 20-day body length experiments, we have determined that exposure to TDCIPP affects *Artemia franciscana*. The LC_{50} for a 48 hr TDCIPP exposure was determined to be 37.1 ± 1.3 μM. Acute exposure (48 h) to 20.0 μM did not affect *A. franciscana* body length but did elicit a metabolic change. Chronic exposure to 0.50 μM TDCIPP caused decreased body length in *A. franciscana* exposed for 20 days and elicited a metabolic response. Principal component analysis revealed variance between acute and chronic exposure along PC1 (36.4%) and between control and TDCIPP along PC2 (17.4%). One-way ANOVA indicated that 19 metabolites were significantly affected by TDCIPP exposure; namely metabolites of the osmolyte class, including betaine, phosphocholine, gadusol, taurine, glycerol and trehalose – metabolites that are essential osmoprotectants in extremophile species. Other pathways that may be perturbed by TDCIPP exposure include one carbon, glycine, serine, threonine, and glycerophospholipid metabolism.

Graphical Abstract

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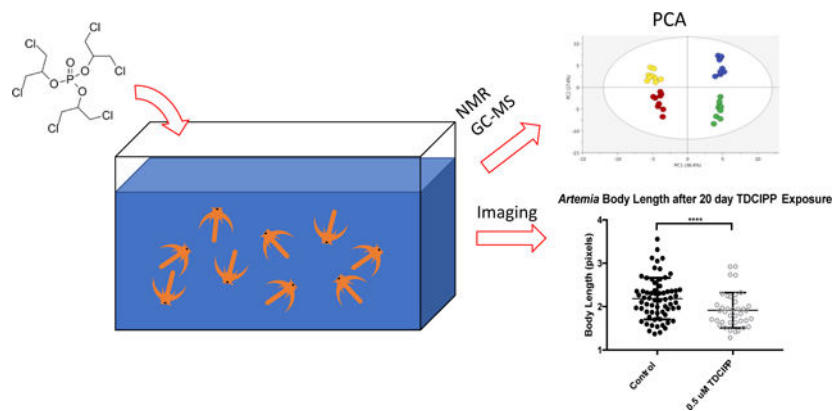
Conflicts of Interest

The authors declare that there are no competing interests.

Appendix A: Supplementary Information

Supplementary data to this article can be found online.

1. Introduction

The organophosphate flame retardant, tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), is an emerging contaminant that is pervasive in indoor and outdoor environments due to high production and widespread use (van der Veen et al. 2012). TDCIPP and its primary metabolite, BDCIPP, have been detected in baby products, indoor dust, human urine, and natural waters, which raises concerns over the impact of constant exposure (van der Veen 2012; Betts 2013, Stapleton et al. 2011). Studies have shown that TDCIPP exposure may cause endocrine disruption, neurotoxicity, carcinogenicity, infertility and developmental toxicity (Feng et al. 2016; Carignan 2017, Li et al. 2015; European Union 2008; WHO 1998). TDCIPP in natural waters is not widely reported (Andresen et al. 2004; van der Veen et al. 2012). Several coastal cities in China report detectable levels of TDCIPP with a mean concentration of 0.254 nM, but little is known of the impact on aquatic life (Hu et al. 2014; Zhong et al. 2018).

Risk assessments from the WHO and EU report that TDCIPP is very persistent in the environment and that fish species, such as killifish, rapidly metabolize this compound (van der Veen 2012; WHO 1998; European Union 2008). However, studies with zebrafish embryos and *Daphnia magna* report that TDCIPP affects development (van der Veen 2012; Li et al. 2015; Kupsco et al. 2017; Dasgupta et al. 2017). Studies with zebrafish embryos have used developmental and genotoxicity assays and targeted genomics analysis to determine that TDCIPP affects DNA methylation during embryonic development (Volz et al. 2016; McGee et al. 2012; Kupsco et al. 2017). Han et al. used *D. magna* as a model to analyze fecundity, growth, and gene transcription and determined that TDCIPP decreased fecundity and body length and affected pathways related to protein synthesis, endocytosis, and metabolism (Li et al. 2015). While targeted studies with aquatic species have revealed more about TDCIPP toxicity, untargeted studies may provide further insight into global metabolic effects. In order to further elucidate the mode of action of TDCIPP in aquatic

organisms, we hope to complement these targeted studies using untargeted ^{1}H NMR and GC-MS metabolomics analysis of the effect of TDCIPP exposure on the *A. franciscana* metabolome.

We have developed imaging and environmental metabolomics analyses using *A. franciscana* as a model organism. *A. franciscana* is a saltwater aquatic crustacean that is native to inland saltwater lakes but is often used for ecotoxicity assays representing the marine environment. As *A. franciscana* are closely related to *D. magna*, we hypothesized that TDCIPP would similarly affect *A. franciscana* growth and metabolism. The metabolic response of *A. franciscana* was measured using ^{1}H NMR and GC-MS metabolomics. NMR is a rapid, robust, and quantitative technique that requires minimal sample preparation (Barding et al. 2012; Larive et al. 2015; Nagana Gowda et al. 2015). Polar metabolites require derivatization for GC-MS analysis, but this technique has low limit of detection and well-established libraries that make it a great complementary technique (Barding et al. 2013; Fiehn et al. 2008). We monitored changes to an organism's small-molecule metabolite profile before and after exposure to TDCIPP. Then data reduction and statistical analysis was applied to extract meaningful information about the potential mode of action and identify biomarkers of stress corresponding to TDCIPP exposure.

The aims of this study were to determine the effect of TDCIPP exposure on *A. franciscana* mortality and growth, characterize the metabolic response of *A. franciscana* to chronic and acute TDCIPP exposure, and apply Principal Component Analysis (PCA) to identify small molecule biomarkers of exposure and metabolic pathways perturbed by chronic and acute TDCIPP exposure.

2.   Materials and Methods

2.1.   Determination of TDCIPP LC$_{50}$

The lethal concentration of TDCIPP for 50% of the naupliar *A. franciscana* population (LC$_{50}$) was determined for a 48 h exposure. *A. franciscana* cysts (Brine Shrimp Direct, Ogden, UT) were hatched in 300 mL tanks containing 35 g/L Oceanic Natural Sea Salt Mix (Amazon.com, Seattle, WA) with 0–300 μM TDCIPP (Sigma Aldrich, St. Louis, MO) and 0.1% dimethylsulfoxide (DMSO) (EMD, Burlington, MA) as a vehicle. During the hatch period, the cysts were exposed to constant light and aeration. Nauplii were transferred to aquariums containing 20 mL salt water with TDCIPP with 20 *A. franciscana* per aquarium and 10 aquariums per dose exposed to a 16 h:8 h light:dark cycle (Morgan et al., 2019). The number of living and dead shrimp in each aquarium was recorded to calculate the mortality rate. Mortality plots were constructed in GraphPad Prism 7 (San Diego, CA).

2.2.   Sublethal acute exposures to TDCIPP for metabolomics analysis

Acute and chronic exposures to TDCIPP were conducted. For the acute exposure, *A. franciscana* cysts (1 oz) were hatched for 24 h in 1-L control sea salt or 20 μM TDCIPP with 0.1% DMSO-*d6* (Sigma Aldrich) sea salt. The hatched nauplii were divided into 6 aquariums containing 300 mL media, 3 control and 3 treated, with constant aeration and a 16:8 h light cycle. After the 48-h exposure, the nauplii were transferred to 2 mL microvials

(3 samples per aquarium, 9 samples per dose) and flash frozen in liquid nitrogen. For the chronic exposure, *A. franciscana* cysts (1 oz) were hatched for 24 h in 1-L control sea salt or 0.5 μM TDCIPP (0.1% d6-DMSO) sea salt and then divided into 6 aquariums. The nauplii were transferred to fresh solution and fed algae after the first 96 hours of the exposure period. After 1 week, the nauplii were transferred to 2 mL microvials (3 samples per aquarium, 9 samples per dose) and flash frozen in liquid nitrogen.

The protocols for metabolite extraction and NMR and GC-MS sample preparation are described in Morgan et al. 2019. Briefly, the metabolites were extracted with 1:1:1 deuterated methanol/water/chloroform and homogenized with a vortex mixer (Eppendorf, Hauppauge, New York) and zirconia beads (Biospec Products, Bartlesville, OK). The solvents were evaporated by speed vacuum (ThermoFisher Scientific, Waltham, MA) and stored at −80 °C until analysis. All samples were prepared concurrently.

2.3. Sublethal chronic exposure to 0.5 μM TDCIPP for imaging

A. franciscana cysts (1 oz) were hatched for 24 h in 1 L control sea salt or 0.50 μM TDCIPP (0.1% DMSO) sea salt. The hatched nauplii were divided into 20 aquariums, 10 treated and 10 control, containing 20 mL media and 20 nauplii per aquarium. The media was exchanged every 48 h. After the first 96 h of exposure, we began feeding algae to the *A. franciscana* by adding it to the fresh media. The number of living and dead *A. franciscana* was recorded each time, the media was exchanged and the dead *A. franciscana* were removed from the aquarium. At the end of the 20-day exposure, the living *A. franciscana* were fixed with 4 % paraformaldehyde (Spectrum, Gardena, CA) and 10 mM phosphate-buffered saline.

2.4. *Artemia franciscana* body length measurements

Fixed *A. franciscana* were stained with Rhodamine 6-G (JT Baker Chemical Co, Phillipsburg, NJ) and transferred to a microplate, one specimen per well. For the 48 h exposure, a 384-well plate was used, and for the 20-day exposure a 96-well plate was used. An image acquisition protocol was optimized on an ImageXpress Micro (IXM) XLS Widefield High-Content Screening System equipped with MetaXpress 6.0.3.1658 (Molecular Devices, Sunnyvale, CA). A 4× objective (384-well plate) or a 2× objective (96-well plate) and TRITC filter cube was used to acquire one frame per entire well. Images were then used to quantify survival and body length similar to previously described protocols (Leet et al. 2014; Volz et al. 2015). *A. franciscana* images were exported to ImageJ (National Institute of Health, Bethesda, MD) and body length measurements were taken from the head to the tail along the thorax and abdomen. An average body length (pixels) was calculated for control and exposed *A. franciscana*.

2.5. NMR acquisition and processing

Dried metabolite extracts were reconstituted in 200 μL 50 mM phosphate buffer (pD 7.45) in D_2O (D, 99.9%) (Cambridge Isotope Laboratories, Tewksbury, MA) containing 0.4 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonic acid-d_6 (DSS-d_6) and 0.2 mM ethylenediaminetetraacetic acid-d_{16} (EDTA) (Cambridge Isotope Laboratories, Tewksbury, MA). ^1H NMR spectra were acquired with a Bruker Avance III NMR spectrometer (Billerica, MA) equipped with a 5 mm TCI CryoProbe operating at 700.23 MHz. ^1H NMR

spectra were measured using a 1D NOESY pulse sequence (noesypr1d) with presaturation during the 120 ms mixing time and 2 s relaxation delay. Spectra were acquired at 25°C with 256 FIDs coadded, 32 dummy scans, and a 3.0 s acquisition time. A spectral width of 11.6808 ppm was used with 32768 complex data points acquired using digital quadrature detection (Morgan et al 2019; Griffith et al 2017, 2018).

Spectra were processed using Bruker Topspin 3.2 to manually phase and reference each spectrum to the resonance of DSS-d_6 (0 ppm). Spectral deconvolution and line fitting were performed using MestReNova 11 (Mestrelab Research, Escondido, CA). FIDs were apodized by multiplication with an exponential function equivalent to 1.5 Hz line broadening, zero-filled to 131072 points, and baseline corrected using a Whittaker Smoother function set to autodetect. Peak fitting was conducted using a generalized Lorentzian peak shape, a lower width constraint of 0 Hz, an upper width constraint of 30 Hz, position constraint within ± 5%, maximum number of fine iterations of 100, and local minima filter of 0. The peak fitting results were exported to Excel (Microsoft Office 2017) and the results of each spectrum were normalized to the sum of the total area between 0.8–9.0 ppm, excluding the region between 4.6–5.2 ppm. One well-resolved resonance for each metabolite was used for statistical analysis.

2.6. GC-MS acquisition and processing

The protocols for the derivatization, GC separation, and MS analysis were reported in previous publications (Barding et al. 2013; Griffith et al. 2018, Morgan et a.l 2019). A 2-μL aliquot of 0.4 mg/mL triclosan (Sigma-Aldrich) in chloroform was added to each GC-MS sample as a derivatization surrogate. The samples were derivatized by adding 20 μL of 20 mg/mL methoxyamine (Sigma-Aldrich, St. Louis, MO) in pyridine (Thermo Scientific, Bellefonte, PA) for sugar cyclization and mixing at 300 rpm for 90 min at 37°C. Silylation reactions were conducted with a 90-μL aliquot of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) containing 1% trimethylchlorosilane (TMCS) (Thermo Scientific, Bellefonte, PA) that was added to each sample and reacted for 30 min at 37°C. A 2-μL aliquot of a 0.4 mg/mL C24 fatty acid methyl ester (FAMES) standard was added to each sample to reference retention times and immediately sealed with a crimp cap. Chloroform blank samples and quality control samples containing a 2-μL FAME aliquot comprised of 0.8 mg/mL C8, C9, C10, C12, C14, and C16 and 0.4 mg/mL C18, C20, C22, C24, C26, C28, and C30 in 110 μL chloroform were also prepared. Twenty experimental samples, a blank, and a FAMEs standard were prepared for each day of GC-MS analysis.

A blank and a FAMEs standard were run every 20 experimental samples to evaluate consistency. The nine replicate samples for each dose were analyzed sequentially as a batch. Samples were injected in pulsed splitless mode on an Agilent J&W DB-5MS UI 30 m × 0.25 mm × 0.25 μm column (Santa Clara, CA) using an Agilent 7890A gas chromatograph coupled to a Waters GCT Premier mass spectrometer. Samples were introduced at initial oven temperature of 60°C held for 1 min, ramped at 10°C/min to a final temperature of 320°C with a final 5-min hold. The injector, transfer line, and source were maintained at 230°C, 320°C, and 220°C, respectively, and the liner was changed after every 25 injections.

Instrument operations were controlled by Waters MassLynx software version 4.1 (Waters Corporation, Milford, MA).

The data were collected in the Waters file format (*.raw) and converted to NetCDF (*.cdf) for compatibility with the Automated Mass Spectral Deconvolution and Identification System (AMDIS, NIST, Gaithersburg, MD). Deconvolution parameters were set to a component width of 17 scans, high resolution, high sensitivity, and medium shape. Retention indices (RIs) were calculated for each sample by AMDIS using an internal standard library and calibration standard library. Experimental samples were calibrated to the FAMEs standard run on the same day of analysis. Compounds were identified using the Golm Metabolome Database (Max Planck Institute of Molecular Plant Physiology, Potsdam, Germany) and NIST 2017 Mass Spectral Library (National Institute of Standards, Gaithersburg, MD).

MarkerLynx XS (Waters Corporation) was used for data preprocessing to collect integration values for identified metabolites. Peaks were detected without smoothing from an initial retention time of 7.00 min and a final retention time of 32.00 min, with a low-mass cutoff of 73.5 Da, a high-mass cutoff of 600 Da, and a mass accuracy of 0.10 Da. A peak-to-peak baseline noise value of 1.0, a marker intensity threshold of 25 counts, and a mass and retention time window of 0.1 Da/min, with 3.0 noise elimination were used. The results were exported to Excel where the retention times and mass were matched with identified metabolites. One mass-retention time pair with the corresponding area for each metabolite was taken for data normalization and statistical analysis. For metabolites with more than one silylation product, the most abundant mass-retention time pair for each retention time was summed to obtain one value for each metabolite. The mass-retention time pair with the highest relative abundance was chosen to represent each metabolite and this value was normalized to the total spectral area.

2.7. Metabolomics statistical methods

NMR and GC-MS results for each sample were combined in SIMCA (SIMCA 14.1, Umetrics, Malmo, Sweden) for multiblock PCA. Univariate statistical analysis was performed using GraphPad Prism. Boxplots were constructed, and the normalized data was processed using one-way ANOVA with Tukey's post-hoc. The R package PAPi was used for pathway analysis of *Artemia* metabolites and their measured responses to TDCIPP exposure (Aggio 2014). Spreadsheets were constructed according to the specifications of the PAPi package with metabolite identity, 2 sample treatments (control and 100 ppm Roundup®), and sample replicates in separate columns. PAPi queries the Kyoto Encyclopedia of Genes and Genomes database (KEGG, Kyoto University, Japan) to first convert metabolite names to KEGG codes and then to extract biochemical pathway information. An activity score is calculated from the number of metabolites identified from each pathway and the relative abundance of each metabolite in a sample. A line graph of the activity score of significant pathways ($p < 0.05$) is plotted with the control group and Bonferroni corrected p-values set as a reference.

3. Results and Discussion

A. franciscana were subjected to acute and chronic TDCIPP stress to identify effects on growth and development and metabolic perturbation resulting from exposure.

3.1. Determination of the LC$_{50}$ for 48 h TDCIPP exposure

The LC$_{50}$ for 48-h TDCIPP exposure was determined to be 37.1 ± 1.3 μM TDCIPP (0.1% DMSO) (Figure 1). The time frame includes a 24-h period during which the *A. franciscana* cysts are hatched in TDCIPP media. This lethal concentration is approximately 10× less toxic than the reported toxicities for *D. magna* (van der Veen and de Boer 2012). This value was determined to identify a starting point for studying sublethal TDCIPP stress in *A. franciscana*. The remaining exposures were conducted at 20 μM and 0.50 μM TDCIPP for acute and chronic assays, respectively.

3.2. Impact of acute and chronic TDCIPP exposure on *A. franciscana* mortality and development

Acute exposure to 0–20.0 μM TDCIPP caused no significant difference in *A. franciscana* body length (Figure S1). However, chronic exposure to 0.50 μM TDCIPP decreased body length significantly (Figure 2). Mortality was also higher in TDCIPP-exposed *A. franciscana* over the 20-day exposure. The experiment started with 200 *A. franciscana*, by day 20 only 109 remained alive. From the TDCIPP group, 41 out of 100 were living, and from the control group, 68 out of 100 were living. Initially, the chronic exposure to 5 μM TDCIPP were tested, but 100% mortality was observed within 14 days. Naupliar and juvenile *A. franciscana* were identified in both the control and treated specimens (Figure S2), but there were 10% more juvenile *A. franciscana* in the control condition which likely contributed to a larger body length average in the controls versus the *A. franciscana* exposed to TDCIPP. This suggests that the ability of *A. franciscana* to molt may be impeded by TDCIPP exposure.

3.2. Multi- and univariate statistical analysis of TDCIPP-induced metabolite shifts

The ^{1}H NMR and GC-MS *A. franciscana* metabolite profile was reported in a previous publication (Morgan et al. 2019). Representative ^{1}H NMR spectra of extracts of control and TDCIPP-exposed *A. franciscana* are shown in Figure S3. The spectra are labeled to highlight the resonances of several significant metabolites including gadusol, glycine, taurine, homarine, and glucose. A representative GC-MS spectrum is shown in Figure S4, with the C24 internal standard and triclosan surrogate peaks labeled. Not all metabolites reported in the *A. franciscana* metabolome were quantified in this study due to the smaller number and age of the specimens. The extracts of 1-week old *A. franciscana* have slight differences in metabolite profile because they have started feeding on algae rather than relying on their own yolk stores.

Metabolite data represented by peak-fitted ^{1}H NMR resonances and GC-MS ion counts, normalized by total spectral area, were used for multi- and univariate analysis. The variance in metabolite abundance for multiblock-PCA of acute and chronic TDCIPP exposure is best shown by PC 1 (36.4%) and PC 2 (17.4%) (Figure 3). PCA of acute and chronic TDCIPP

exposure indicates variance in metabolite abundance for each condition. Separation along PC1 corresponds to differences in the metabolite profile between 48-h and 1-week old specimens. The loading plot (Figure 3b) indicates that inosine, glutamine, methionine, glutamate, taurine, and homarine contribute to the separation of the samples from the 1-week exposure along PC1, and betaine, choline, lysine, arginine, and alanine contribute to the segregation of the 48-h exposure samples along PC1. Glucose, ornithine and taurine contribute to the separation of the TDCIPP exposure samples along PC1, and threonine, tryptophan, and leucine contribute to the segregation of the control group along PC1. Univariate analysis was used to complement the multivariate analysis and clarify the individual metabolite changes for these four groups to evaluate the significance of those changes (Saccenti et al. 2014).

One-Way ANOVA identified metabolites that increased or decreased significantly ($p < 0.05$) from the acute (48 h, 20 μM TDCIPP) and chronic (1 week, 0.5 μM TDCIPP) exposures. For the acute exposure, aspartate, phenylalanine, glycine, phosphocholine, betaine, taurine, gadusol, glycerol and trehalose levels increased, while methanol, methionine, and leucine levels decreased. For the chronic exposure, the levels of glucose, phosphocholine, betaine, taurine, and gadusol increased, whereas tyrosine, serine, threonine, aspartate, glutamate, phenylalanine, glycerol, trehalose, and glycerophosphocholine decreased (Figure 4). For NMR, glycerophosphocholine was not detected in 48 h-old *A. franciscana* and resonance overlap by glycerol prevented the quantification of glycine for 1-week old *A. franciscana*. For GC, taurine was not detected in 48-h old *A. franciscana* and methionine was not detected in 1-week old *A. franciscana*. There remains one unidentified peak in the GC chromatogram at 15.59 min that did change significantly from the 1-week exposure. We were not successful in determining the compound responsible for this feature.

3.3. Biochemical interpretation of acute and chronic TDCIPP exposure

In agreement with studies on zebrafish and *D. magna*, our study finds that chronic exposure to sublethal TDCIPP impedes development (Volz et al. 2016; McGee et al. 2012; Li et al. 2015). Over a 20-day exposure, we observed decreased body length in TDCIPP-exposed *Artemia* compared to the control group. We were unable to collect samples for metabolomics analysis of the 20-day exposure due to low survival of specimens in both the control and treated conditions; however, samples taken after 1 week of exposure to 0.50 μM TDCIPP reveal metabolic perturbation that may lead to the decreased growth that was observed downstream. During the 20-day exposure, each specimen was expected to molt several times in order to grow and develop to adulthood (Abatzopoulos, T.J. 2002, Clegg, J 2002). The lack of growth in the TDCIPP-exposed *A. franciscana* may indicate an effect on the molt cycle (Figure 2). Acute exposure (48 hr) to 20 μM TDCIPP did not have an impact body length but the organisms are not expected to molt during this time (Figure S1).

Dormant *Artemia* cysts are preloaded with all the necessary components for initial life stages (Clegg, J 2002; Warner et al. 2013). These cysts are also impervious to environmental conditions and will only activate once conditions are suitable for hatching (Clegg, J 2002; Abatzopoulos, T.J 2012). The cysts were hatched in media containing TDCIPP, and the hatch rate was unaffected by TDCIPP exposure. Studies with zebrafish indicate that TDCIPP

affects early embryogenesis (McGee et al. 2012). Embryogenesis was already completed by the time the cyst was formed, so embryogenesis was unaffected (Ezquieta et al. 1985). In a study with *D. magna*, fecundity and growth were affected in subsequent generations following maternal TDCIPP exposure, which may result from an impact on *D. magna* embryogenesis (Li et al. 2015). Therefore, additional studies are needed to assess the effects of maternal TDCIPP exposure on reproduction (oviparity vs ovoviparity), growth rate, fecundity, and metabolome of future generations to elucidate the impact on embryogenesis in *A. franciscana*.

Pathway activity profiling using the PAPi R package was conducted to connect metabolite shifts to the global biochemical mode of action of TDCIPP exposure (Aggio et al. 2014). Pathway analysis is useful for hypothesis generation, but it is not robust enough for *Artemia* metabolomics because there is no reference *A. franciscana* genome and it has many novel metabolites; therefore, conclusive results are not possible. However, many pathways related to amino acid metabolism, lipid metabolism, sugar metabolism, and oxidative stress were identified as affected pathways from acute and chronic TDCIPP exposure (Figure S5, S6).

Acute exposure did have considerable impact on the expression of several metabolites, namely osmolytes such as glycerol, betaine, phosphocholine, taurine, gadusol, and trehalose. Acute exposure to TDCIPP may cause osmotic stress, which triggers increased levels of glycerol and trehalose. Glycerol and trehalose play an important role in *A. franciscana* for both development and stress protection (Clegg, J 2002; Warner et al. 2013; Teets et al. 2013; Yancey 2005). In the encysted embryo, energy is stored in the form of trehalose. As the embryo emerges and develops through the naupliar stages, trehalose is converted to glucose with the help of the proteolytic trehalase enzyme (Ezquieta et al. 1985). Trehalase and the other proteolytic enzymes that are involved in *A. franciscana* development are highly sensitive to environmental conditions, such as temperature, oxygen levels, and pH. Trehalose also serves as a substrate for the synthesis of glycerol and glycogen. In some extremophile organisms, glycerol and trehalose serve as osmoprotectants and cryoprotectants to protect internal structures of the organism from extreme conditions, such as temperature, dehydration, and salinity (Bundy et al. 2003). In contrast, glycerol, trehalose, and glycerophosphocholine were decreased in *A. franciscana* experiencing chronic exposure to TDCIPP, indicating that they had been depleted from exposure. Glucose levels increased with chronic TDCIPP exposure, indicating that trehalose may have been converted to glucose at higher rates.

Phosphocholine, taurine, gadusol, and betaine levels were increased with chronic and acute exposure to TDCIPP. Gadusol is a mycosporin-like amino acid (MAA) that has been categorized as a natural sunscreen in marine species because it absorbs UV radiation (Carreto et al. 2011; Brotherton et al. 2015; Osborn et al. 2015; Grant et al. 1985; Arbeloa et al. 2010). MAAs are also related to salinity, thermal, and desiccation stress. A study with *Artemia* in Lake Urmia observed bioaccumulation of MAAs with UV radiation and salinity stress; therefore, we hypothesize that gadusol accumulation is related to an effect on osmoregulation (Khosravi et al. 2013). *A. franciscana* is adapted to hypersaline environments and is an excellent osmoregulator; therefore, the high expression of these metabolites is likely a contributing factor in the higher lethal concentration for TDCIPP in

A. franciscana versus *D. magna*, killifish and goldfish (van der Veen et al. 2012; Li et al. 2015; Andresen et al. 2004; Clegg, J. 2002).

The metabolites that were significantly ($p < 0.05$) affected by TDCIPP exposure are also involved in one carbon metabolism, glycine, serine, and threonine metabolism, and glycerophospholipid metabolism. Glycerophosphocholine and glycerol decreased while phosphocholine increased. This indicates that glycerophospholipid metabolism may be downregulated from chronic TDCIPP exposure, which may result in an alteration to energy storage mechanisms and lipid structures (van der Veen et al. 2012; Li et al. 2015; Kanehisa et al. 2000). One carbon metabolism involves metabolic processes where methyl groups are transferred using folate cofactors to many biochemical pathways (Locasale et al. 2013; Fox et al. 2008). Methanol is an intermediary of one carbon metabolism and downregulation of this metabolite may indicate perturbation of this pathway in response to oxidative stress. Phosphocholine is a precursor to choline in the glycine, serine, threonine, metabolic pathway and betaine is another intermediary, the measured changes to these metabolites may be an indication that combating TDCIPP-induced toxicity is an energy priority over amino acid metabolism (Locasale et al. 2013; Kanehisa et al. 2000). These pathways and metabolites are interrelated and the measured patterns in metabolite shifts may indicate a perturbation in *Artemia franciscana* experiencing acute or chronic TDCIPP exposure.

4. Conclusion

In conclusion, this study provides new insights into the effects of a widely used flame retardant on a non-target organism, *A. franciscana*. Environmental monitoring has demonstrated widespread occurrence of TDCIPP, raising concerns about the impact on aquatic life. Using ^{1}H NMR and GC-MS metabolomics and 20-day body length experiments, we have determined that chronic exposure to environmentally relevant concentrations of TDCIPP affects *A. franciscana* development, which may indicate an effect on their molt cycle. TDCIPP exposure triggered a significant response in the abundance of metabolites that are essential osmoprotectants in extremophile species and in pathways that related to amino acid and glycerophospholipid metabolism. Future studies with *A. franciscana* should focus on effects of maternal TDCIPP exposure on reproduction, growth rate, fecundity, and metabolome of future generations in order to determine if similar affects are observed in *A. franciscana*, zebrafish embryos, and *D. magna* (Li et al. 2015; Volz et al. 2016).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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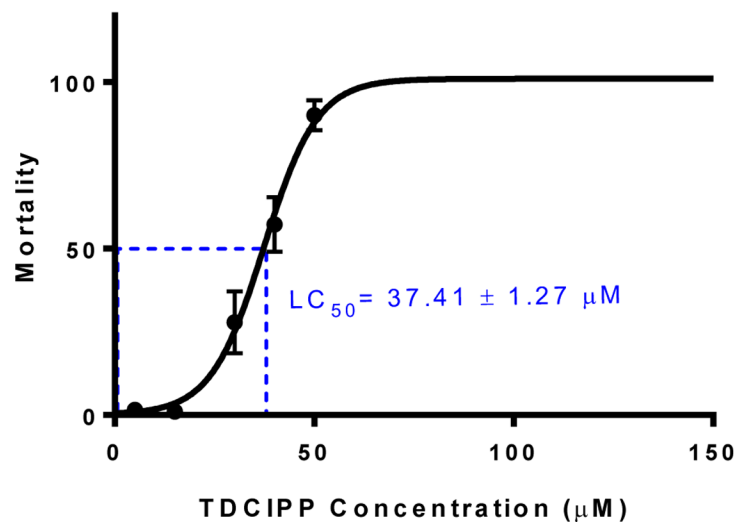
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Highlights

- *Artemia franciscana* growth was impeded by a 20-day exposure to 0.50 μM TDCIPP

- Multiblock-PCA demonstrated TDCIPP dose and time-dependent grouping for PC 1 (36.4 %) and PC 2 (17.4 %)

- One-way ANOVA identified 19 metabolites that were significantly affected by TDCIPP exposure

- Metabolites related to osmoregulation, one carbon, amino acid, and glycerophospholipid metabolism were affected
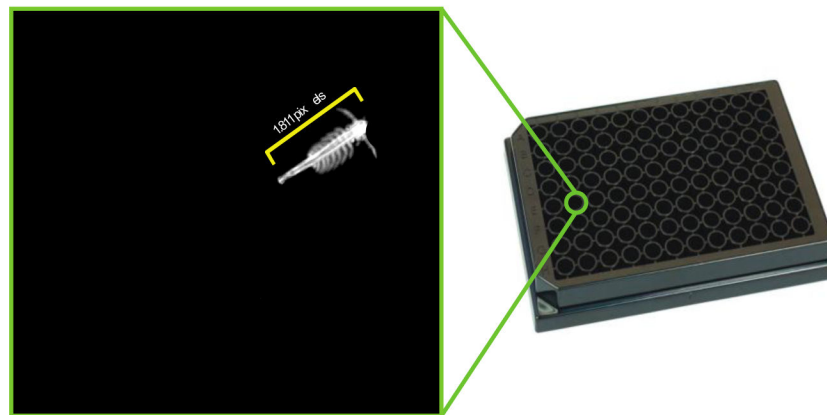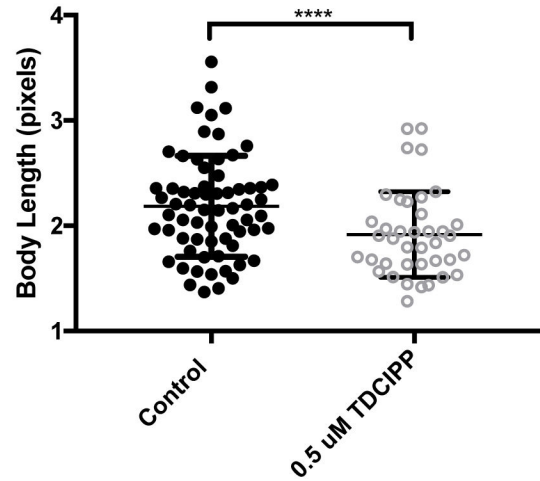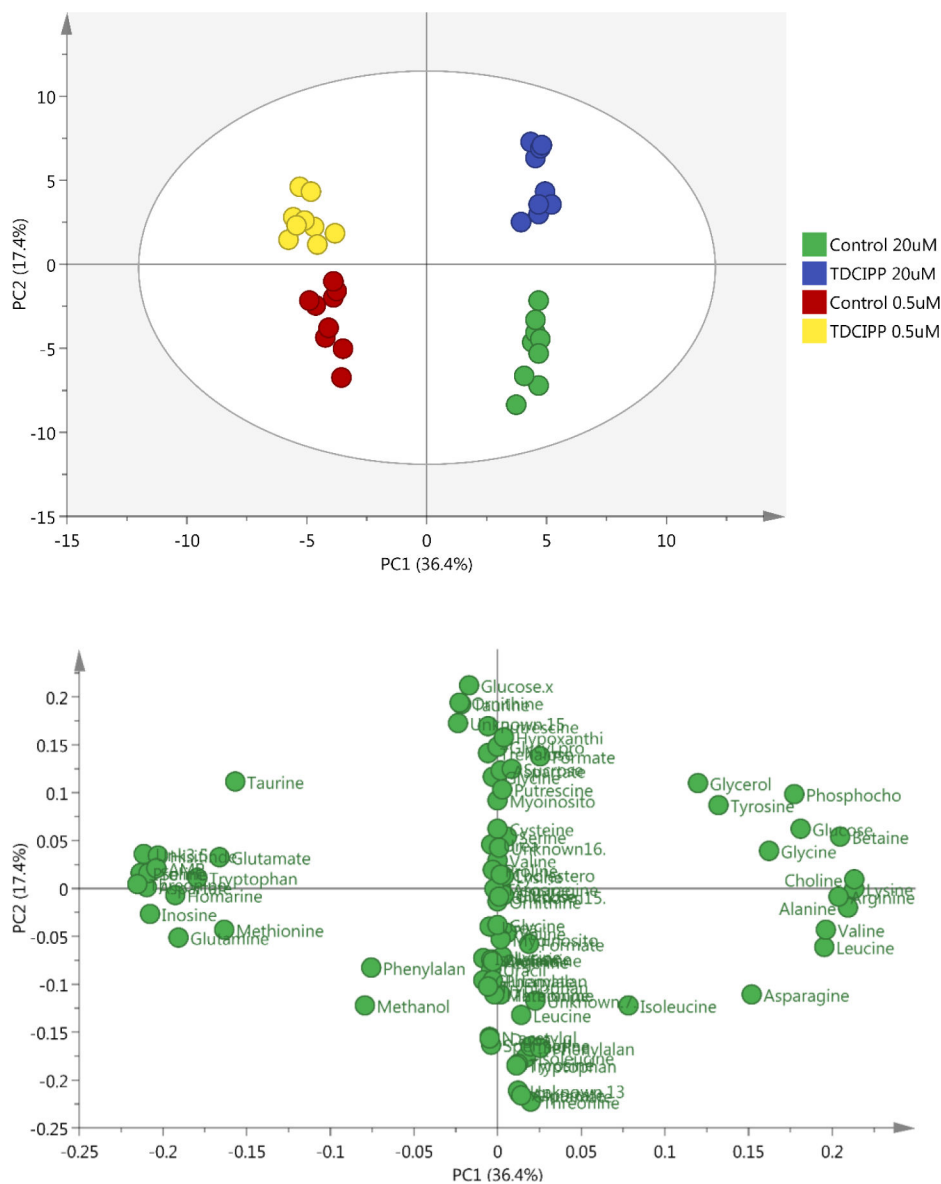
Figure 1.
Artemia mortality over a 48 h TDCIPP exposure ranging from 0–300 μM. The LC$_{50}$ was determined to be 37.1 ± 1.3 μM.

Figure 2.
Body length measurements for 20-day *A. franciscana* exposed to 0.5 μM TDCIPP. The body length for TDCIPP exposed *A. franciscana* are significantly shorter than the body length for Control *Artemia* (**** *p* <0.0001). Body length measurements (measured in pixels) are taken from the top of the head to the tip of the tail.

Figure 3.
Multiblock-PCA Score Plot (a) for acute (48 h, 20 μM) and chronic (1 week, 0.5 μM) exposure to TDCIPP. The variance between control and TDCIPP-stressed *A. franciscana* is best explained by PC 1 (36.4 %) and PC 2 (17.4 %) with model fit R2X (cum) = 0.688542 and Q2 (cum) = 0.359558. The loading plot (b) indicates how metabolites contribute to each component. Data points are labeled by metabolite identity as determined by GC-MS and ^{1}H NMR.
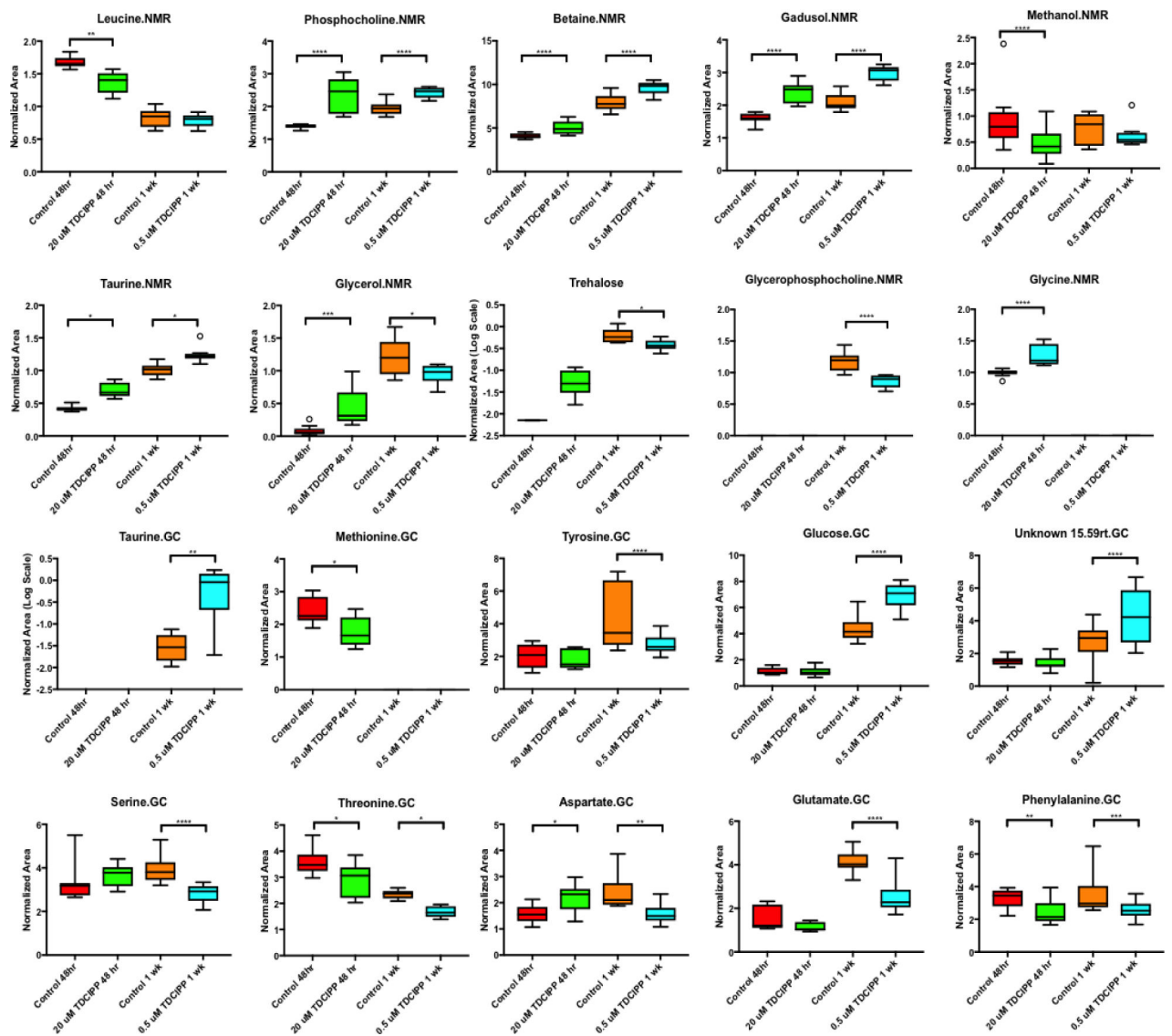
Figure 4.

Univariate box plots indicating how significant variables change with acute (20 μM, 48 hr) and chronic (0.5 μM, 1 wk) TDCIPP exposure. Variables are labeled by metabolite name and instrument used for detection. One-way ANOVA with Tukey's HSD was used to identify significant differences between control and dosed. (*$p < 0.1$, **$p < 0.01$, ***$p < 0.001$**** $p < 0.0001$).