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Early Zebrafish Embryogenesis Is Susceptible to Developmental TDCPP Exposure

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Running Title: Early embryogenesis and TDCPP exposure

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Abbreviations:

APE, Aryl phosphate ester

CPE, Chlorinated phosphate ester

FFRP, Furniture Flame Retardancy Partnership

gDNA, Genomic DNA

hpf, Hours post-fertilization

OPFR, Organophosphate-based flame retardant

TCEP, Tris(2-chloroethyl) phosphate

TCPP, Tris(1-chloro-2-propyl) phosphate

TDCPP, Tris(1,3-dichloro-2-propyl) phosphate

V6, 2,2-bis (chloromethyl) propane-1,3-diyl-tetrakis (2-chlorethyl) bis (phosphate)

ABSTRACT

Background: Chlorinated phosphate esters (CPEs) are widely used as additive flame retardants for low-density polyurethane foams, and have frequently been detected at elevated concentrations within indoor environmental media.

Objectives: To begin characterizing the potential toxicity of CPEs on early vertebrate development, the objective of this study was to assess the developmental toxicity of four CPEs used in polyurethane foam – tris(1,3-dichloro-2-propyl) phosphate (TDCPP), tris(2-chloroethyl) phosphate (TCEP), tris(1-chloro-2-propyl) phosphate (TCPP), and 2,2-bis (chloromethyl) propane-1,3-diyl-tetrakis (2-chlorethyl) bis (phosphate) (V6).

Methods: Using zebrafish as a model for vertebrate embryogenesis, we first screened the potential teratogenic effects of TDCPP, TCEP, TCPP, and V6 using a developmental toxicity assay. Based on these results, we focused on identification of susceptible windows of developmental TDCPP exposure as well as evaluation of uptake and elimination of TDCPP and bis(1,3-dichloro-2-propyl)phosphate (BDCPP, the primary metabolite) within whole embryos. Finally, as TDCPP-specific genotoxicity assays have, for the most part, been negative *in vivo* and zygotic genome remethylation is a key biological event during cleavage, we investigated whether TDCPP altered the status of zygotic genome methylation during early zebrafish embryogenesis.

Results: Overall, our findings suggest that the cleavage period during zebrafish embryogenesis is susceptible to TDCPP-induced delays in remethylation of the zygotic genome, a mechanism that may be associated with enhanced developmental toxicity following initiation of TDCPP exposure at the start of cleavage.

Conclusions: Our results suggest that further research is needed to better understand the effects of a widely used and detected CPE within susceptible windows of early vertebrate development.

INTRODUCTION

In 2004, the commercial polybrominated diphenyl ether (PBDE) mixture known as PentaBDE – a widely used brominated flame retardant (FR) – was voluntarily phased out in the United States due to concerns about persistence, bioaccumulation, and toxicity (Rahman et al. 2001; Tullo 2003). Prior to phase-out, PentaBDE was the primary FR used in the US to meet Technical Bulletin 177 (TB117) (State of California 2000) – a mandated flammability standard for polyurethane foam within upholstered furniture and baby products classified as juvenile furniture (Stapleton et al. 2011). In 2005, in order to maintain compliance with this standard, the US Environmental Protection Agency (EPA) Furniture Flame Retardancy Partnership (FFRP) identified 14 FR formulations as potential alternatives to PentaBDE-based formulations (USEPA 2005). With the exception of one formulation, all FR formulations identified by EPA contained one or both major classes of organophosphate-based FRs (OPFRs) – chlorinated phosphate esters (CPEs) and aryl phosphate esters (APEs).

Similar to PBDEs, OPFRs are additive (non-reactive) FRs that tend to migrate from end-use products into indoor environmental media (e.g., dust) (USEPA 2005). Due to increased use as PentaBDE replacements for household furniture and baby products (Stapleton et al. 2009; Stapleton et al. 2011), OPFRs have been detected at concentrations comparable to and, in some cases, higher than total PBDE concentrations in household dust (Marklund et al. 2003; Stapleton et al. 2009). For example, tris(1,3-dichloro-2-propyl) phosphate (TDCPP) – a widely used CPE and the focus of this study – has been detected at geometric mean concentrations of 1,880 ng/g within house dust (Meeker and Stapleton 2010). While TDCPP was detected and measured in human adipose tissue (maximum = 260 ng/g) (LeBel and Williams 1983; LeBel et al. 1989) in samples from the 1980s, exposure of the general population has likely increased – particularly in

the US – since TDCPP is a primary replacement for the phased-out PentaBDE commercial mixture (Stapleton et al. 2009; Stapleton et al. 2011). Presently, levels of TDCPP in house dust are comparable to PBDEs (Stapleton et al. 2009) but, unlike PBDEs, there are currently no methods available to quantify serum TDCPP concentrations. Since TDCPP is expected to be rapidly metabolized based on studies in adult rodents (Nomeir et al. 1981), recent studies are seeking to characterize exposure by monitoring TDCPP metabolites within urine (Cooper et al. 2011). Overall, these data suggest that chronic human exposure to TDCPP and other OPFRs following leaching from treated products is common within the US. Therefore, a better understanding of the potential developmental effects of OPFRs is needed since infants and children tend to (1) ingest more house dust than adults due to crawling and mouthing behaviors and (2) be exposed up to five times the acceptable daily OPFR intake level due to use in residential furniture (Babich 2006). However, to date most studies have focused on the genotoxicity and neurotoxicity of CPEs and APEs, respectively, in adult animals, while little attention has been placed on the developmental toxicity of both major classes of OPFRs.

To begin characterizing the potential toxicity of OPFRs on early vertebrate development, we used zebrafish as an animal model to evaluate the potential teratogenic effects of four CPEs recently identified and detected in polyurethane foam collected from baby products (Stapleton et al. 2011) – tris(1,3-dichloro-2-propyl) phosphate (TDCPP), tris(2-chloroethyl) phosphate (TCEP), tris(1-chloro-2-propyl) phosphate (TCPP), and 2,2-bis (chloromethyl) propane-1,3-diyl-tetrakis (2-chlorethyl) bis (phosphate) (commercially sold as V6). Based on these screening assays, we found that static exposure of zebrafish embryos from 5.25 hours post-fertilization (hpf) (50% epiboly) to 96 hpf to TCEP, TCPP, or V6 concentrations as high as 50 μM resulted in no significant effects on embryonic survival or development, while static exposure during the

same developmental window to TDCPP concentrations of 8 μM and above resulted in a significant increase in mortality and developmental malformations. Based on these initial results, we then focused on identification of susceptible windows of developmental TDCPP exposure using the following exposure scenarios: 1) 0.75 hpf (2-cell) to 2 hpf (64-cell), followed by incubation within clean water to 96 hpf; 2) 0.75 hpf (2-cell) to 96 hpf; and 3) 5.25 hpf (50% epiboly) to 96 hpf. To evaluate uptake and elimination during these exposure scenarios, TDCPP and bis(1,3-dichloro-2-propyl)phosphate (BDCPP, the primary metabolite of TDCPP) concentrations within whole embryos were quantified using LC/MS-MS. Finally, as TDCPP-specific genotoxicity assays have, for the most part, been negative *in vivo* (Bloom 1984; Brusick et al. 1980; Cifone 2005; Morales and Matthews 1980) and zygotic genome remethylation is a key biological event during cleavage (Mhanni and McGowan 2004), we investigated whether TDCPP altered the status of zygotic genome methylation during early zebrafish embryogenesis.

METHODS

Animals. Adult wild-type (5D) zebrafish were raised and maintained on a 14-h:10-h light:dark cycle within a five-shelf stand-alone recirculating system (Aquatic Habitats, Apopka, FL) containing photoperiod enclosures and conditioned reverse osmosis (RO) water (~27-28°C). Adult females and males were bred off- or on-system using breeding traps suspended within 1- or 3-L tanks, respectively, to allow spawned eggs to settle to the tank bottom. For all experiments described below, newly fertilized eggs were staged according to previously described methods (Kimmel et al. 1995). All fish were handled, treated humanely, and treated with regard for alleviation of suffering in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols at the University of South Carolina – Columbia.

Chlorinated phosphate ester (CPE) exposures. Tris(1,3-dichloro-2-propyl) phosphate (TDCPP, 99% purity based on in-house GC-EI-MS analysis), tris(2-chloroethyl) phosphate (TCEP, 97% purity), and tris(1-chloro-2-propyl) phosphate (TCPP, 96% purity) were purchased from ChemService (West Chester, PA), Sigma-Aldrich (St. Louis, MI), and Pfaltz & Bauer (Waterbury, CT), respectively. A commercial mixture of 2,2-bis (chloromethyl) propane-1,3-diyl-tetrakis (2-chlorethyl) bis (phosphate) (V6) was purchased from Hongming Auxiliaries CO., LTD (Jiande, Zhejiang Province, China). Stock solutions were prepared by dissolving chemicals in HPLC-grade dimethylsulfoxide (DMSO). For each experiment, working solutions were prepared fresh by spiking stock solutions into embryo media (EM) (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$), resulting in 0.1% DMSO within all vehicle control and treatment groups.

We first performed static range-finding tests to determine the median lethal concentration (LC_{50}) of TDCPP, TCEP, TCPP, and V6. Starting at 5.25 hours post-fertilization (hpf) (50% epiboly), 20 viable wild-type (5D) embryos per replicate were exposed under static conditions to vehicle (0.1% DMSO) or water-borne TDCPP (0.05-50 μM), TCEP (0.05-50 μM), TCPP (0.05-50 μM), or V6 (0.05-50 μM) within at least two replicate solvent- and RO-rinsed 40-mL glass beakers per treatment until 96 hpf (24 hours post-hatch). All embryos were incubated within 14 mL of treatment solution at 28°C under a 14-h:10-h light:dark cycle. At 96 hpf, gross morphology assessments were conducted under transmitted light, and percent mortality per beaker was averaged across replicates within each treatment.

As TDCPP was the only CPE tested to induce toxicity within developing zebrafish embryos at concentrations <50 μM, we then identified developmental windows sensitive to TDCPP exposure by treating zebrafish embryos (20 per replicate) to vehicle (0.1% DMSO) or

water-borne TDCPP (0.5-9 μM) within three replicate beakers per treatment for each of the following static exposure scenarios: 1) 0.75 hpf (2-cell) to 96 hpf; 2) 2.25 hpf (128-cell) to 96 hpf; 3) 5.25 hpf (50% epiboly) to 96 hpf; 4) 10 hpf (Bud) to 96 hpf; and 5) 24 hpf (Prim-5) to 96 hpf. In addition, embryos (20 per replicate) were exposed during cleavage (0.75 to 2 hpf), blastula (2.25 to 5 hpf), gastrula (5.25 to 10 hpf), segmentation (10 to 24 hpf), or pharyngula (24 to 48 hpf) alone in triplicate glass beakers, and incubated in vehicle control media (0.1% DMSO) pre- and post-exposure until 96 hpf. At 96 hpf, all surviving larvae were evaluated under transmitted light for the following developmental abnormalities: decreased body length, craniofacial malformations, trunk curvature, tail malformations, pericardial edema, and yolk sac edema. Percent mortality and malformations per beaker were averaged across replicates for a total sample size of three beakers per treatment. Lastly, 20 embryos per replicate were exposed to vehicle (0.1% DMSO) or 50 μM TCEP, TCPP, or V6 within two replicate glass beakers per treatment from 0.75 to 96 hpf to confirm that exposures initiated prior to gastrula did not enhance the developmental toxicity of these three CPEs.

TDCPP and BDCPP analysis within whole embryos. TDCPP and bis(1,3-dichloro-2-propyl)phosphate (BDCPP, the primary metabolite) concentrations within vehicle and TDCPP-treated whole zebrafish embryos were quantified using procedures similar to those previously published (Stapleton et al. 2009). Similar to procedures described above, embryos (20 per replicate) were treated with vehicle (0.1% DMSO) or 3 μM TDCPP within nine replicate beakers per treatment for each of the following static exposure scenarios: 1) 0.75 hpf (2-cell) to 2 hpf (64-cell) followed by incubation in vehicle until 24 hpf (Prim-5); 2) 0.75 hpf (2-cell) to 24 hpf (Prim-5); and 3) 2.25 hpf (128-cell) to 24 hpf (Prim-5). At 2, 10, and 24 hpf, embryos were transferred from three replicate beakers to three 2-mL amber vials, snap-frozen in liquid

nitrogen, and stored at -80°C until analysis. Frozen embryos were spiked with 100 µL each of deuterated TDCPP (D15-TDCPP) and deuterated BDCPP (D10-BDCPP) as internal standards, and 0.5 mL acetonitrile (J.T. Baker, Phillipsburg, NJ) was added. Samples were homogenized, sonicated for 20 min, and centrifuged at 10,000xg for 1 min. The supernatant was removed, and the extraction was repeated two additional times, combining all supernatants. The supernatant was concentrated to near-dryness under N_2, resuspended in 400 µL 1:1 methanol:water, filtered through a 0.2-µm nylon membrane, and spiked with deuterated triphenyl phosphate (D27-TPP, Isotech, Miamisburg, OH) and deuterated diphenyl phosphate (D10-DPP) to assess recovery of the internal standards. D15-TDCPP, D10-BDCPP, and D10-DPP were synthesized by Dr. Vladimir Belov (Max Planck Institute for Biophysical Chemistry, Goettingen, Germany).

Samples were analyzed by LC/MS-MS on an Agilent 1200 series LC connected to an Agilent 6410B triple quadrupole MS detector with an electrospray ionization source. Chromatographic separation was performed on a Kinetex XBC18 column (100 mm x 2.1 mm; 2.6 µm) (Phenomenex) using a methanol-water gradient as previously described (Cooper et al. 2011). Analytes were detected using multiple reaction monitoring (MRM) in positive (TDCPP, D15-TDCPP and D27-TPP) and negative (BDCPP, D10-BDCPP and D10-DPP) ionization modes (see Supplemental Material, Table S1). Method detection limits (MDLs) were defined as three times the standard deviation of lab blanks (if present) or three times the noise. MDLs for TDCPP and BDCPP were 0.51 ng and 0.04 ng, respectively. Recoveries averaged 80±15% and 87±14% for the internal standards, D15-TDCPP and D10-BDCPP, respectively.

Genomic DNA methylation analysis using restriction enzymes. Methylation-dependent restriction analysis was used as a genome-wide, screening-level approach to determine whether TDCPP altered zygotic genome methylation during embryogenesis. Similar to procedures

described above, embryos (20 per replicate) were exposed from 0.75 hpf (2-cell) to 24 hpf (Prim-5) to vehicle (0.1% DMSO) or 3 μM TDCPP under static conditions within nine replicate beakers per treatment. At 2, 10, and 24 hpf, 60 embryos were pooled from triplicate beakers and total genomic DNA (gDNA) was extracted using a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) per manufacturer's instructions. Following elution in 40 μL of nuclease-free water, total gDNA concentrations and 260/280 ratios were quantified using a NanoDrop ND-2000 spectrophotometer. All gDNA samples were stored at 4°C until restriction analysis. This experiment was repeated two additional times, resulting in three independent gDNA samples per time-point and treatment.

Methylation-insensitive (HindIII, MspI, NcoI, and NsiI) and methylation-sensitive (AatII, HpaII, NotI, and SalII) restriction endonucleases were purchased from New England Biolabs (Ipswich, MA). MspI (methylation-insensitive) and HpaII (methylation-sensitive) share identical recognition sites (5'-CCGG-3'), and were included to eliminate the possible influence of recognition sites on methylation-independent restriction digestion rates. Using a 25-μL reaction volume, total gDNA (1 μg) was digested for 70 min at 37°C with 10 U of methylation-insensitive or methylation-sensitive restriction endonuclease and optimal 1X NEBuffer per manufacturers instructions. Digested gDNA samples from each treatment group and time-point were separated on 0.5% agarose gels containing ethidium bromide and visualized using a UVP BioSpectrum Digital Gel Imaging system (Upland, CA).

Statistics. To estimate the LC_{50} following static TDCPP exposure from 5.25 to 96 hpf, a four-parameter concentration-response curve was fit to mean percent mortality data using log-transformed TDCPP concentrations within Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). For developmental toxicity and analytical chemistry data, a general linear model (GLM) analysis

of variance (ANOVA) ($\alpha = 0.05$) was used within SPSS Statistics 19.0 (IBM Software, Inc., Armonk, NY) to identify significant overall effects compared to vehicle controls. For toxicity data, pair-wise Tukey-based multiple comparisons of least square means were performed to identify significant within-exposure window effects and/or within-treatment effects compared to vehicle controls or 5.25- to 96-hpf exposure windows, respectively. For analytical chemistry data, pair-wise Tukey-based multiple comparisons of least square means were performed to identify significant within-time-point effects and/or within-treatment effects compared to vehicle controls or 2-hpf time-points, respectively.

RESULTS

TDCPP induces developmental toxicity during zebrafish embryogenesis. To evaluate the relative teratogenic effects of four different but structurally related CPE flame retardants (Figure 1A), we treated zebrafish embryos from 5.25 to 96 hpf to concentrations ranging from 0.05 to 50 μM using static exposure conditions. Based on the highest concentration tested (50 μM), exposure to TCEP, TCPP, and V6 resulted in no significant effects on mortality (Figure 1B), gross developmental malformations, delayed hatching, or obvious signs of impaired locomotion under transmitted light compared to vehicle controls (data not shown). However, static exposure to 50 μM TDCPP initiated at 5.25 hpf resulted in 100% mortality by 96 hpf (Figure 1B), with a median lethal concentration (LC_{50}) of approximately 8.5 μM (Figure 1C). Based on preliminary assessments of locomotion (under transmitted light conditions), acetylcholinesterase (AChE) activity (within whole body homogenates), and secondary motoneuron axon morphology (within intact specimens) at 96 hpf (data not shown),

developmental exposure to all four CPEs examined in this study did not adversely impact these endpoints compared to vehicle controls.

TDCPP exposure during cleavage enhances developmental toxicity. To evaluate potential sensitive windows of embryonic development, we initiated TDCPP exposure at the beginning of five different stages of zebrafish embryogenesis (0.75, 2.25, 5.25, 10, and 24 hpf). As initiation of TDCPP exposures at 2.25, 10, and 24 hpf, as well as exposures within blastula (2.25 to 5 hpf), gastrula (5.25 to 10 hpf), segmentation (10 to 24 hpf), or pharyngula (24 to 48 hpf) alone, did not enhance the toxicity of TDCPP compared to exposures from 5.25 to 96 hpf (data not shown), we relied on toxicity data derived from 5.25- to 96-hpf exposures as a reference for examining the potential for enhanced toxicity resulting from exposures initiated at the start of cleavage (0.75 hpf) or restricted to cleavage alone (0.75 to 2 hpf). Representative images of developmental stages used to define these exposure windows are shown within Figure 2A.

Relative to exposures initiated at 5.25 hpf (50% epiboly), static exposure to 3 μM TDCPP from 0.75 hpf (2-cell) to 96 hpf resulted in a significant increase in mortality and developmental abnormalities (Figures 2B and 2C). Moreover, TDCPP exposures restricted to the cleavage period – 0.75 hpf (2-cell) to 2 hpf (64-cell) – resulted in similar effects on survival and development compared to static exposures from 0.75 hpf (2-cell) to 96 hpf (Figures 2B and 2C). Embryos surviving static exposure to TDCPP from 5.25 to 96 hpf, 0.75 to 96 hpf, or 0.75 to 2 hpf exhibited a range of abnormal phenotypes that were first visible at initiation of the hatching period (48 hpf) regardless of the exposure scenario. Figure 3 shows representative images of the relative severity of these phenotypes following static exposure to 3 μM from 0.75 to 96 hpf. Lastly, exposure to 50 μM TCEP, TCPP, or V6 from 0.75 to 96 hpf resulted in no significant

effects compared to exposures from 5.25 to 96 hpf (data not shown). Overall, these data collectively suggest that the cleavage period is susceptible to developmental TDCPP exposure, and that TDCPP-induced malformations may be a result of random, non-targeted effects at the genomic-level.

TDCPP uptake and BDCPP formation are not associated with enhanced toxicity during cleavage. TDCPP and BDCPP concentrations were quantified within whole embryos at 2, 10, and 24 hpf following exposure to vehicle from 0.75 to 2 hpf, or 3 μM TDCPP from 0.75 to 2 hpf, 0.75 to 24 hpf, or 2.25 to 24 hpf. TDCPP levels within embryos exposed from 0.75 to 24 hpf or 2.25 to 24 hpf increased with time and were significantly higher than levels within embryos only exposed during cleavage (0.75 to 2 hpf) (Figure 4). However, TDCPP levels within embryos from cleavage-only exposures were non-detectable by 24 hpf following transfer to vehicle control water at 2 hpf (Figure 4). Whole-embryo BDCPP concentrations were two- to three-orders of magnitude lower than TDCPP concentrations (Figure 4), suggesting that TDCPP is not readily metabolized to BDCPP within zebrafish embryos, or is further metabolized to unanalyzed metabolites such as 1,3-dichloro-2-propanol.

TDCPP does not affect cell morphology during cleavage. To determine whether TDCPP affected cell morphology during early embryogenesis, embryos exposed to vehicle or 3 μM TDCPP were imaged under transmitted light at six different stages during cleavage (0.75 to 2 hpf). TDCPP had no obvious effect on cell cycle during cleavage, as progression through the cleavage period was concurrent with vehicle-treated embryos (Figure 5). In addition, overall embryo size and cell morphology (size, shape, and viability) within TDCPP-treated embryos were similar to vehicle controls (Figure 5), suggesting that TDCPP does not result in cytotoxicity or cellular abnormalities within the cleavage period of zebrafish embryogenesis.

TDCPP alters genomic DNA methylation during cleavage. By comparing banding patterns following digestion with methylation-insensitive and methylation-sensitive restriction endonucleases, we revealed that TDCPP altered the status of zygotic gDNA methylation at 2 hpf but not 10 or 24 hpf. When digested with methylation-insensitive restriction endonucleases, gDNA was fully digested and no banding pattern differences were observed at any time-point in TDCPP-treated embryos relative to vehicle controls (Figure 6). At 10- and 24-hpf, gDNA extracted from TDCPP-treated embryos was incompletely digested by all methylation-sensitive restriction endonucleases tested, and banding patterns were similar to vehicle-treated controls (Figure 6). However, gDNA extracted from TDCPP-treated embryos – but not vehicle-treated embryos – at the end of cleavage (2 hpf) was completely digested by methylation-sensitive restriction endonucleases, suggesting that normal gDNA methylation at 2 hpf was absent in TDCPP-treated embryos. These data suggest that exposure to TDCPP during cleavage delays remethylation of the zygotic genome in zebrafish embryos, an effect that may be associated with an increased prevalence of malformations and mortality observed for exposures initiated at the start of cleavage (0.75 hpf).

DISCUSSION

The objectives of this study were to begin investigating the effects of CPEs during vertebrate embryogenesis. Using zebrafish as an animal model, we first demonstrated that (1) static exposure from 5.25 to 96 hpf to TCEP, TCPP, or V6 concentrations as high as 50 μM resulted in no significant effects on embryonic development or survival (Figure 1); (2) static exposure from 5.25 to 96 hpf to concentrations of 8 μM TDCPP and above resulted in a significant increase in mortality (LC_{50} = 8.5 μM) compared to vehicle controls (Figure 1); (3)

relative to exposures initiated at 5.25 hpf, static exposure to 3 μM TDCPP from 0.75 hpf (2-cell) to 96 hpf, or 0.75 hpf (2-cell) to 2 hpf (64-cell), resulted in an increase in mortality and developmental abnormalities (Figure 2); and (4) embryos surviving static TDCPP exposure exhibited a range of abnormal phenotypes (Figure 3) that were first visible at initiation of the hatching period (48 hpf). Following characterization of TDCPP toxicity, we showed that TDCPP uptake and BDCPP formation were not associated with enhanced toxicity following exposures that included the cleavage period of development (Figure 4). Moreover, TDCPP exposure did not result in obvious impacts on cell cycle, overall embryo size, or cell morphology during cleavage (Figure 5), suggesting that TDCPP mediates toxicity at the sub-cellular- or genomic-level during this stage of embryogenesis. As TDCPP-specific genotoxicity assays have, for the most part, been negative *in vivo* (Bloom 1984; Brusick et al. 1980; Cifone 2005; Morales and Matthews 1980) and zygotic genome remethylation is a key biological event during cleavage (Mhanni and McGowan 2004), we investigated whether TDCPP altered the status of gDNA methylation, and showed that normal gDNA methylation at the end of cleavage (2 hpf) was absent in TDCPP-treated embryos (Figure 6). Overall, our findings suggest that the cleavage period during zebrafish embryogenesis is susceptible to TDCPP-induced delays in remethylation of the zygotic genome, a mechanism that may be associated with enhanced developmental toxicity following initiation of TDCPP exposure at the start of cleavage.

Despite close structural similarities, TDCPP was the only CPE that, even at high concentrations (50 μM), induced a significant increase in gross malformations and mortality within developing zebrafish embryos (relative to vehicle controls) following exposure from 5.25 to 96 hpf. An absence of developmental TCEP, TCPP, and V6 toxicity may be due to differences in embryonic uptake and accumulation rates across the four CPEs. Within intact non-

dechorionated zebrafish embryos, there is a strong positive correlation between chemical hydrophobicity and the prevalence and magnitude of developmental toxicity, where hydrophobic chemicals (log $K_{ow} > 2$) tend to be more toxic than hydrophilic chemicals (log $K_{ow} < 2$) (Padilla et al. 2012). Out of all four CPEs tested, TDCPP was the most hydrophobic (log $K_{ow} = 3.65$). However, it is unlikely that hydrophobicity alone accounted for the lack of developmental TCEP, TCPP, and V6 toxicity during zebrafish embryogenesis, as (1) the log K_{ow} for TCPP and V6 are 2.59 and 2.83, respectively; (2) very high concentrations (50 μM) were used as an upper-limit concentration within our screening assay; and (3) non-dechorionated zebrafish embryos were immersed within treatment solution from ~72 to 96 hpf following hatching. Rather, the unique structure of TDCPP likely accounts for enhanced biological affinity and toxicity during embryogenesis.

Although TDCPP induced a significant increase in mortality at 8 μM and above when exposures were initiated at 5.25 hpf (50% epiboly), TDCPP was significantly more toxic when exposures were initiated at the start of cleavage or restricted to cleavage alone. Moreover, the developmental toxicity of TDCPP following exposure from 0.75 hpf (2-cell) to 96 hpf, or from 0.75 hpf (2-cell) to 2 hpf (64-cell), was nearly identical, suggesting that cleavage was the most susceptible stage for developmental TDCPP exposure. To determine whether this window of sensitivity was due to differences in TDCPP uptake, yolk sac retention, or increased metabolism, we analyzed whole zebrafish embryo extracts for TDCPP and the primary metabolite BDCPP. For embryos exposed to 3 μM from 0.75 to 24 hpf under static conditions, our results confirmed time-dependent uptake and accumulation of TDCPP within embryos following passive movement across the embryonic protective barrier (chorion) from aqueous treatment solutions. Importantly, higher TDCPP concentrations within whole embryos were not associated with

enhanced toxicity, as embryos exposed within cleavage alone contained no detectable levels of TDCPP by 24 hpf following transfer to clean water at 2 hpf. In addition, our data show that concentrations of BDCPP – the primary metabolite of TDCPP in rodents (Nomeir et al. 1981) – were neither elevated within any of the exposure scenarios nor associated with enhanced toxicity. Overall, our analytical data suggest that (1) TDCPP uptake and elimination is rapid within zebrafish embryos; (2) BDCPP is not the predominant metabolite detected from 2 to 24 hpf; and (3) TDCPP and/or other unanalyzed metabolites are responsible for inducing developmental toxicity within zebrafish embryos.

Our study showed that embryonic exposure to TDCPP from 0.75 to 96 hpf resulted in a range of abnormal phenotypes with varying degrees of relative severity, suggesting that TDCPP does not target a particular organ but rather induces random, non-targeted effects at the genomic-level. Although we expected that TDCPP would affect cell proliferation and morphology during cleavage, our gross assessments of cleavage-stage embryos under transmitted light revealed no visible differences between vehicle controls and TDCPP-treated embryos, suggesting that TDCPP-induced toxicity was likely at the sub-cellular- or genomic-level. Interestingly, zebrafish embryos possess neither active G_1 or G_2 cell cycle checkpoints (Duffy et al. 2005) nor apoptosis signaling pathways (Ikegami et al. 1997) until mid-gastrula (~7 hpf). As such, we suspect that TDCPP exposure induces irreversible sub-cellular or genomic damage that is not manifested until late embryogenesis. While mechanisms of TDCPP toxicity are probably similar within later stages of embryogenesis, active sub-cellular and/or DNA repair mechanisms present following the mid-gastrula period may contribute to partial mitigation of toxicity observed following exposure initiation at 5.25 hpf.

As TDCPP-specific genotoxicity assays have, for the most part, been negative *in vivo* (Bloom 1984; Brusick et al. 1980; Cifone 2005; Morales and Matthews 1980), we used a screening-level, genome-wide restriction analysis approach to determine whether the susceptibility of cleavage was associated with adverse effects on zygotic genome methylation. Interestingly, we found that gDNA within TDCPP-treated embryos – but not vehicle-treated embryos – at the end of cleavage was completely digested by methylation-sensitive restriction endonucleases, suggesting that normal gDNA methylation at 2 hpf was absent in TDCPP-treated embryos. Similar to mammals, zebrafish embryos progress through the maternal-to-zygotic transition (MZT) in two phases: (1) rapid degradation of maternally loaded transcripts and (2) minor and major waves of zygotic genome activation (Tadros and Lipshitz 2009). In zebrafish embryos, the MZT commences near the end of cleavage and terminates at approximately mid-blastula (3 hpf). Prior to the MZT, the zygotic genome of zebrafish embryos undergoes rapid demethylation immediately following fertilization, and steady remethylation from the onset of cleavage to mid-blastula (Mhanni and McGowan 2004). Remethylation is critical for zygotic genome activation and normal somatic development, as exposure of zebrafish embryos to 5-azacytidine (5-azaC) – a potent inhibitor of DNA methylation – during cleavage alone results in gDNA hypomethylation and developmental abnormalities, including shortened or loss of tail, block-shaped somites, and enlarged pericardial cavity (Martin et al. 1999). Therefore, our data suggest that, similar to 5-azaC, TDCPP exposure during early embryogenesis may affect remethylation of the zygotic genome within zebrafish embryos either via direct effects on gDNA and/or indirect effects due to inhibition of DNA methyltransferase activity – a mechanism that is consistent with our findings that (1) cleavage is more susceptible to TDCCP exposure compared

to later stages of embryogenesis and (2) developmental TDCPP exposure results in variable within-treatment phenotypes.

CONCLUSIONS

In summary, our data collectively suggest that the cleavage period during zebrafish embryogenesis is susceptible to TDCPP-induced delays in remethylation of the zygotic genome, a mechanism that may be associated with enhanced developmental toxicity following initiation of TDCPP exposure at the start of cleavage. Interestingly, while TCEP, TCPP, and V6 are structurally similar to TDCPP, the unique structural properties of TDCPP appear to account for enhanced biological affinity and toxicity during zebrafish embryogenesis. While the relevance of our findings to prenatal human exposures within indoor environments is currently uncertain, this study raises questions about the potential health risks of a widely used and detected CPE-based OPFR to early human development. Therefore, our results suggest that further research is needed to better understand the mechanisms of TDCPP-induced toxicity within susceptible windows of vertebrate embryogenesis, as well as the potential health risks of TDCPP exposure to developing human embryos.

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FIGURE LEGENDS

Figure 1. TDCPP induces developmental toxicity during zebrafish embryogenesis. (A) Four structurally related CPE-based FRs screened for toxicity during zebrafish embryogenesis. All four CPEs were recently identified and detected in polyurethane foam collected from baby products (Stapleton et al. 2011). (B) Mean percent mortality (± standard deviation) at 96 hpf following static exposure from 5.25 hpf (50% epiboly) to 96 hpf to vehicle (0.1% DMSO) or water-borne CPE (50 μM). Asterisk (*) denotes significant treatment effects ($p<0.05$) relative to vehicle controls. N = two beakers per treatment. (C) Four-parameter concentration-response curve fit to mean percent mortality (± standard deviation) at 96 hpf following static TDCPP exposure from 5.25 hpf (50% epiboly) to 96 hpf. N = five beakers per treatment for 3 and 4 μM TDCPP, and two beakers per treatment for the remaining TDCPP treatments.

Figure 2. TDCPP exposure during cleavage enhances developmental toxicity. (A) Brightfield images of normal zebrafish stages used to define exposure windows in Panels B and C. (B) Mean percent mortality (± standard deviation) and (C) mean percent malformed (± standard deviation) at 96 hpf following static exposure to TDCPP from 5.25 to 96 hpf, 0.75 to 96 hpf, or 0.75 to 2 hpf. For exposures from 0.75 to 2 hpf, embryos were incubated in vehicle (0.1% DMSO) from 2 to 96 hpf. All surviving 96-hpf larvae that exhibited a range of abnormal phenotypes were included within percent malformation data. Asterisk (*) denotes significant within-exposure window effects ($p<0.05$) relative to vehicle controls. Crosses (†) denote significant within-treatment effects relative to exposures initiated at 5.25 hpf. N = three beakers per treatment.

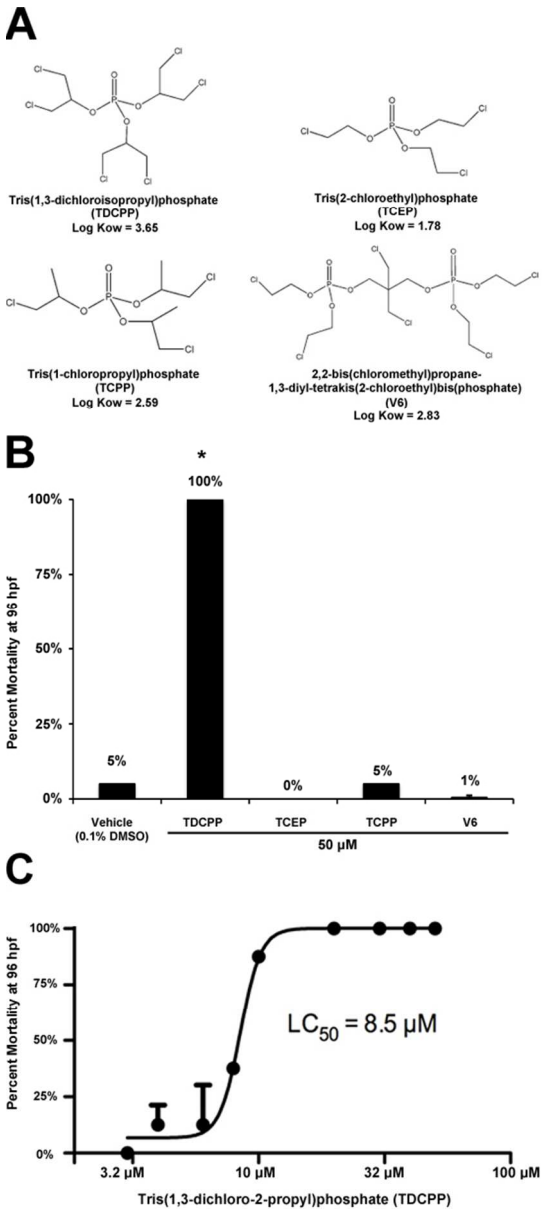
Figure 3. TDCPP exposure results in variable embryonic phenotypes. Zebrafish embryos were treated with vehicle (0.1% DMSO) or 3 μM TDCPP from 0.75 hpf (2-cell) to 96 hpf and assessed for gross malformations under transmitted light at 96 hpf. Images are representative of within-treatment phenotypes observed within surviving larvae following developmental TDCPP exposure. Mild = trunk curvature and/or tail malformations; moderate = trunk curvature, tail malformations, craniofacial malformations, and decreased body length; severe = trunk curvature, tail malformations, craniofacial malformations, decreased body length, pericardial edema, and yolk sac edema.

Figure 4. TDCPP uptake and BDCPP formation are not associated with enhanced toxicity during cleavage. Mean ng TDCPP or BDCPP (± standard deviation) detected within homogenates of 20 whole embryos exposed in triplicate to 3 μM TDCPP. Asterisks (*) denote significant within-exposure window differences in TDCPP or BDCPP concentration ($p<0.05$) relative to vehicle controls. Crosses (†) denote significant within-treatment differences in TDCPP or BDCPP concentrations relative to 2-hpf embryos. Note that TDCPP levels within embryos exposed from 0.75 to 2 hpf alone were non-detectable by 24 hpf. N = three replicate embryo pools per treatment and time-point.

Figure 5. TDCPP does not affect cell morphology during cleavage. Brightfield images of six different stages within cleavage for embryos treated with vehicle (0.1% DMSO) or 3 μM TDCPP under static conditions. Note that TDCPP exposure did not appear to have adverse impacts on gross cell morphology (size, shape, viability) or cell cycle progression during cleavage.

Representative images of 60 embryos treated with vehicle or TDCPP are shown.

Figure 6. TDCPP alters genomic DNA methylation during cleavage. Genomic DNA (gDNA) extracted from TDCPP-treated embryos – but not vehicle-treated embryos – at the end of cleavage (2 hpf) was completely digested by methylation-sensitive restriction endonucleases (white arrows in upper-right gel), suggesting that normal gDNA methylation at 2 hpf was absent in TDCPP-treated embryos. Representative gels from three independent gDNA samples per time-point and treatment are shown.



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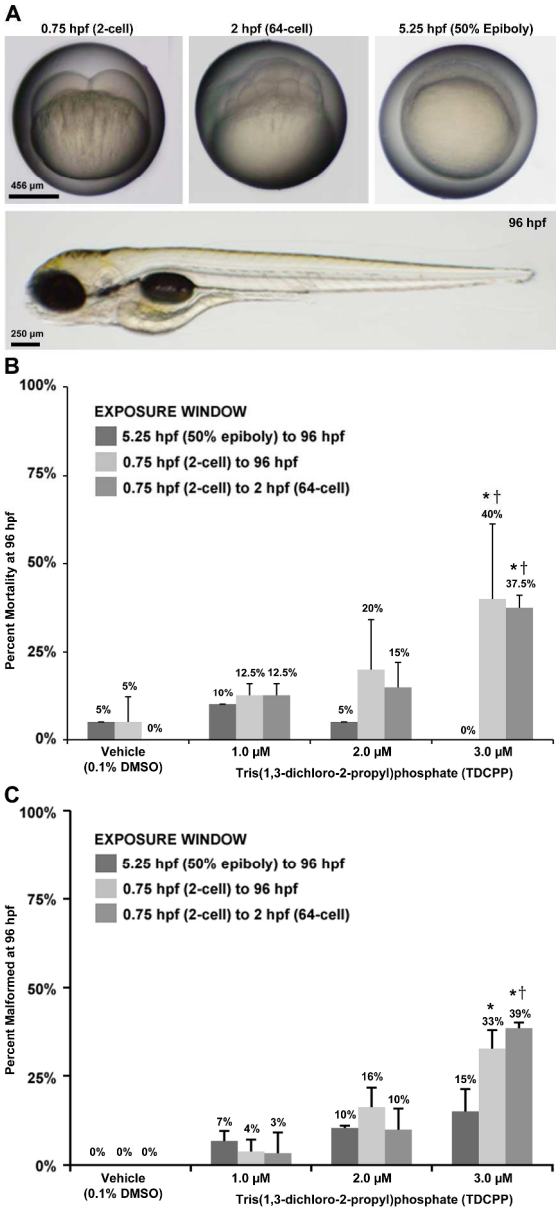

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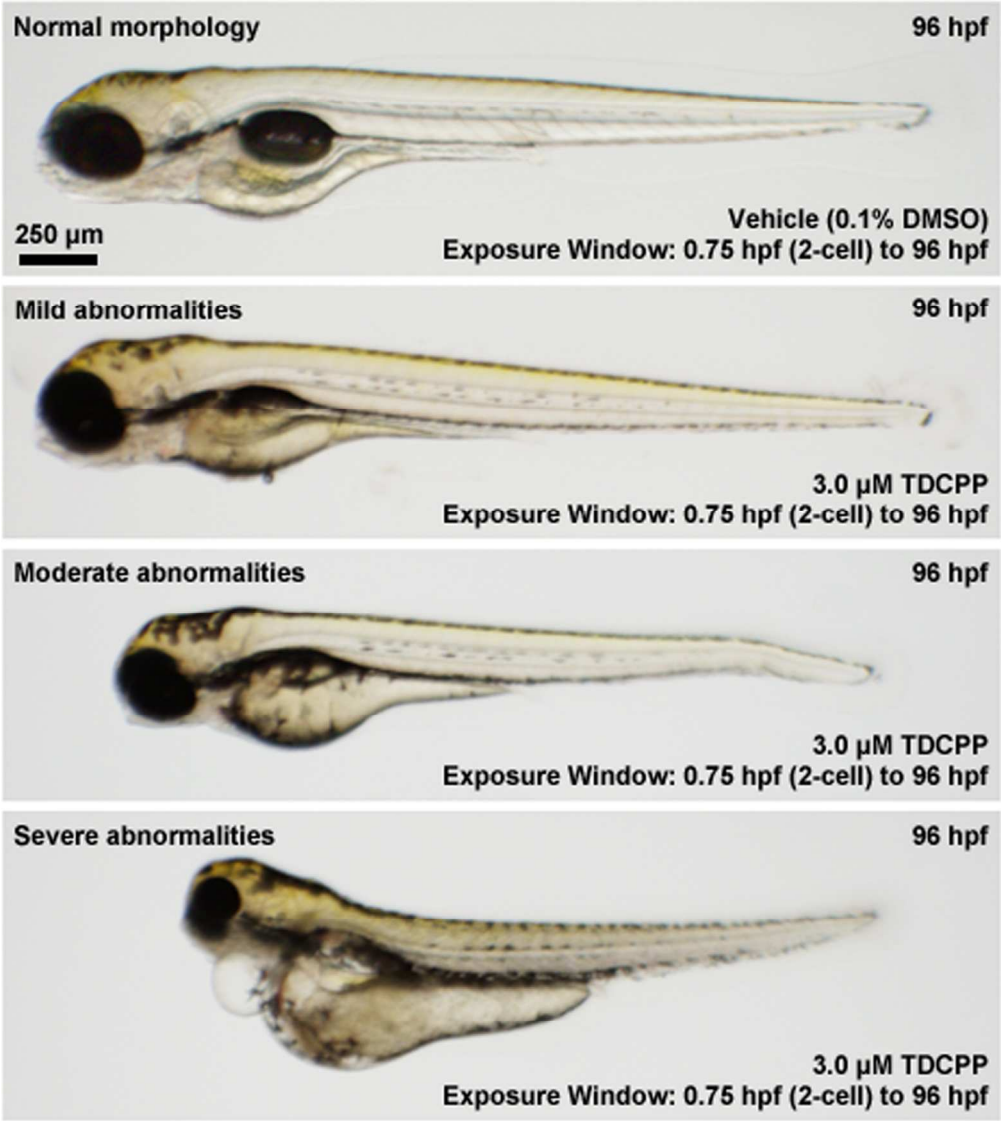

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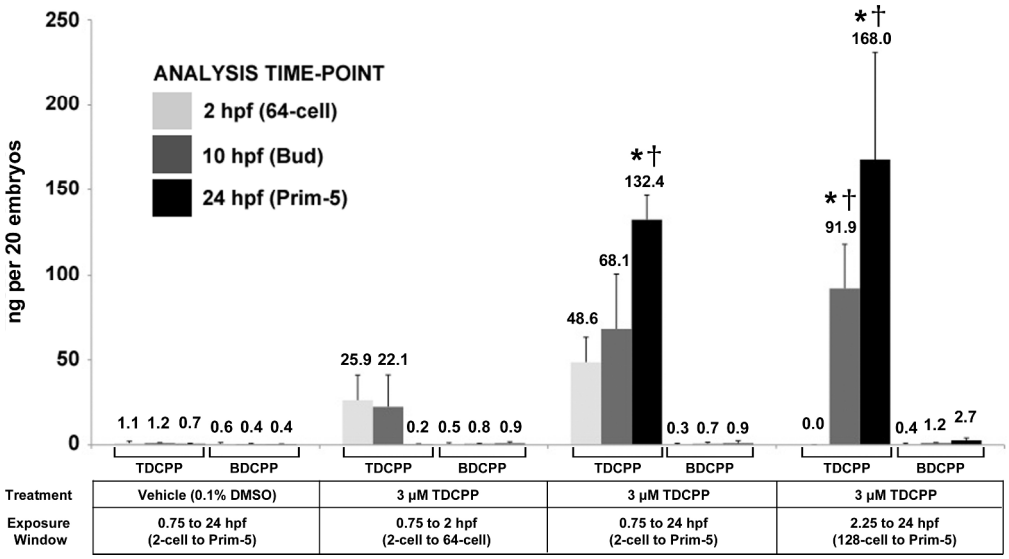

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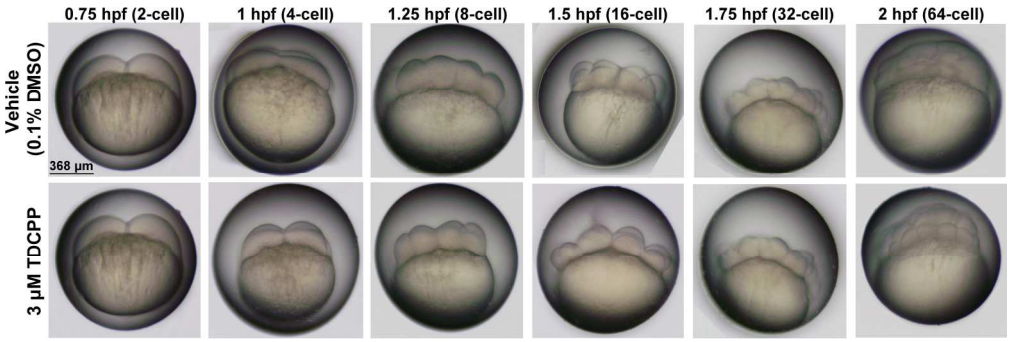

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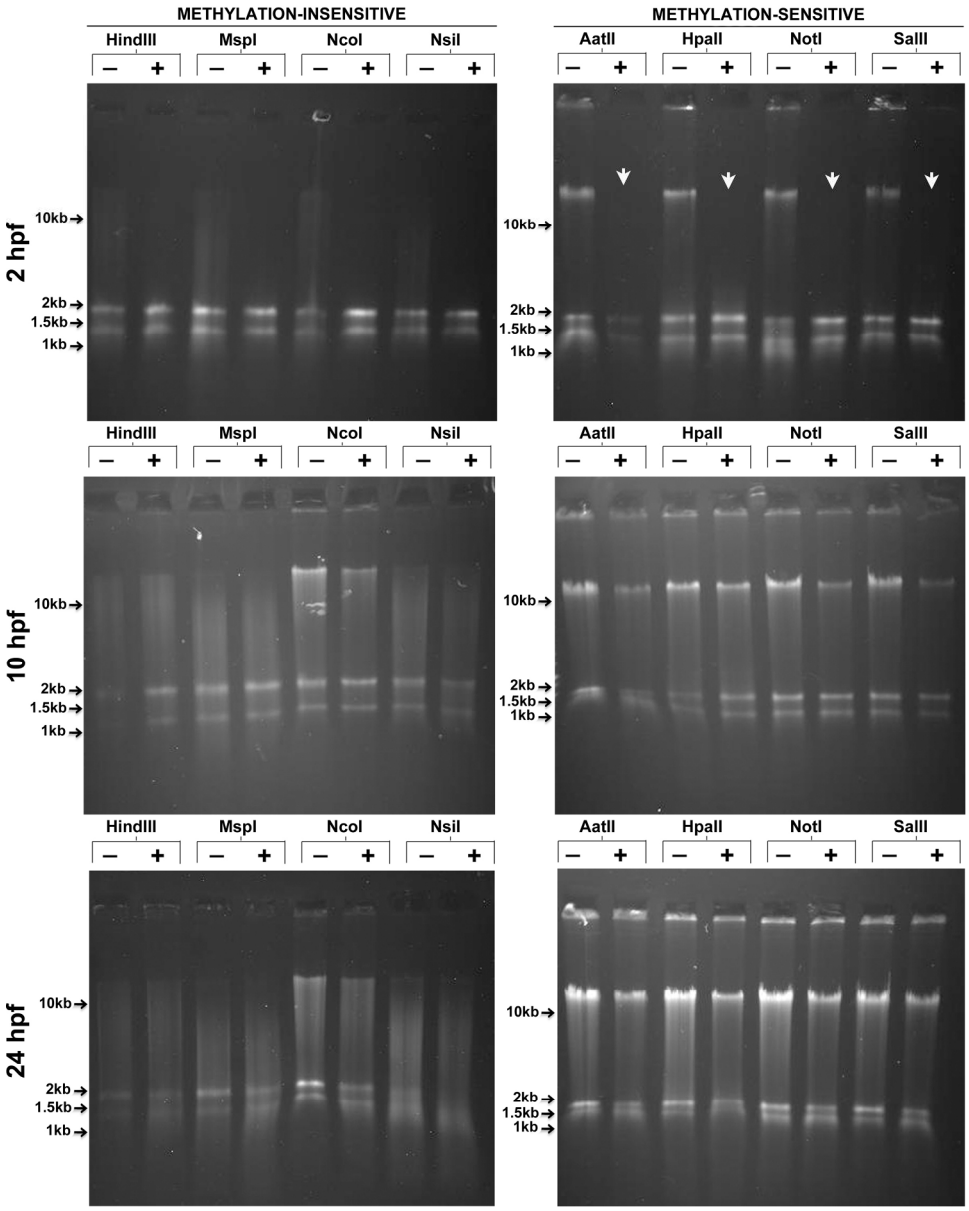

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