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Detection of endocrine disrupting chemicals in *Danio rerio* and *Daphnia pulex*: Step-one, behavioral screen

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HIGHLIGHTS

- 24-hr exposure protocol utilized for development of high throughput assay.
- Evaluated effects of 9 contaminants of emerging concern on swimming behavior.
- Distance or angular change altered in *D. pulex* (6 out of 9), *D. rerio* (4 out of 9).
- Behavioral response and sensitivity to CECs varied across species.
- Behavioral assay identified sub-lethal concentrations for genomic studies (step 2).

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ABSTRACT

Anthropogenic surface and ground water contamination by chemicals is a global problem, and there is an urgent need to develop tools to identify and elucidate biological effects. Contaminants of emerging concern (CECs) are not typically monitored or regulated and those with known or suspected endocrine disrupting potential have been termed endocrine disrupting chemicals (EDCs). Many CECs are known to be neurotoxic (e.g., insecticides) and many are incompletely characterized. Behavioral responses can identify chemicals with neuroactive properties, which can be relevant to EDC mechanisms (e.g., neuroendocrine disturbances). Two freshwater species, *Daphnia pulex* and *Danio rerio*, were evaluated for swimming behavior alterations resulting from 24-hr exposure to 9 CECs: triclosan, triclocarban, chlorpyrifos, dieldrin, 4-nonylphenol, bisphenol-A, atrazine, metformin, and estrone. This is the first step in the development of a bioassay for detecting estrogenic and/or anti-androgenic activity with the goal to evaluate complex mixtures of uncharacterized contaminants in water samples. The second step, described in a subsequent report, examines transcriptome alterations following chemical exposure. Significant differences in the swimming behavior response and sensitivity were found across chemicals within a species and across species for a given chemical in this unique optical bioassay system. In the concentration ranges studied, significant behavioral alterations were detected for 6 of 9 CECs for *D. pulex* and 4 of 9 CECs for *D. rerio*. These results underscore the utility of this bioassay to identify behavioral effects of *sublethal* concentrations of CECs before exploration of transcriptomic alterations for EDC detection.

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1. Introduction

As many as 1500 new chemical entities are estimated to be synthesized each year (USGAO, 2017). Many of these chemicals are found in the environment and worldwide surface and ground water contamination from urban, agricultural, and industrial sources are well documented (Halling-Sorenson et al., 1998; Ternes, 1998; Daughton and Ternes, 1999; Kolpin et al., 2002a; Kummerer, 2009; Lapworth et al., 2012; Yang et al., 2012; Sorensen et al., 2015; Bradley et al., 2018; Li et al., 2018).

Chemical contaminants that are not routinely regulated or monitored and are known or suspected to cause adverse ecological or human health effects are referred to as *emerging contaminants* or *contaminants of emerging concern* (CECs) by the United States Geological Survey (USGS, 2019). A large number of the structurally diverse CECs found in water are known or suspected endocrine disrupting chemicals (EDCs). These include pesticides, pharmaceuticals, flame retardants, plasticizers, detergent metabolites (e.g., alklyphenols), steroids and steroid hormones, and other organic waste products (Kolpin et al., 2002b; Kohno et al., 2018).

EDCs interfere with normal hormone homeostatic mechanisms, which can include enzyme and receptor-mediated mechanisms, synthesis or degradation of hormones or hormone receptors, and the regulation of or timing of hormone release (Mihaich et al., 2017; Sifakis et al., 2017). Endocrine disruption in aquatic vertebrates has been reported to occur in association with environmental xenobiotic exposure (Desforges et al., 2010; Hayes et al., 2011; Schultz et al., 2013; Orlando and Ellestad, 2014; Kohno et al., 2018). Feminization of vertebrates, such as male fish, is frequently reported in association with CEC contaminated surface water and this has been attributed to estrogenic or anti-androgenic EDCs as a probable causal factor (Hayes et al., 2011; Jobling et al., 2006; Kavanagh et al., 2004; Kidd et al., 2007; Kiyama and Wada-Kiyama, 2015; Rempel and Schlenk, 2008; Vajda et al., 2011). There is also evidence for endocrine disruption in humans from environmental exposure (Agopian et al., 2013; Cheek et al., 1998; Gore et al., 2015; Sifakis et al., 2017). The estrogenic activity of individual CECs has been reported (e.g., Andersen et al., 2002; Campbell et al., 2006; Sanborn and Yu, 1973; Zhao et al., 2014; Yu et al., 2015; Watanabe et al., 2017).

Anthropogenic water contamination with complex chemical mixtures is a global phenomenon (Ternes, 1998; Kolpin et al., 2002b; Williams et al., 2009; Wilson and Schwarzman, 2009; Diamond et al., 2015; Petrie et al., 2015; Burket et al., 2018; Guruge et al., 2019). Identifying chemicals most responsible for significant biological impacts is very challenging given the complex mixture of constituents, the potential for additive or synergistic effects (Wang et al., 2017), and the ability of some compounds to bioaccumulate or biomagnify (McLeod et al., 2015). Some of the CECs that are suspected to be estrogenic or anti-androgenic (e.g., atrazine, triclosan, triclocarban, metformin, dieldrin, chlorpyrifos) may act through alternate molecular pathways that do not include steroid receptor binding (Ahn et al., 2008; Chung et al., 2011; Crago and Klaper, 2011; Kiyama and Wada-Kiyama, 2015; Serra et al., 2018). High throughput biological assays can be used to detect endocrine disrupting properties of xenobiotics, including estrogenic and anti-androgenic effects of CECs. For example, Nishihara et al. (2000) used a high throughput yeast two-hybrid assay to detect estrogen receptor activity in a diverse array of 517 chemicals relative to 10% of activity of a 10^{-7} M 17β-estradiol standard, and found that CECs exhibited a wide range of estrogenic activity. Chemicals relevant to the study by Nishihara et al. (2000) that showed very weak estrogen receptor activity (concentrations > than 1×10^{-4} M relative standard) included 4-nonylphenol, atrazine, dieldrin, and chlorpyrifos-methyl (metformin, triclosan and triclocarban were not studied). To detect EDC activity associated with alternate molecular pathways, high throughput bioassays which include more components of native biological systems, from cells to whole organisms, are needed. A few recent reports have underscored the potential of whole-transcriptome profiling of ecological species as a method to evaluate complex chemical mixtures of concern (Basu et al., 2019; Ewald et al., 2020).

The evaluation of EDC activity in water is further complicated by the fact that exposure may involve: (1) complex and incompletely characterized mixtures, (2) species-dependent expression of different estrogen receptor isoforms, (3) species-dependent differences in sensitivity to EDCs, and (4) chemical interactions that influence the endocrine disrupting potential of a mixture (Backhaus, 2016; Kohno et al., 2018; Wang et al., 2017). Aquatic animal-based bioassay systems can complement *in vitro* or *in situ* techniques and enable the detection of endocrine disrupting mechanisms that may otherwise be missed by assays using expressed receptor systems and the results may more readily translate to aquatic ecosystems.

Since exposure to some CECs or EDCs (e.g., insecticides) can affect the development and/or function of the nervous system (Eddins et al., 2010; Rauh et al., 2012; Abreu-Villaca and Levin, 2017), it is also important to assess the behavioral effects of these chemicals as an index of potential neurotoxic effects, including altered neurobehavioral development. The hypothalamic-pituitary axis is the central command center for the endocrine system in vertebrates and the importance of nervous and endocrine system interactions is well established for many invertebrates (Hartenstein, 2006). In addition, the acquisition of behavioral data provides a reference for examining the relative sensitivity of behavioral versus non-behavioral responses through comparison of exposure dose or concentration (e.g., aquatic animals) and can provide insight into the nature of water samples containing unknown chemical contaminants.

We examined the effects of 24-hr exposure to nine different known or suspected endocrine disrupting CECs: estrone, bisphenol-A, 4-nonylphenol, dieldrin, chlorpyrifos, atrazine, metformin, triclosan, and triclocarban in two well studied model organisms, a vertebrate, *Danio rerio* (zebrafish), and an invertebrate, *Daphnia pulex* (waterflea). Although *Daphnia* have an ecdysteroid system that differs from the vertebrate steroid hormone system, they are very sensitive to environmental change (Colbourne et al., 2011) and are used extensively to test the toxicity of chemicals like pesticides (US-EPA, 2002). *Daphnia pulex*, a keystone species (Altshuler et al., 2011; Zuykova et al., 2018), was used as a chemical biosensor in this study (e.g., see Antczak et al., 2013) with relevance to freshwater ecosystems, and this enabled comparisons of invertebrates using a ecdysteroid system (Miyakawa et al., 2018) to the vertebrate steroid-based hormone system. *Danio rerio* served as a model vertebrate with relevance to other aquatic and terrestrial vertebrates including humans (Ablain and Zon, 2013; Balasubramanian et al., 2019). The effects of CEC exposure on these two model organisms was measured as alterations in swimming behavior. This study demonstrates the potential utility of a two-species live animal bioassay system to detect water contaminants that may have (1) neuroactive components and/or (2) estrogenic-like activity caused by estrogen-receptor or non-estrogen receptor mediated mechanisms. The study is part of a two-organism based bioassay system under development that combines behavioral and genomic approaches as a two-step process to provide biological endpoints that enable the assessment of EDC activity in water, complement analytical chemistry, and serve as a tool for evaluating mitigation efforts.

2. Materials and methods

2.1. Animals

2.1.1. Daphnia pulex

Daphnia pulex were collected from a pond at the Michigan State University Kellogg Biological Station in 2008. The culture has been maintained at WSU in COMBO media (Kilham et al., 1998) at a temperature of 21 ± 0.5 °C in 2L glass beakers with a light:dark cycle of 16:8 h. The animals were fed 3 times a week with 50/50 mixture algae composed of *Ankistrodesmus falcatus* and *Desmodesmos* and the COMBO medium was replaced twice per week. Adult *D. pulex* used in experiments were obtained by filtering the culture through a plastic mesh and capturing adult animals that were a minimum of 1.4 mm in length (Zein et al., 2014).

2.1.2. Danio rerio

Danio rerio (AB lineage) adult zebrafish were maintained in reverse osmosis (RO) water buffered with Instant Ocean salts (60 mg/L; Aquarium Systems Inc, Mentor, OH, USA) on a 14:10 h light/dark cycle. Temperatures were maintained at 27°C-29 °C and pH at 7–7.5. Fish were fed a mixture of Zeigler Adult Zebrafish Diet (Zeigler Bros. Inc, Gardners, PA, USA), Spirulina Flake Fish Food (Ocean Star International, Snowville, UT, USA), and 300–500 μm Golden Pearls (Aquatic Foods Inc, Fresno, CA, USA) two times daily.

Embryos (2 hpf) were collected, cleaned, and bleached (0.6% solution) from spawns (1 male: 2 females). They were then transferred to a fresh solution containing RO water and 60 mg/L Instant Ocean salts in glass petri dishes and raised (28 °C; 14:10 h light/dark cycle) with daily water changes. 96 hpf zebrafish larvae were utilized in all bioassays.

Larval zebrafish were euthanized at 120 hpf via an immersion bath with 0.4 g/L tricaine methanesulfonate (MS-222) and 0.66 g/L sodium bicarbonate, followed by the addition of a drop of 6.25% bleach as an adjunctive method of euthanasia (AVMA Guideline for the Euthanasia of Animals: 2013 Edition). Animal use protocols were approved by the Wayne State University Institutional Animal Care and Use Committees, according to the National Institutes of Health Guide to the Care and Use of Laboratory Animals (Protocol No. 16-03-054).

2.2. Chemicals and solutions

A structurally diverse set of nine different chemicals known or purported to possess potential estrogenic- or anti-androgenic activity (estrogenic-like activity) were selected for study (see Kiyama and Wada-Kiyama, 2015; Crago and Klapper, 2018; Rochester et al., 2017; Serra et al., 2018). The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The CAS numbers and purity for them are as follows (CAS; purity): 4-n-nonylphenol (104-40-5; analytical standard), atrazine (1912-24-9; 98.1%) bisphenol-A (80-05-7; 100%), chlorpyrifos (2921-88-2; 99.8%), dieldrin (200-484-5; 97.8%). Estrone (53-16-7; ≥99%) was purchased from Spectrum Chemicals (Gardena, CA) and metformin hydrochloride (1115-70-4; 100%), triclocarban (101-20-2; 100%), triclosan (3380-34-5; 100%) were purchased from USP (Rockville, MD). Stock solutions were made in culture media (metformin) or the solvents, acetone (4-nonylphenol, atrazine, bisphenol-A, chlorpyrifos, dieldrin, triclocarban, triclosan) or DMSO (estrone) based on the solubility of the compound. The initial concentration range selected for the chemicals studied was based on reports of toxicity in the literature, particularly estimates of lethality where available (e.g., LC_{50}) for *D. pulex* or *D. rerio* (Arboleda et al., 2013; Bendis and Relyea, 2014; Wang et al., 2017; Chow et al., 2013; Zein et al., 2014, 2015). The goal was to include a high sublethal concentration for 24 h exposure and then make 2-fold serial dilutions to test for behavioral effects. Greater spacing between concentrations was used for atrazine (10-fold dilutions), estrone (10-fold dilutions), and metformin (5-fold dilutions) to explore a broader concentration range since atrazine did not appear to be as acutely toxic as the other chemicals (Gorge and Nagel, 1990; Wang et al., 2017), estrone was not expected to be acutely toxic even at concentrations well above physiological levels in fish, and the toxicity of metformin in these aquatic animals did not appear to be reported in the literature.

The concentration of the stock solution was generally 10 mM except for atrazine, which was 100 mM. The concentration of acetone or DMSO was not more than 0.05% in the highest concentration used for all chemicals evaluated except for atrazine, which was 0.1% acetone for the highest concentration. All the stock solutions were made as 10 mM or 100 mM (atrazine) concentrations and were then serially diluted with the appropriate media (COMBO for *D. pulex* or fish media for *D. rerio*) to make the test concentrations. The concentration of vehicle in the control for each experiment matched the highest concentration used in the exposure group.

2.3. Behavior Assay

2.3.1. Exposure system

Depending on the species studied, single adult female *D. pulex* or 4dpf zebrafish embryos were loaded into each individual well of 24-well plates (Falcon polystyrene 24-well plate, Fisher Scientific, Waltham, MA, USA) using a plastic water dropper. The *D. pulex* were transferred into the plates at room temperature (~20 °C). Zebrafish embryos were maintained at 28 °C in fish media on a warming pad prior to placing the animals into the 24-well plates. Video recording of behavior commenced within 5 min after application of all test solutions on the 24-well plate and was recorded every 10 min for 24 h. Animals were maintained at constant temperature for the duration of an experiment by sandwiching the 24-well plate between a translucent temperature control flow cell made from glass (below) and a separate heated plexiglass cover (above; see Fig. 1).

To maintain constant media temperature in the 24-well plates, a Polyscience (Niles, IL, USA) water bath circulator (model: AD07R-20-A11B) with Polycool HC-50 coolant was used to cool or warm the temperature flow cell as needed. The plexiglass cover prevented media evaporation and fogging was eliminated by gentle warming with a nichrome wire. This experimental setup enabled the maintenance of appropriate temperatures for *D.* pulex: (21° ± 0.2C) and *D. rerio* (28° ± 0.2C) within the 24-well plate for 24-hr experiments.

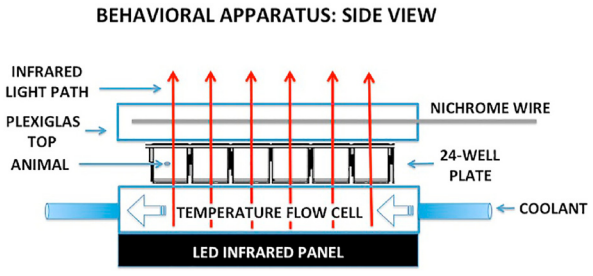

Fig. 1. A side view of the behavioral apparatus used to measure swimming behavior of the aquatic animals is shown. Constant temperature for *D. pulex* (21 °C) and *D. rerio* (28 °C) is maintained in the 24-well plate containing the animals in media by a glass temperature flow cell underneath and a Plexiglas top warmed with a nichrome wire on top. Infrared light is transmitted by a panel below the temperature flow cell and the silhouettes of the swimming animals is recorded by a digital camera using a telecentric lens.

An infrared LED light panel (Edmund Scientific, Barrington, NJ, USA) was placed underneath the temperature flow cell for backlighting animals in order to track animal movement (see Fig. 1). Animal swimming behavior was recorded using a Lumenera (Ottawa, Ontario, CA) digital camera (Model: Infinity 35-1URM) that was connected to an Opto-engineering (Houston, TX, USA) telecentric lens (Model TC 12 120) equipped with an infrared filter (850 nM band pass, Advanced Illumination).

One species and one chemical were studied at a time and the location of specific treatments within the 24-well plate was randomized for each experiment using an online routine (random.org). The 24-well plates were prepared with 6 different concentrations including the appropriate vehicle control and 4 replicates. The concentration of the vehicle control was based on the highest test concentration. An animal was placed in each well and various concentrations of potential toxicants were then randomly administered to each well resulting in a total volume of 3.3 ml per well. Well location (1–24) was recorded and plate number (1,2) was evaluated during statistical analysis. The time from the beginning of the application of toxicants to the 24-well plate to the beginning of video recording was less than 5 min.

2.3.2. Video Recording and Optical Tracking

The duration of the video recordings for each species was based on the relative level of baseline activity of the animals. Five second recordings were used for *D. pulex* (more active) and 20 s was used for *D. rerio* (less active). A camera speed of 28 frames per second (fps) was used for all recordings and this generated 148 frames per video file for *D. pulex* and 592 frames per video file for *D. rerio.* Recordings were made every 10 min over the 24-hr exposure period. These 144 videos were collected and analyzed using Image Pro Premier software (Media cybernetics, Rockville, MD, USA).

Image analysis by the Image Pro Premier software of the recordings associated with each 24-well plate generated an Excel spreadsheet with two variables calculated for the 148 frames in each video: distance (mm) and angular change (degrees). The distance moved was measured by the software across 2 successive frames and angular change was measured as the change in angle occurring in the third frame out of set of three successive frames. The angular change in direction (degrees) is determined by the animal location in the third frame relative to the line created by the two points representing animal locations in the first two frames (see Zein et al., 2014). An Excel macro was then used as a data reduction step, and it calculated two values for each recording period: the *maximum* accumulated distance (mm) and the *mean* angle (mean angular change in degrees).

Twenty-nine videos were selected from the 144 recordings to represent the time-course for behavioral effects observed in each species over the 24-hr exposure period. Out of the 144 videos generated, videos were selected every 10 min for the first 3 h, then every 2 h for the next 6 h, and finally every 4 h for the last 12 h. Greater time-resolution was required for the behavioral responses during the first 3 h of the time-course because the responses tended to occur relatively rapidly, and the later time-points provided information on the duration and longevity of responses and 24-h survival (see Zein et al., 2014, 2015), a requirement for subsequent genomic studies.

2.3.3. Design and statistics

A separate two-way analysis of variance (ANOVA) was used for the analysis of behavioral results obtained from each aquatic organism. The experiments conducted included: 9 chemicals x 6 concentrations (including vehicle control) x 2 organisms, for a total of 108 defined exposures. Two 24-well plates were used to test the responses of each organism to every one of the 9 chemicals, giving a total sample size of 8 animals per chemical concentration (see Video Recording and Optical Tracking**,** above). Depending on outcome, additional concentration ranges were studied for selected chemicals (see chlorpyrifos, dieldrin, metformin, triclocarban; Table 1). When including both species, the total number of concentration-response curves examined was 14 (e.g., see Table 2) and a total of 1344 animals were utilized for the behavioral assay. The dependent variables were maximum accumulated distance and mean angle. The independent factors were concentration (6 levels including vehicle control with concentration 0) and the repeated measure, time (29 time points over 24-hr period). A Least Significant Difference (LSD) test was used for planned comparisons between controls and chemically treated organisms for each dependent variable whenever a significant concentration or time x concentration F-ratio from ANOVA was significant. A P-value of less than 0.05 was considered significant in all inferential tests.

3. Results

The results obtained from all chemical exposures are listed in alpha-numeric order in Tables 1 and 2. Table 1 lists the P-values obtained from ANOVA with concentration as one factor and time as the repeated measure. Each cell in Table 1 indicates the P-value for the concentration effect (upper value) and concentration × time interaction (lower value) obtained from ANOVA for each chemical for the two species. Table 2 is organized in a similar fashion, but instead of P-values it lists the specific concentrations identified as eliciting significant changes in swimming behavior using the LSD test.

All of the chemicals listed elicited a significant behavioral response, with the exception of bisphenol-A and estrone. Significant effects of 24-hr exposure on the swimming behavior of *D. pulex* at concentrations less than 8 μM was found for 4-nonylphenol, chlorpyrifos, dieldrin, and triclosan. *D. rerio* were less sensitive to the organophosphate insecticide, chlorpyrifos, than *D. pulex* with no behavioral effects up to 1 μM exposure, but *D. rerio* were more sensitive to the organochloride insecticide, dieldrin, where lethality was observed at concentrations above 2.5 μM and stimulation of swimming behavior was observed at concentrations as low as 0.25 μM. *D. pulex* swimming behavior was stimulated at a higher concentration range of 2.5–62.5 μM dieldrin without lethality over the 24-hr period. Similar to *D. pulex, D. rerio* swimming behavior was also significantly affected by 4-nonylphenol and triclosan at concentrations below 8 μM, but in contrast to *D. pulex,* which was not affected, *D. rerio* swimming behavior was affected by exposure to metformin at concentrations less than 8 μM. Effects on swimming behavior is described for each chemical in the text below and in Figs. 2–5, addressing the outcome for *D. pulex* first, then *D. rerio.* The figures depicting significant behavioral effects at more than one concentration below 8 μM are organized by the type of chemical exposure, beginning with the alkylphenol, 4-nonylphenol (Fig. 2), then the two insecticides, chlorpyrifos and dieldrin (Fig. 3), a pharmaceutical, metformin (Fig. 4), and the personal care product, triclosan (Fig. 5).

The time-course for effects of 4-nonylphenol is shown in Fig. 2. The time-course for effects of the remaining chemicals is not shown (Figs. 3–5) to simplify presentation of results. Since maximum accumulated distance and mean angle are mathematically related (more intense turning behavior most often results in less distance covered), the shape of the concentration-response curves for these two variables often tends to appear reciprocal, with high values of accumulated distance corresponding to lower mean turning values and vice versa.

Table 1
Statistical analysis of CEC effects on swimming behavior: ANOVA with repeated measures[1]. [P-values in each cell from top to bottom: concentration effect, concentration x time[1] effect].

Contaminants of Emerging Concern (concentration range studied)	*D. pulex* Distance	*D. pulex* Angle	*D. rerio* Distance	*D. rerio* Angle
4-Nonylphenol (0.25 μM–4 μM)	P < 0.05 P > 0.20	P < 0.001 P < 0.001	P > 0.10 P < 0.001	0.05 < P < 0.10 P < 0.001
Atrazine (0.16 μM–100 μM)	P < 0.001 P > 0.50	P > 0.05 P > 0.50	P > 0.20 P < 0.01	P > 0.50 P < 0.005
Bisphenol A (4 μM–64 μM)	P > 0.20 P > 0.50	P > 0.50 P > 0.10	P > 0.10 P > 0.20	0.05 < P < 0.10 0.05 < P < 0.10
Chlorpyrifos (0.00031 μM–0.005 μM)	P > 0.50 P > 0.50	P > 0.50 P > 0.50	_	_
Chlorpyrifos (0.015 μM–0.25 μM)	P < 0.001 P < 0.05	P < 0.001 P < 0.001	_	_
Chlorpyrifos (0.062 μM–1.0 μM)	_	_	P > 0.50 P > 0.50	P > 0.20 P > 0.20
Dieldrin (0.0625 μM–1 μM)	_	_	P < 0.001 P < 0.001	P < 0.01 P < 0.05
Dieldrin (0.10 μM–62.5 μM)	P < 0.01 P > 0.50	P > 0.50 P < 0.005	> 2.5 μM Lethal	> 2.5 μM Lethal
Estrone (0.0062 μM–0.1 μM)	P > 0.10 P > 0.50	P > 0.20 P > 0.50	_	_
Metformin (0.01 μM–100 μM)	P > 0.50 P > 0.50	P > 0.50 P > 0.20	P ~ 0.072 P > 0.50	P < 0.05 P > 0.20
Metformin (25 μM–400 μM)	P > 0.20 P > 0.50	P > 0.20 P > 0.50	_	_
Triclocarban (0.0062 μM–0.1 μM)	P > 0.50 P > 0.50	P > 0.20 P ~ 0.051	P > 0.50 P > 0.50	P > 0.50 P > 0.20
Triclocarban (0.5 μM–8 μM)	P > 0.50 P ~ 0.059	P > 0.50 P < 0.05	Lethality 25–50% across all conc.	Lethality 25–50% across all conc.
Triclosan (0.125 μM–8 μM)	P < 0.05 P < 0.05	P < 0.05 P > 0.50	P > 0.10 P < 0.05	P > 0.50 P > 0.20

(–) indicates experiment not performed.

Table 2
Concentrations of CECs having a significant effect on swimming behavior.

Contaminants of Emerging Concern (concentration range studied)	*D. pulex* Distance	*D. pulex* Angle	*D. rerio* Distance	*D. rerio* Angle
4-Nonylphenol (0.25 μM–4 μM)	2.0, 4.0 μM	0.25–4.0 μM	0.25, 0.5 μM	0.25, 0.5, 4.0 μM
Atrazine (0.16 μM–100 μM)	20–100 μM	N.D.	4 μM	4 μM
Bisphenol A (4 μM–64 μM)	N.D.	N.D.	N.D.	N.D.
Chlorpyrifos (0.00031 μM–0.005 μM)	N.D.	N.D.	_	_
Chlorpyrifos (0.015 μM–0.25 μM)	0.062–0.25 μM	0.062–0.25 μM	_	_
Chlorpyrifos (0.062 μM–1.0 μM)	_	_	N.D.	N.D.
Dieldrin (0.0625 μM–1 μM)	_	_	0.25–1.0 μM	0.062, 0.25, 0.5, 1 μM
Dieldrin (0.10 μM–62.5 μM)	2.5–62.5 μM	62.5 μM	> 2.5 Lethal	> 2.5 Lethal
Estrone (0.0062 μM–0.1 μM)	N.D.	N.D.	_	_
Metformin (0.01 μM–100 μM)	N.D.	N.D.	0.01, 1, 10 100 μM	0.01, 10, 100 μM
Metformin (25 μM–400 μM)	N.D.	N.D.	_	_
Triclocarban (0.0062 μM–0.1 μM)	N.D.	N.D.	N.D.	N.D.
Triclocarban (0.5 μM–8 μM)	8 μM	8 μM	Lethal (all)	Lethal (all)
Triclosan (0.125 μM–8 μM)	0.5–4 μM	2–4 μM	0.125–0.25 μM	N.D.

N.D. indicates not significantly different. (–) indicates experiment not performed.

3.1. 4-nonylphenol

The time-course for behavioral responses to the alkylphenol, 4-nonylphenol, over 24 h are shown for *D. pulex* and *D. rerio* on the right side of Fig. 2. The overall 24 h mean values for concentration-response are shown on the left side. Exposure of *D. pulex* to 4-nonylphenol elicited a concentration-dependent decrease in maximum accumulated distance at 2 μM and 4 μM concentrations (Upper panel - Fig. 2 A and B, Tables 1 and 2). There was a significant concentration-dependent increase in mean angle, particularly at the 2 μM and 4 μM concentrations (Fig. 2C, Table 2). Based on the significant concentration by time interaction, this increase was sustained across the time course 30 min after exposure (Fig. 2 D, Table 1). Note that the measures for maximum accumulated distance and mean angle tend to have opposite concentration-dependent changes in direction relative to each other (Upper panel – Fig. 2 A and C).

There was no overall concentration-dependent effect on maximum accumulated distance detected in *D. rerio* when considering the concentration factor alone (Table 1). However, there was a significant concentration × time interaction, with the 0.25 and 0.5 μM concentrations being significantly lower than controls based on the LSD test (Lower panel - Fig. 2 E, Tables 1 and 2). The highest concentration, 4 μM, exhibited a periodic short duration stimulatory effect on swimming behavior over the first 2 h that can be seen as successive increasing and decreasing mean values for maximum accumulated distance before subsiding to control levels after approximately 3 h of exposure (Lower panel - Fig. 2 F). Although there was a non-significant trend for a concentration-dependent effect of 4-nonylphenol on mean angle in

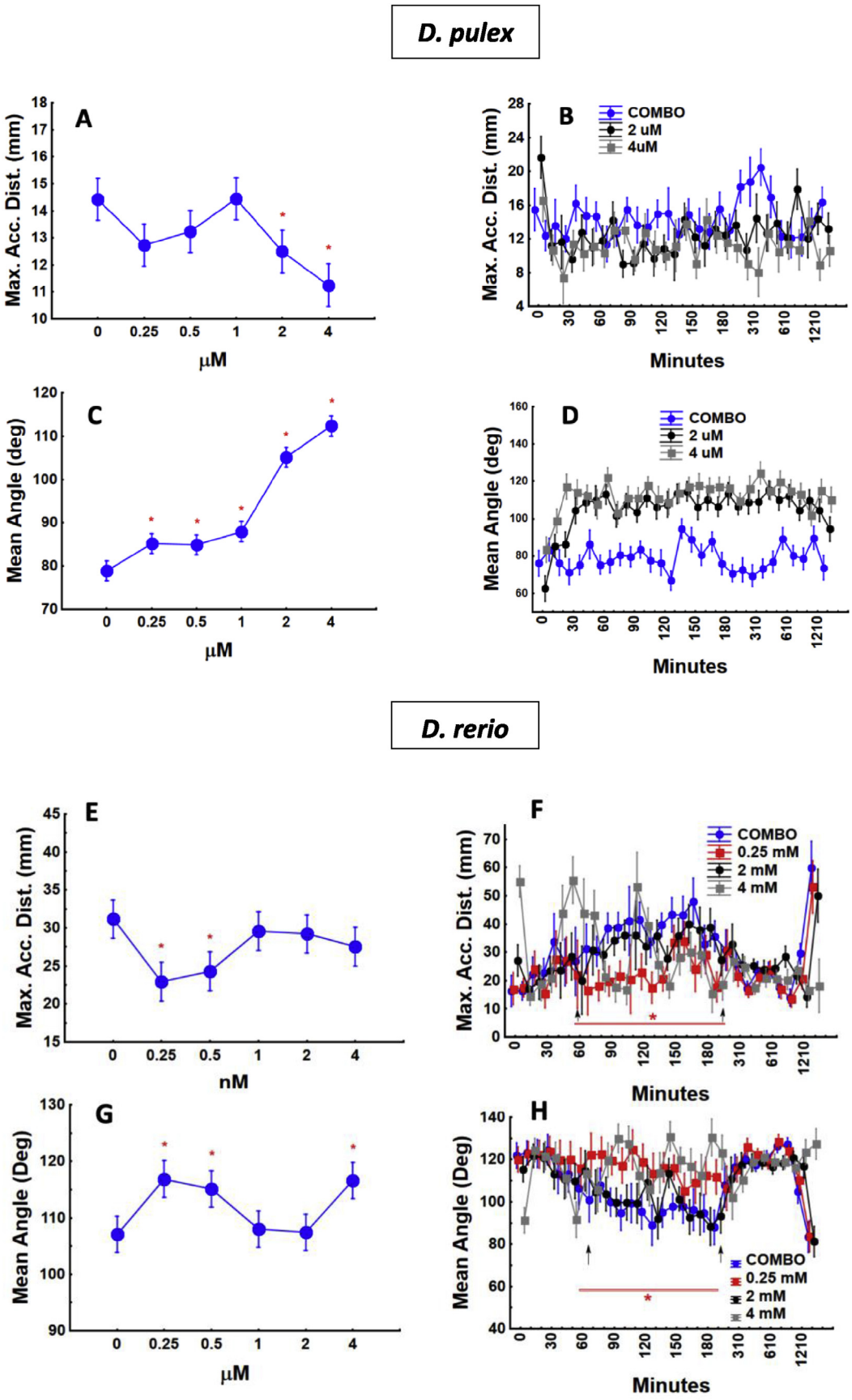

Fig. 2. The behavioral effects of the alkylphenol, 4-nonylphenol, on *D. pulex* (upper four panels, A to D) and *D. rerio* (lower four panels, E to H). Panel A and E show the concentration-dependent effects on maximum accumulated distance and panels B and F depicts the time course for the concentration-dependent effects on maximum accumulated distance. Panel C and G show the concentration-dependent effects on mean angle and panel D and H show the time-course for the concentration-dependent effects on mean angle. Only selected concentrations are shown in the time-course graphs (B, D, F, H) for clarity. The symbols represent mean and standard error and the asterisks represent significant differences from control using the LSD test. The redlines and asterisks associated with the time-course data for *D. rerio* (lower panels F & H) indicate significant differences between control and 0.25 µM 4-nonylphenol treatment using contrast analysis ($P < 0.05$).

D. rerio, the concentration × time interaction was significant and the LSD test detected significant elevations in mean angle for the 0.25, 0.5, and 4 μM concentrations (Lower panel - Fig. 2 G and H, Tables 1 and 2). Again, note that the measures for maximum accumulated distance and mean angle tend to have opposite concentration-dependent changes in direction relative to each other (Lower panel – Fig. 2 E and G).

3.2. Atrazine

The herbicide, atrazine, elicited a concentration-dependent effect on maximum accumulated distance in *D. pulex*, with behavioral stimulation seen as an increase in maximum accumulated distance only at the two highest concentrations, 20 μM and 100 μM. The effects of atrazine on maximum accumulated distance was not found to be dependent on time, and it did not have any significant effects on mean angle (Tables 1 and 2, see supplemental information).

In *D. rerio*, atrazine did not elicit a significant concentration-dependent effect on maximum accumulated distance, but there was a significant concentration x time effect on maximum accumulated distance (Table 1). Similarly, atrazine did not elicit a concentration-dependent effect on mean angle, but there was a significant concentration × time interaction effect (Table 1). The significance of the concentration × time interactions for both maximum accumulated distance and mean angle was associated with the influence of only one concentration, 4 μM (Table 2, see supplemental information).

3.3. Bisphenol-A

Bisphenol-A, a plastic precursor, did not affect maximum accumulated distance or mean angle of *D. pulex* at any concentration studied (Tables 1 and 2, supplemental information). Similarly, for *D. rerio*, bisphenol-A did not elicit any effects on maximum accumulated distance (Tables 1 and 2). There was a non-significant trend for effects of bisphenol-A on mean angle. Therefore bisphenol-A did not significantly affect swimming behavior in either species at concentrations up to 64 μM.

3.4. Chlorpyrifos and dieldrin

Chlorpyrifos and dieldrin are organophosphate and organochlorine insecticides, respectively, and are known to be neurotoxic. In *D. pulex,* chlorpyrifos did not have any effects on swimming behavior in the concentration range of 0.31 nM–5 nM (see Table 1). In the higher concentration range of 15.6–250 nM, chlorpyrifos elicited a concentration- and time-dependent decrease in maximum accumulated distance and increase in mean angle in *D. pulex* (Fig. 3 A and B, Tables 1 and 2). Immobility of *D. pulex* was observed at 125 nM after approximately 3 h and at 250 nM after approximately 2 h. *D. pulex* were immobilized within 5 min after any concentrations greater than 250 nM. In sharp contrast to the results in *D. pulex* described above, a higher concentration range of chlorpyrifos, 62.5 to 1000 nM, of chlorpyrifos did *not* elicit any effects on the swimming behavior of *D. rerio* (see Table 1 and supplemental information).

For the dieldrin concentration range of 0.1–62.5 μM, a concentration-dependent increase in maximum accumulated distance was observed for 2.5 μM, 12.5 μM, 62.5 μM in *D. pulex*, but these concentration-dependent effects were not dependent on time (Fig. 3 C, Tables 1 and 2). However, there was only a time-dependent effect of concentration of dieldrin on mean angle which cannot be observed in Fig. 3 D (see Table 1). In contrast to *D. pulex*, dieldrin exposure resulted in a robust concentration- and time-dependent increase in the maximum accumulated distance and change in mean angle in *D. rerio* (Fig. 3E and F, Tables 1 and 2). This effect of dieldrin on *D. rerio* occurred over a lower concentration range of 0.0625–1 μM than tested in *D. pulex* and concentrations higher than 2.5 μM were found to be lethal. The effect of dieldrin on mean angle in *D. rerio* initially elicited an increase at the low concentration of 0.0625 nM and then mean angle decreased in the higher concentration range of 0.25–1.0 μM (Fig. 3 F, Tables 1 and 2). Behavioral stimulation of *D. rerio* occurred earlier in the time-course at higher concentrations than it did for the lower concentrations. For example, the peak stimulation for 1 μM was seen at approximately 40 min, at 40–50 min for 0.5 μM, and approximately 100 min for 0.25 μM. The highest concentration (1 μM) produced a decrease in mean angle within 10 min.

3.5. Estrone

Estrone (6.25–100 nM) did not affect maximum accumulated distance or mean angle in *D. pulex* (Table 1). The effects of estrone on *D. rerio* behavior are addressed in a separate study.

3.6. Metformin

Metformin in the concentration range of 0.01–400 μM did not elicit any effect on swimming behavior in *D. pulex* (Table 1, see supplemental information). In *D. rerio,* there was a non-significant trend for metformin exposure (0.01–100 μM) to produce a concentration-dependent decrease in maximum accumulated distance, but the time × concentration interaction effect was not significant. The LSD test identified lower accumulated distance at the 0.01, 1, 10 and 100 μM concentrations as the main contributor to the trend (Fig. 4 A, Tables 1 and 2). However, the effect on maximum accumulated distance in *D. rerio*, was associated with a significant concentration-dependent increase in mean angle (Fig. 4 B), but no significant concentration × time interaction. Although metformin did not elicit significant behavioral effects in *D. pulex* over the concentrations studied, it did alter swimming behavior of *D. rerio* at a concentration as low as 10 nM.

3.7. Triclocarban

Triclocarban did not significantly affect maximum accumulated distance or mean angle of either species in the 6.25–100 nM concentration range. When a higher 0.5–8 μM range was tested, there was only a significant concentration × time interaction in *D. pulex*. Contrast analysis indicated that the highest 8 μM concentration increased mean angle ($P < 0.05$) in *D. pulex* after 3 h of exposure. Excluding control, there was 25–50% lethality in *D. rerio* across all concentrations in this concentration range (see Tables 1 and 2, supplemental information). Therefore, *D. rerio* did not exhibit altered swimming behavior within the lower sub-lethal 6.25–100 nM concentration range and *D. pulex* did not exhibit any alterations in swimming behavior in concentration range from 0.0062 up to 4 μM.

3.8. Triclosan

Triclosan stimulated swimming behavior in *D. pulex* and produced both a concentration- and time-dependent increase in maximum accumulated distance (Fig. 5 A, Tables 1 and 2). At 24-hrs there was no longer significant stimulation of swimming detected for any concentration of TCS. The mean angle was decreased by TCS in a concentration-, but not a time-dependent manner in *D. pulex* (Fig. 5 B, Tables 1 and 2). The decrease in mean angle was associated with an increase in maximum accumulated distance.

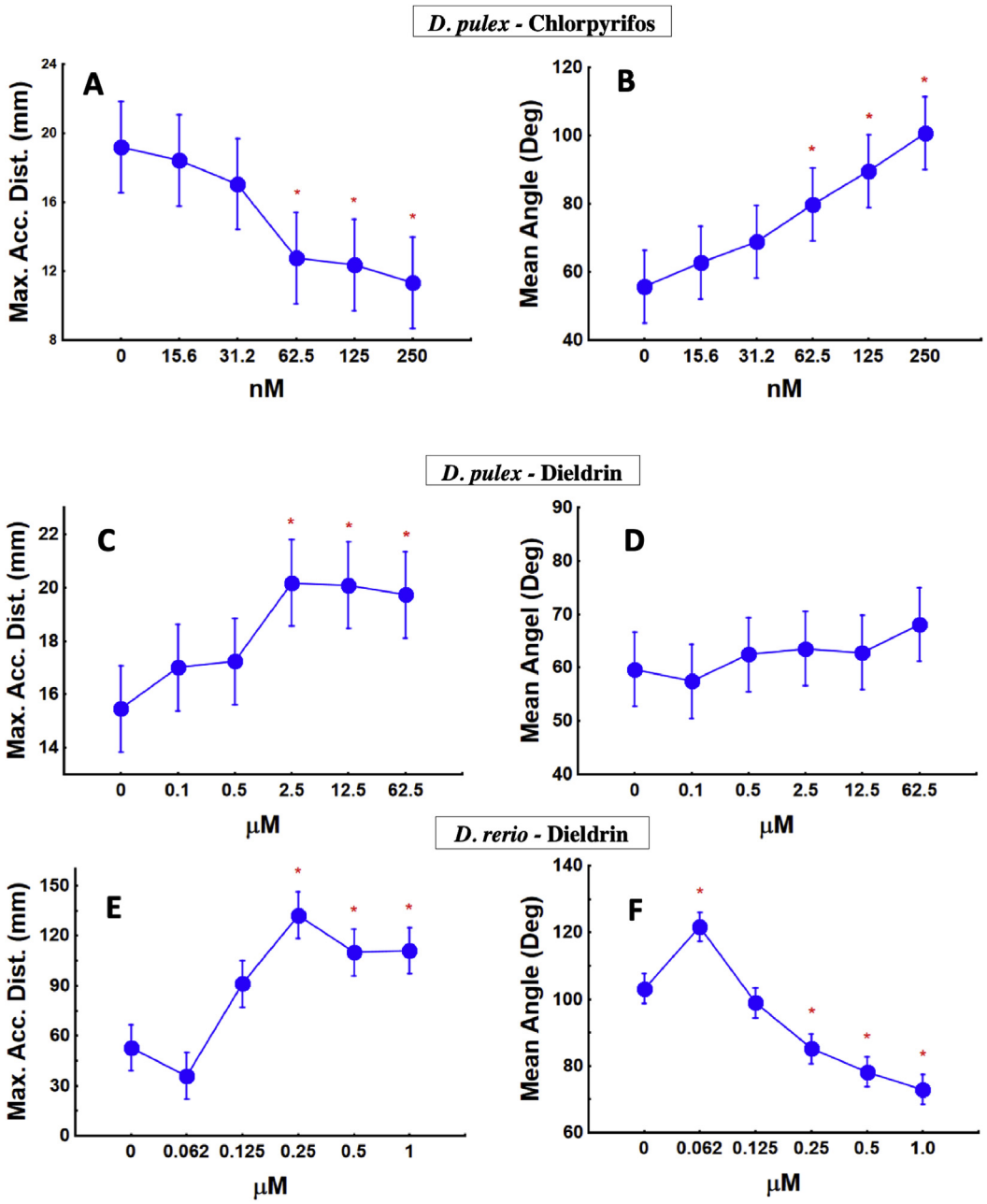

Fig. 3. The behavioral effects of the insecticides, chlorpyrifos on *D. pulex* (upper panels A and B), and dieldrin on both *D. pulex* and *D. rerio* (lower panels C–F) are depicted. The concentration-dependent effects of chlorpyrifos (panel A) and dieldrin (panel C) on maximum accumulated distance, and chlorpyrifos (panel B) and dieldrin (panel D) on mean angle are shown for *D. pulex*. The concentration-dependent effects of dieldrin on maximum accumulated distance and mean angle are shown for *D. rerio* in Panels E and F, respectively. The symbols represent mean and standard error and the asterisks represent significant differences from control (LSD test).

Although triclosan did not elicit a concentration-dependent effect on maximum accumulated distance in *D. rerio*, there was a significant time x concentration effect (Fig. 5C, Table 1). Based on the LSD test, the accumulated distance for the 0.125 and 0.25 concentrations were significantly higher than control (Table 2). The mean angle was not changed by triclosan in *D. rerio* (Fig. 5 D, Table 1). The lower values for mean angle were associated with elevated maximum accumulated distance, consistent with a stimulatory effect on swimming behavior (Fig. 5C and D).

4. Discussion

Many of the CECs examined in this study have previously been shown to alter *D. pulex* (Zein et al., 2014b, 2015b; Huang et al., 2018; Liu et al., 2019) and embryonic *D. rerio* behavior (Ton et al., 2006; Saili et al., 2012; Horzmann et al., 2018). Some of the pesticides, such as the insecticides chlorpyrifos and dieldrin, are known to target the nervous system (Costa et al., 2008; Rizzati et al., 2016) and other non-pesticide CECs, such as 4-nonylphenol and triclosan, have neurotoxic properties (Jie et al., 2013; Szychowski et al., 2019). Behavioral screening for chemically induced alterations in swimming behavior by the nine structurally diverse CECs evaluated in the study: 1) ensures that sublethal effects can be observed over the 24 h exposure period, (2) characterizes the behavioral activity of a range of sublethal concentrations (50% of the chemical/species combinations showed behavioral activity at less than 8 µM), (3)

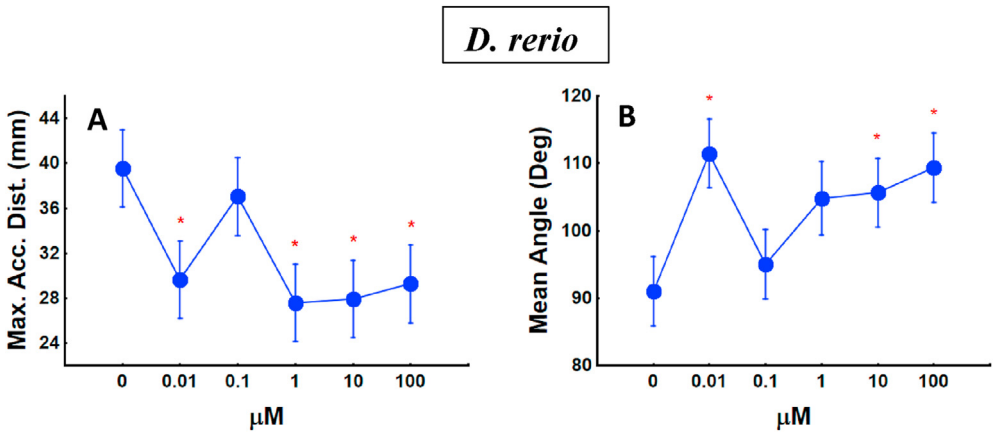

Fig. 4. The concentration-dependent effects of the pharmaceutical, metformin, on *D. rerio* are shown. Panels A depicts the effects on maximum accumulated distance and panel B shows the concentration-dependent effects on mean angle. The symbols represent mean and standard error and the asterisks represent significant differences from control (LSD test).

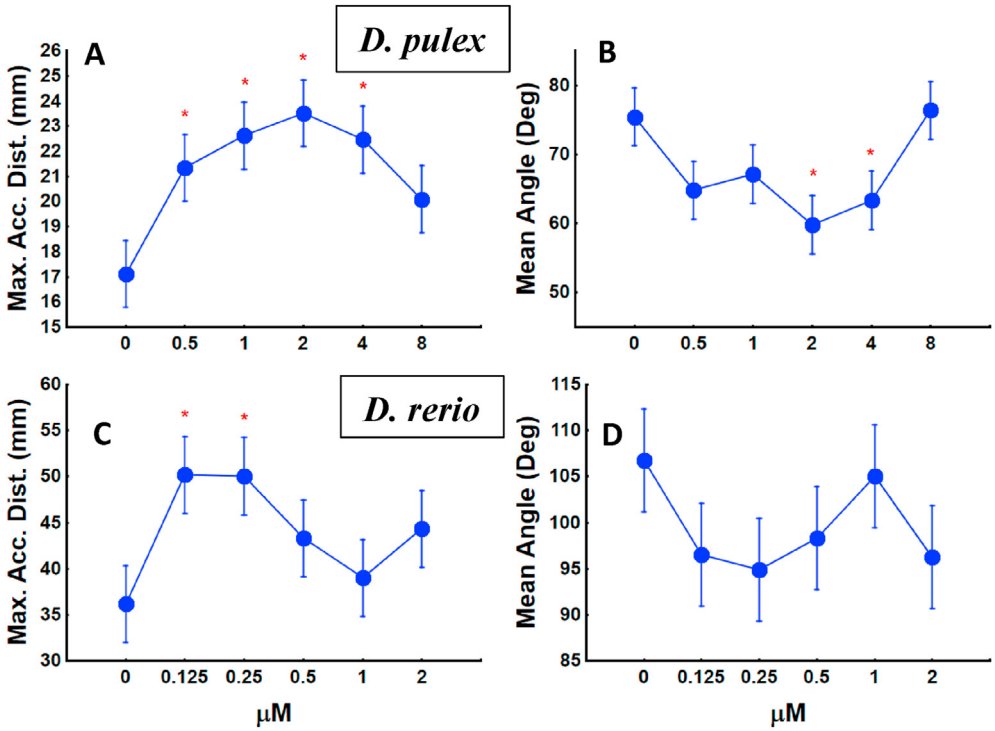

Fig. 5. The effects of the personal care product, triclosan on *D. pulex* (panels A and B) and *D. rerio* (Panels C and D). Panels A and C depict concentration-dependent effects on maximum accumulated distance and panels B and D depict concentration-dependent effects on mean angle. The symbols represent mean and standard error and the asterisks represent significant differences from control (LSD test).

provides an initial index for comparing the relative sensitivity of concentration-dependent behavioral responses to transcriptomic responses, (4) provides additional chemical identification criteria based on concentration-dependent behavioral responses (absent or present; increase or decrease in the two swimming parameters), and (5) helps to establish guidelines for the evaluation of chemical contaminants with unknown or unreported properties. Therefore, this approach enables an initial toxicological assessment of water samples containing uncharacterized complex mixtures (e.g., wastewater effluent) and provides critical data for making decisions about *sublethal* concentration-ranges to be examined in subsequent transcriptomic assays. The 24-hr exposure period was chosen as a standard consistent with the goal of creating a high throughput assay system. The overarching goal is development of a high-throughput whole-animal EDC detection model to examine *sublethal* concentrations of CECs and detect endocrine disrupting activity that is dependent on (*D. rerio*), or independent of, estrogen receptor activity (*D. rerio* or *D. pulex*). The first step of this approach is to utilize an optically based behavioral assay system suitable for studying more than one species.

When the concentration-dependent effects of the CECs on behavior were compared across species, 6 out of 9 CECs for *D. pulex* and 4 out of 9 CECs for *D. rerio* were found to significantly alter the swimming behavior in the concentration ranges studied. It is possible, although less likely, that significant behavioral changes could occur at concentrations lower than those which were

studied. Although this information would be of value, the pursuit of this question is not consistent with the main goal of the assay system, to detect estrogenic-like activity at sublethal chemical concentrations, and would require additional behavioral measures beyond simply quantifying swimming behavior to refute a negative outcome of no significant behavioral change. The concentration-dependent behavioral responses of *D. rerio* to metformin, 4-nonylphenol, and triclosan appear to be non-monotonic, suggesting more complex concentration-response relationships. Exposure periods longer than 24-hrs might detect behavioral changes at lower concentrations. However, increasing the duration of exposure would decrease assay throughput and detract from the utility of the assay system to detect EDC activity in a large number of water samples.

Chlorpyrifos is an example of a CEC where an expanded concentration range (0.31–250 nM) was studied in *D. pulex* because of its known toxicity as an insecticide and acetylcholinesterase inhibitor, and reported lack of stimulation of swimming behavior in *D. pulex* over a range of 16–250 nM (Zein et al., 2014). Two other acetylcholinesterase inhibitors, physostigmine and diazinon have been shown to stimulate swimming behavior at lower concentrations and inhibit swimming behavior at higher concentrations (Zein et al., 2014, 2015). In the present study, no stimulation of *D. pulex* swimming behavior by chlorpyrifos was observed from 0.31 to 250 nM and only inhibitory effects on swimming behavior were detected above 31 nM. The reason for the apparent difference in the ability of these three acetylcholinesterase inhibitors to stimulate swimming behavior (increase in maximum accumulated distance) may be related to toxicokinetic factors with chlorpyrifos having the highest K_{OW} of the three chemicals (Christensen et al., 2009; Harper et al., 2009; National Center for Biotechnology Information; Zein et al., 2014), or other differences in chemical properties. It is noteworthy to point out that prenatal chlorpyrifos exposure has been reported to be associated with brain anomalies in children (Rauh et al., 2012), and it is not clear if this is due to targeting acetylcholinesterase or other known secondary targets. Secondary targets reported for chlorpyrifos include non-acetylcholinesterases (Casida et al., 2004), the aromatic hydrocarbon receptor (Takeuchi et al., 2008), and cytochrome P-450 enzymes (Hodgson and Rose, 2007). Usmani et al. (2006) have reported that chlorpyrifos can inhibit estradiol metabolism by CYP1A2 and CYP3A4. Chlorpyrifos exposure has been reported to be estrogenic in *D. rerio* and induces vitellogenin production in male fish (Manjunatha and Philip, 2016), but it appears to be only a weak estrogen receptor agonist with estrogenic-like effects that involves other mechanisms (Moyano et al., 2020). The estrogenic activity of chlorpyrifos may not be mediated by simply binding to the estrogen receptor since it is a weak estrogen receptor agonist (Nishihara et al., 2000), inhibits estradiol metabolism (Usmani et al., 2006), and induces proliferation of human breast cancer cells through mechanisms that include aromatic hydrocarbon receptor activation (Moyano et al., 2020).

The design of these experiments enables the assessment of genomic responses at *sublethal* concentrations, including the targeting of concentrations below those where behavioral responses were observed. In this study we focused on the range of sublethal concentrations where altered swimming activity could be detected. This information has been used as a guide for deciding what concentration range to use for exploring potential transcriptomic alterations. The strategy was to target a relatively high sublethal concentration, no higher than 10 μM, as the highest concentration for the transcriptomic studies, and then do serial dilutions to drop under the concentration range used in the behavioral screen. Successively lower logarithmically-related concentrations are included in a series of dilutions (e.g., 10-fold dilution). Consistent with the intent of developing high throughput assays, these exposures are also limited to a 24-hr period known to sublethal based on the behavioral screen.

Comparing the responses of *D. pulex*, an invertebrate with an ecdysteroid- and juvenile-hormone based system, with *D. rerio,* a vertebrate with a non-ecdysteroid-based steroid hormone system, creates an assay system with contrasting biology for the detection of chemical signals in water. Given the evolutionary conservation of estrogen receptors among vertebrates which also includes some invertebrates (Jones et al., 2017; Keay and Thornton, 2009), and the rising concern about endocrine disruption (Colborn et al., 1993; Kolpin et al., 2002; Schug et al., 2013) that can affect vertebrate (Barber et al., 2012) and invertebrate populations (Zou, 2020), the inclusion of *D. pulex* as a chemical biosensor extends the ecological relevance of our assay system for aquatic ecosystems. *Daphnia* possess the ecdysteroid- and juveinile-hormone systems, but do not have estrogen receptors (Thomson et al., 2009). Since *D. rerio* do have estrogen receptors, this two- organism combination creates a contrast based on the presence or absence of the estrogen receptor. In order to maintain high throughput, we tested *D. pulex* and *D. rerio* in the same behavioral assay system and this required the use of animals at different stages of development, adult *D. pulex* and four-day-old *D. rerio*. A later developmental exposure period for *D. rerio* would more likely impact sexual development of the fish (Arcand-Hoy and Benson; 1998; Segner, 2009; Baker et al., 2013). Of the two organisms utilized, *D. rerio* is the organism best suited to detect those estrogenic chemical signals in water that have the potential to affect aquatic vertebrates. As a keystone species with relevance to freshwater ecosystems, *D. pulex*, provides a contrasting hormonal system that does not include the estrogen receptor. Based on our unpublished results, *D. pulex* still shows remarkable biosensor-like genomic sensitivity for all nine of these CECs, including estrone, in the ppt concentration range. The composition of this two-organism bioassay system is aligned with the "Tier 4" whole animal assessment found within the protocol for detecting endocrine disrupting activity described by Schug et al. (2013).

Connecting specific exposure concentrations to altered phenotypes is necessary to provide evidence of endocrine disrupting potential of a given CEC. Estrogenic-like effects on *D. rerio* phenotype have been reported for most of these compounds (Bautista et al., 2018; Hill and Janz, 2003; Manjunatha and Philip, 2016; Segner et al., 2003; Torres-Duarte et al., 2012; Van den Belt et al., 2004; Wang et al., 2016). Although this study did not examine any developmental or reproductive outcomes resulting from exposure that would represent endocrine disruption (e.g., altered vitellogenin expression, feminized males, altered sex-ratio), such studies will be necessary to connect exposure concentrations with specific changes in gene expression and specific morphological outcomes for later developmental periods in *D. rerio*. This step will be vital to the development of our bioassay system for detecting the estrogenic and/or anti-androgenic properties of water samples that are contaminated by chemical mixtures. The use of aquatic organisms to screen for estrogenic and/or anti-androgenic effects of CEC exposure *in vivo* provides the potential to detect both receptor-mediated and non-receptor mediated endocrine disrupting effects.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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