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Bioaccumulation of estrogenic hormones and UV-filters in red swamp crayfish (*Procambarus clarkii*)

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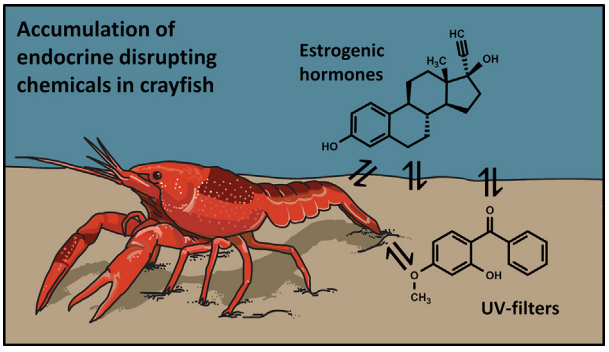
HIGHLIGHTS

- *Procambarus clarkii* readily accumulated endocrine disrupting chemicals.
- 4-methylbenzylidene camphor and octocrylene accumulated in crayfish fecal matter.
- 2-ethylhexyl-4-methoxycinnamate and homosalate had highest bioaccumulation factors.
- Octocrylene bioaccumulation factor lower than expected from its hydrophobicity
- Chemical analysis overpredicted estrogenicity compared to yeast estrogen screen.

GRAPHICAL ABSTRACT



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ABSTRACT

Estrogenic hormones and organic ultraviolet-filters (UV-filters) have attracted increased attention as endocrine disrupting chemicals (EDCs) due to their potent estrogenicity and widespread occurrence in the environment. This study investigated the accumulation of three estrogenic hormones and five UV-filters in red swamp crayfish (*Procambarus clarkii*). Exposure experiments were conducted for 42 days with a mixture of EDCs at two environmentally-relevant design concentrations (*i.e.*, 500 and 5000 ng L^{-1}). The aqueous-phase EDC concentrations decreased over time and were re-established every two days. Within 14 days of exposure, the five UV-filters were measured at 2.2 to 265 ng g^{-1} (dry weight) in crayfish tail tissue. Only one estrogenic hormone, 17β-estradiol, was detected in the crayfish at 10.4–13.5 ng g^{-1}. No apparent changes were observed for EDC concentrations in the tail tissue over the next four weeks of exposure. The apparent bioaccumulation factors for the EDCs ranged from 23 L (kg tail tissue, dry weight)$^{-1}$ for 4-methylbenzylidene camphor to 1050 L (kg tail tissue, dry weight)$^{-1}$ for 2-ethylhexyl-4-methoxycinnamate. EDC input was stopped after 42 days, and the more hydrophobic UV-filters (*i.e.*, octocrylene, 2-ethylhexyl-4-methoxycinnamate, homosalate) were found to be persistent throughout a 14-d elimination period. A lyticase-assisted yeast estrogen screen demonstrated that the residual estrogenic activity of water samples aligned with (or was lower than) predictions from targeted chemical analysis. These results suggest that the transformation products did not contribute significant estrogenicity, although further analysis of endocrine disruption outcomes in crayfish is recommended.

1. Introduction

Endocrine disrupting chemicals (EDCs) are exogenous compounds that cause adverse health effects in organisms or their progeny.

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Numerous examples of reproductive abnormalities have been observed in aquatic vertebrates exposed to EDCs (Gibson et al. 2005; Jobling et al. 1998; Jobling et al., 2006; Kidd et al. 2007; Kloas et al., 2009; Pollock et al. 2010). For example, Jobling et al. (1998) reported that the occurrence of intersex in wild populations of riverine fish (*Rutilus rutilus*) correlated with exposure to hormonally-active substances in wastewater effluent. In a seven-year whole lake experiment, Kidd et al. (2007) described the collapse of fish populations (*Pimephales promelas*) after chronic exposure to 5–6 ng L^{-1} of the 17α-ethinylestradiol (EE2) synthetic estrogen, which is more potent and persistent than natural estrogens. In addition, EDCs are known to exert oxidative stress (Gismondi 2018) and alter immune response (de Melo et al. 2019) in invertebrates. Among EDCs, estrogenic hormones and organic ultraviolet-filters (UV-filters) have attracted increased attention due to their potent estrogenic activity (Johnson and Williams 2004; Welshons et al. 2003), widespread use in pharmaceuticals and personal care products (Ash and Ash 2004; Shaath and Shaath 2005), and ubiquitous presence in aquatic and marine environments (He et al. 2019; Hopkins and Blaney 2016; Mitchelmore et al. 2019). Due to their continuous introduction into the environment, these EDCs can be considered pseudo-persistent. Hopkins and Blaney (2016) reported that UV-filter concentrations in raw wastewater, wastewater effluent, and surface water were as high as 20.2, 16.0, and 7.3 μg L^{-1}, respectively. Most estrogens and UV-filters are moderately hydrophobic (*i.e.*, log D = 3–7) and known to bioaccumulate in aquatic vertebrates (Blüthgen et al. 2014; Gago-Ferrero et al. 2013; Gago-Ferrero et al. 2015), but the uptake of these EDCs and the resulting impacts on aquatic invertebrates are not well understood (Meredith-Williams et al. 2012; Ricciardi et al. 2016).

Three priority estrogens, namely estrone (E1), 17β-estradiol (E2), and EE2, and five widely used UV-filters, including 4-methylbenzylidene camphor (4-MBC), benzophenone-3 (BP-3, or oxybenzone), 2-ethylhexyl-4-methoxycinnamate (EHMC, or octinoxate), homosalate (HMS), and octocrylene (OC), were selected for this study. These compounds were chosen due to their hydrophobicity (log D > 3; ChemAxon, 2018) and potential to accumulate in aquatic organisms (Dussault et al. 2009a; Fent et al. 2010; Gago-Ferrero et al. 2013; He et al. 2019; Huerta et al. 2015; Langford et al. 2015; Ricciardi et al. 2016). E1, E2, EE2, and EHMC were included in the European Union Watch List of chemicals that pose a significant risk to the aquatic environment (European Union 2013; European Union, 2015). BP-3 has been implicated in coral bleaching (Downs et al. 2015; Tsui et al. 2017), and bans on the sale of sunscreens containing BP-3 and EHMC have been approved in Hawaii (2018) and Florida (2019). Previous measurements with the yeast estrogen screen (YES) have confirmed the estrogenic activity of 4-MBC and HMS (Kunz and Fent 2006). HMS is one of the most intensively used UV-filters in the United States (*i.e.*, up to 15% in sunscreen products (FDA, 2007)), but few data are available on environmental occurrence (Hopkins and Blaney 2016). The other UV-filter, OC, has been frequently detected in aquatic organisms (Fent et al. 2010; Gago-Ferrero et al. 2013; He et al. 2017; Langford et al. 2015).

Among invertebrates, crustaceans (*e.g.*, crabs, lobsters, shrimp, and crayfish) deserve special attention due to their widespread abundance in nature, important role in the food web, and consumption by humans. The wide-ranging habitat of crayfish in freshwater systems throughout Africa, Asia, Europe, North America, and South America (Hobbs et al. 1989; Taylor et al. 2007) represents an opportunity to use these organisms as universal bioindicators for estrogens, UV-filters, and other EDCs. As crayfish serve an important ecological function that connects aquatic plants, algae, and small invertebrates to fish, birds, and mammals (Rabeni 1992), bioaccumulation of EDCs in crayfish will provide insight to the trophic transfer of these contaminants. Furthermore, over 50% of crayfish species are endangered or threatened in the United States (Taylor et al. 1996), and the role of endocrine disruption from xenobiotics has not been fully established. Due to human consumption of crayfish, the potential accumulation of EDCs also raises public health concerns. In the United States, the red swamp crayfish (*Procambarus clarkii*) is the principal species consumed by humans, and the State of Louisiana, alone, has approximately 48,000 ha of aquaculture devoted to crayfish farming (FAO, 2005). To inform future (eco)toxicological studies, it is important to quantify accumulation of estrogenic hormones and UV-filters in crayfish.

The objectives of this study were to (1) investigate the partitioning of priority estrogens and UV-filters between water, fecal matter, and crayfish tail tissue (*i.e.*, the edible portion that consists of the aggregate abdomen, telson, and uropod tissues) and (2) elucidate the residual estrogenic activity of the water using a targeted approach with chemical concentrations measured by liquid chromatography with tandem mass spectrometry (LC-MS/MS) and a non-targeted strategy that employed a lyticase-assisted YES assay. These objectives were addressed for environmentally-relevant conditions corresponding to raw wastewater and wastewater effluent to relate partitioning coefficients, bioaccumulation factors, and residual estrogenic activity to real scenarios observed in natural systems.

2. Material and methods

2.1. Chemicals, supplies, and reagents

Ammonium formate (NH_4COOH), ammonium hydroxide (NH_4OH), anhydrous magnesium sulfate ($MgSO_4$), calcium chloride dihydrate ($CaCl_2$•$2H_2O$), hydrochloric acid (HCl), magnesium sulfate heptahydrate ($MgSO_4$•$7H_2O$), sodium carbonate (Na_2CO_3), sodium chloride (NaCl), potassium chloride (KCl), disodium phosphate (Na_2HPO_4), monosodium phosphate (NaH_2PO_4), and sodium hydroxide (NaOH) were obtained from Fisher Scientific (Pittsburgh, PA, USA). These chemicals were used during (i) extraction of estrogens and UV-filters from crayfish fecal matter and tail tissue, (ii) generation of medium-hardness water for use in crayfish tanks, and (iii) preparation of YES assay buffers. Oasis hydrophilic-lipophilic balanced (HLB) cartridges (60 mg, 3 cm^3) were acquired from Waters Corp. (Milford, MA, USA) for solid-phase extraction (SPE) of water samples and cleanup of fecal matter and crayfish tail tissue extracts. LC-MS grade water, methanol (MeOH), and acetonitrile (ACN) were procured from Fisher Scientific for use during LC-MS/MS analysis of estrogens and UV-filters.

Unless otherwise stated, the estrogens and UV-filters of concern, as well as their corresponding isotopically-labeled standards, were purchased from Sigma-Aldrich (St. Louis, MO, USA). E1 and 4-MBC were obtained from Acros Organics (Morris Plains, NJ, USA) and Alfa Aesar (Stoughton, MA, USA), respectively. Physicochemical properties of the analytes are summarized in Table S1 of the Supporting Information (SI). The internal standards EE2-d_4 and 4-MBC-d_4 were procured from CDN Isotopes (Pointe-Claire, Canada). Stock solutions of the estrogens, UV-filters, and their isotopically-labeled compounds were prepared at 400 mg L^{-1} in MeOH. All stock solutions were stored in amber glass containers at −20 °C. The stock solutions for isotopically-labeled standards were diluted to 50% MeOH with deionized (DI) water for use as internal standards during LC-MS/MS analysis.

To achieve the design concentration levels for exposure experiments, appropriate volumes of the estrogen and UV-filter stock solutions were added to medium-hardness water. The composition of the medium-hardness water was adapted from previous studies (Frontera et al. 2011; Grosell et al. 2002). For preparation of 40 L of medium-hardness water, 3.2 g Na_2CO_3 were added to approximately 30 L of DI water. The pH was adjusted to 8.0 with 3 M HCl, then 4 g $CaCl_2$•$2H_2O$ and 0.4 g $MgSO_4$•$7H_2O$ were added to the solution under constant stirring. The pH was amended to 8.0 ± 0.5 with 1 M HCl or 1 M NaOH, and DI water was added to reach a final volume of 40 L. The medium-hardness water was kept at room temperature (*i.e.*, 21 ± 2 °C) for no more than 48 h before use.

2.2. Crayfish cultures

Male and female *P. clarkii* were purchased from Aquatic Research Organisms (Hampton, NH, USA). Each crayfish was rinsed with DI water several times to remove residue from the exoskeleton. The crayfish culture conditions shown in Fig. 1 were based on previous exposure studies (Frontera et al. 2011; Sherba et al. 2000). Specifically, all crayfish were acclimated for one week in medium-hardness water at 21 ± 2 °C with a light-to-dark cycle of 10–14 h. On a daily basis, the pH was manually adjusted to 8.0 ± 0.5 with 1 M HCl or 1 M NaOH. Dissolved oxygen was maintained above 5 mg L^{-1} *via* continuous air bubbling through a diffuser stone. After the acclimation period, 45 male and 45 female active adult crayfish with weights of 20 ± 3 g were selected for the exposure experiment. From this pool, 15 male and 15 female crayfish were distributed into three 15-gal (56.8 L) aquaria. Throughout experimentation, crayfish were fed hormone-free, freeze-dried bloodworms at a rate of 0.1 g $crayfish^{-1}$ day^{-1}. The volume of the medium-hardness water was maintained at 40 L throughout all experiments.

2.3. Experimental design

As indicated in Fig. 1, Tanks 1 and 2 were dosed with a mixture of EDCs containing 500 and 5000 ng L^{-1}, respectively, of each target compound. Tank 3 was a control reactor that was not dosed with estrogens or UV-filters. The two dosing levels, namely 500 and 5000 ng L^{-1}, represented environmentally-relevant conditions, based on previously reported EDC concentrations in wastewater effluent (Cunha et al. 2015; Fayad et al. 2013) and raw wastewater (Cunha et al. 2015; Rodil and Moeder 2008; Rodil et al. 2009; Tsui et al. 2014a), respectively. Composite water samples were collected from five locations of each aquarium (*i.e.*, 10 mL × 5 locations = 50 mL samples). The aqueous-phase concentrations of estrogens and UV-filters in Tanks 1 and 2 were monitored every other day and tended to be lower than the design values due to partitioning and degradation reactions. Using a mass balance approach, Tanks 1 and 2 were dosed with estrogens and UV-filters every two days to re-establish the design conditions. Every four days, 10 L of water was changed in each tank, and the corresponding volume change and mass loss were incorporated into the mass balance analysis using EDC concentrations measured before and after the water change. Based on the mass balance analysis, EDCs were dosed into 10 L of fresh medium-hardness water and then added to the aquaria to re-establish the design concentrations.

During the water change process, crayfish fecal matter was collected by vacuum through a Teflon tube. After overnight settling at room temperature, the water layer from the fecal matter sample was carefully decanted, and the remaining mixture was centrifuged at 6000*g* for 10 min. The solids were collected, freeze-dried, massed, and kept at −20 °C until analysis of estrogens and UV-filters by LC-MS/MS. Note that a small volume of water was present in the fecal matter solids prior to freeze-drying, but according to the water-phase concentrations, water volume, and solids mass, the water contributed a negligible mass of EDCs to the freeze-dried samples.

The exposure experiment was conducted over 42 days, followed by a 14-day elimination period, during which time crayfish remained in the same aquaria but the input of EDCs was stopped to determine their persistence in the water, fecal matter, and crayfish tail tissue phases. No changes in crayfish behavior were observed throughout the exposure and elimination periods, and no crayfish died in the aquaria. Three male and three female crayfish were collected from each tank at days 0, 14, 28, 42, and 56 to measure estrogen and UV-filter concentrations in the tail tissue and examine potential differences in EDC accumulation by sex. In accordance with protocols from previous studies (Hong et al., 2020; Wei and Yang 2016), crayfish were anesthetized on ice for 30 min before dissection. The abdomen, telson, and uropod tissues (*i.e.*, described as the "tail tissue" below) were collected, freeze-dried, massed, and kept at −20 °C until analysis of estrogens and UV-filters by LC-MS/MS.

2.4. Sample pretreatment and LC-MS/MS analysis

The LC-MS/MS analytical methods for estrogens and UV-filters in the water, fecal matter, and tail tissue samples were adopted from previous studies (He et al. 2019; He et al. 2017; Mitchelmore et al. 2019). Briefly, 10-mL water samples were dosed with isotopically-labeled surrogates (10 ng) and processed by SPE with HLB cartridges. Isotopically-labeled surrogates (10 ng) were also added to freeze-dried fecal matter (150 mg) and tail tissue (50 mg) samples and allowed to equilibrate overnight before extraction with a modified Quick, Easy, Cheap,

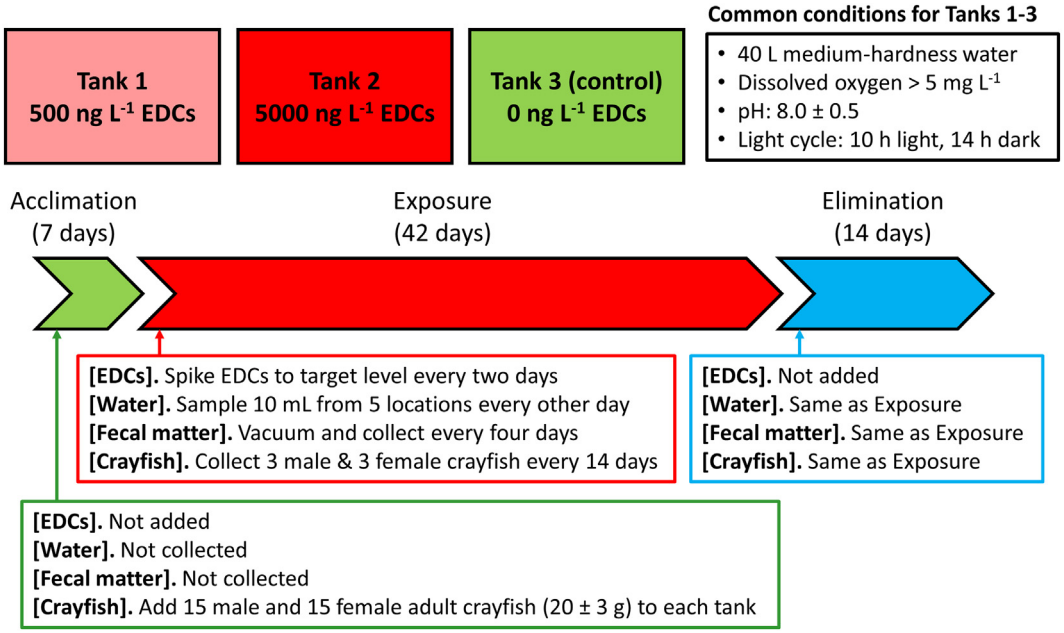

Fig. 1. Schematic of the experimental design and sampling scheme for crayfish exposure tests.

Effective, Rugged, and Safe (QuEChERS) protocol. All samples were extracted and analyzed in triplicate. The final extracts from the SPE and QuEChERS operations were evaporated to dryness under a gentle stream of nitrogen and reconstituted with 0.5 mL MeOH and 0.5 mL 0.1% NH_4OH. The estrogen and UV-filter concentrations in the reconstituted extracts were analyzed by LC-MS/MS (Dionex UltiMate 3000 Rapid Separation LC with Thermo TSQ Quantum Access Max triple quadrupole MS/MS; Waltham, MA, USA). The EDCs were separated along Waters XBridge C18 guard (2.1 × 10 mm, 3.0 μm) and analytical (2.1 × 150 mm, 2.5 μm) columns within 15 min. To improve electrospray ionization of the analytes, 0.1% NH_4OH was added to the LC-MS/MS eluents (He et al. 2017). The two most abundant MS/MS fragment ions were used for quantitation and confirmation.

2.5. Calculation of partition coefficients

The fecal matter-water partition coefficients (K_d in L kg^{-1}) for estrogens and UV-filters were calculated according to Eq. (1).

$$K_d = \frac{C_{fm}}{C_w} \quad (1)$$

In Eq. (1), C_{fm} is the analyte concentration in the fecal matter (ng kg^{-1}, dry weight), and C_w is the analyte concentration in the aqueous phase (ng L^{-1}).

To evaluate the tendency of the estrogenic hormones and UV-filters to accumulate in crayfish tail tissue, bioaccumulation factors (BAFs in L kg^{-1}) were calculated using Eq. (2). Because the lipid content in crayfish tail tissue is approximately 4% of the dry weight (Tricarico et al. 2008), the BAFs as L (kg lipid, dry weight)$^{-1}$ would be approximately 25× higher.

$$BAF = \frac{C_{tt}}{C_w} \quad (2)$$

In Eq. (2), C_{tt} is the EDC concentration in the crayfish tail tissue (ng kg^{-1}, dry weight).

2.6. YES assay protocols

Water samples were analyzed for estrogenic activity using a modified XenoScreen assay. The XenoScreen XL YES kit and the corresponding genetically-modified yeast cells were purchased from Xenometrix (Allschwil, Switzerland). The cells were cultured at 31 °C in standard yeast extract peptone dextrose media with 50 μM $CuSO_4$ (Zhang et al. 2006). When the optical density at 690 nm (OD_{690}) exceeded 2.0 for a 1-cm pathlength, the yeast cells were distributed into a 96-well plate containing serial dilutions of E2 (reference compound), other analyte standards, and experimental samples. The final volume in each well was 160 μL. After an 18-h incubation, microplates were shaken for 60 s using the orbital mixing function of the microplate reader (BioTek Eon, Winooski, VT, USA). After aspirating at least three times, 80 μL of the yeast cell culture was transferred into a plate containing 80 μL of lysis buffer (described below) and incubated at 31 °C for another 24 h. The lysis buffer composition was based on previous reports (Schultis and Metzger 2004; Zhang et al. 2006). Specifically, lyophilized lyticase powder from *Arthrobacter luteus* was dissolved in 50% glycerol (v/v) to form a 2000 U mL^{-1} stock solution. The stock lyticase solution was diluted 10× with the assay buffer, which was prepared by mixing 100 mg chlorophenol red D-galactopyranoside (CPRG), 100 mL Z-buffer (*i.e.*, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM 2-mercaptoethanol at pH 7.0), and 0.1 mL Triton X-100.

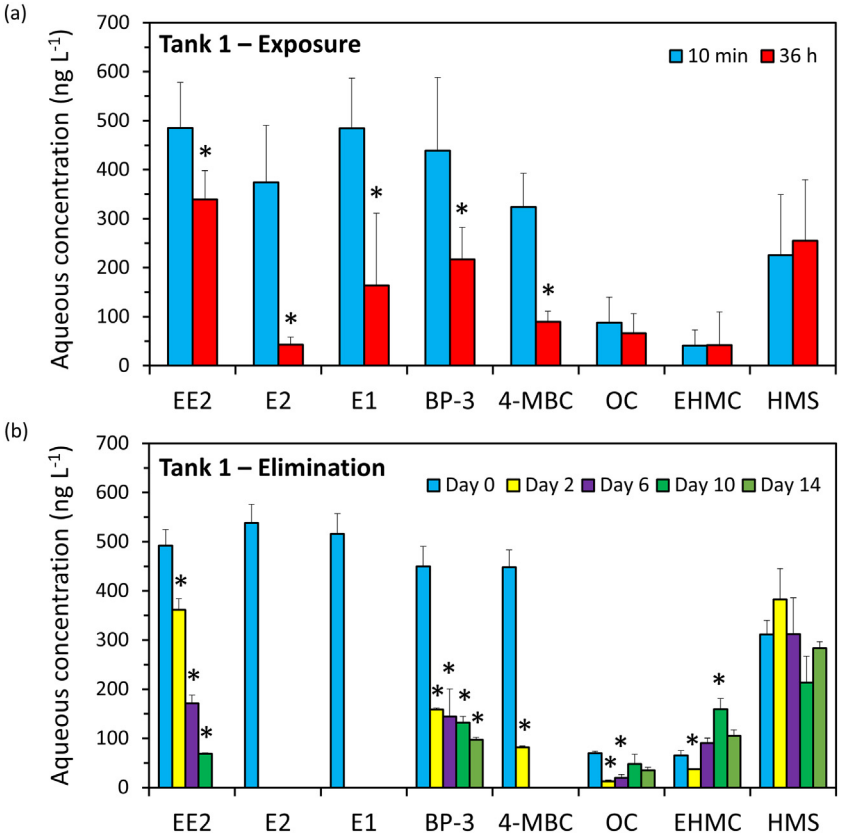

Fig. 2. Mean aqueous-phase concentrations of estrogens and UV-filters in Tank 1 during the (a) 42-d exposure and (b) 14-d elimination periods. Note, * indicates significant differences (*i.e.*, $p < 0.05$) between the (a) 10 min and 36 h data and (b) Day 0 and Day 2, 6, 10, or 14 data; more details on *p* values are provided in Table S2 of the SI. Chemical acronyms are as follows: EE2, 17α-ethinylestradiol; E2, 17β-estradiol; E1, estrone; BP-3, benzophenone-3; 4-MBC, 4-methylbenzylidene camphor; OC, octocrylene; EHMC, 2-ethylhexyl-4-methoxycinnamate; and, HMS, homosalate.

After a 24-h incubation, the total β-galactosidase expression was colorimetrically quantified at 570 nm by measuring the conversion of the yellow CPRG compound into the red CPRG product; note that the absorbance at 570 nm was corrected for diffraction from yeast cells using OD_{690}. The estrogenic activity was calculated using Eq. (3).

$$\text{Estrogenic activity} = \frac{1}{G}\left(\frac{A_{570,E} - OD_{690,E}}{A_{570,C} - OD_{690,C}}\right) \quad (3)$$

In Eq. (3), G is the yeast cell growth factor, $A_{570,E}$ is the absorbance at 570 nm for experimental wells, $OD_{690,E}$ is the optical density at 690 nm for experimental wells, $A_{570,C}$ is the absorbance at 570 nm for control wells, and $OD_{690,C}$ is the optical density at 690 nm for control wells. The yeast cell growth factor was calculated by subtracting the OD_{690} of control wells that contained 1% dimethyl sulfoxide in water from the OD_{690} of experimental wells. With these data, dose-response curves were generated and the effective concentrations for 50% estrogenic activity (EC_{50}) were calculated for individual EDCs and experimental samples. The E2 equivalents (EEQs) of experimental samples were calculated using Eq. (4) (Leusch et al. 2010).

$$EEQ_{YES}\ (ng/L) = \left(\frac{EC_{50,E2}\ (M)}{EC_{50,sample}(\text{unitless})}\right)\left(\frac{272.38\ g}{mol}\right)\left(\frac{10^9\ ng}{g}\right) \quad (4)$$

2.7. Statistical analysis

The aqueous-phase concentration data were expressed as mean ± standard deviation (n = 21 for 10-min and 36-h samples collected in the exposure period; and, n = 3 for samples collected during the elimination period). The tissue-phase concentrations were also reported as mean ± standard deviation (n = 3). All statistical analyses were performed with OriginPro 2016 (OriginLab Corp.; Northampton, MA, USA). Specifically, a Student's *t*-test was applied to the EDC concentrations in the 10-min and 36-h samples to investigate significant differences between EDC concentrations during the exposure period. A one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference test was conducted to compare EDC concentrations during the elimination period. Three-way ANOVAs with post-hoc Tukey's test were performed to determine significant differences in tissue-phase EDC concentrations with sex (*i.e.*, female, male), treatment (*i.e.*, Tanks 1, 2), and exposure time (*i.e.*, Day 14, 28, 42, 56). Differences were considered significant if $p < 0.05$, and results are summarized in Table S2 of the SI.

3. Results and discussion

3.1. Fate of estrogens and UV-filters in the water and fecal matter phases

The average aqueous-phase concentrations of estrogens and UV-filters for the 42-day exposure and 14-day elimination periods in Tanks 1 and 2 are summarized in Figs. 2 and 3, respectively. Samples were collected 10 min and 36 h after dosing EDCs into the aquaria to assess and maintain the design analyte concentrations; therefore, the data were expected to have some inherent variability due to changes in degradation, sorption, and partitioning reactions throughout the exposure and elimination periods. The aqueous- and fecal matter-phase concentrations for each analyte across the full experimental period are

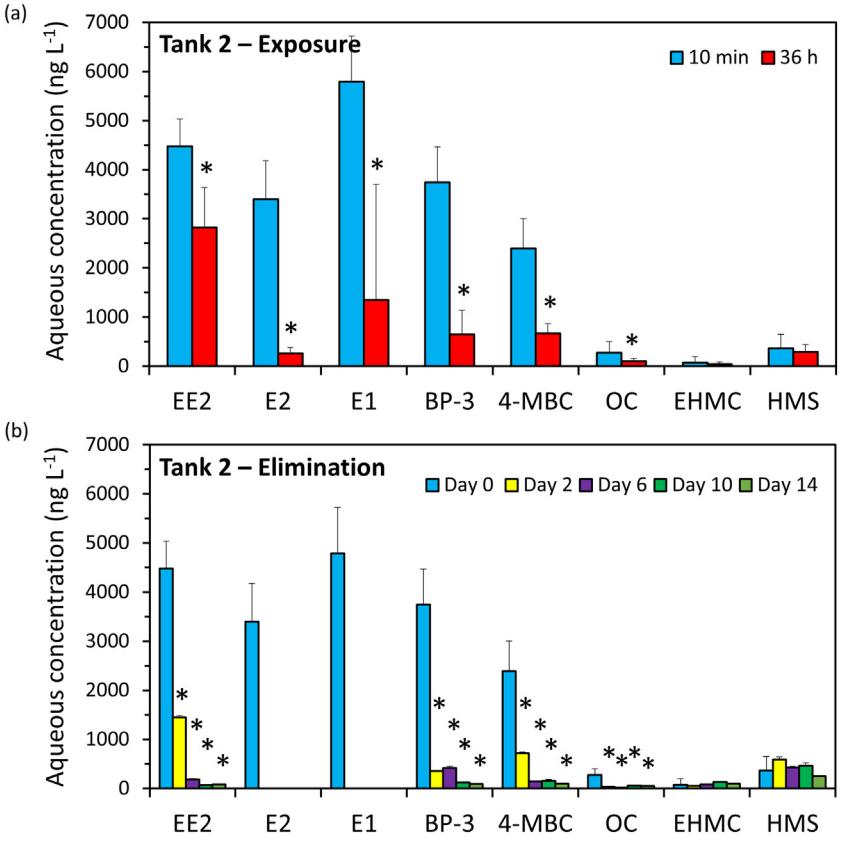

Fig. 3. Mean estrogen and UV-filter concentrations in water from Tank 2 during the (a) 42-d exposure and (b) 14-d elimination periods. Note, * indicates significant differences (*i.e.*, $p < 0.05$) between the (a) 10 min and 36 h data and (b) Day 0 and Day 2, 6, 10, or 14 data; more details on *p* values are provided in Table S2 of the SI. Chemical acronyms are as follows: EE2, 17α-ethinylestradiol; E2, 17β-estradiol; E1, estrone; BP-3, benzophenone-3; 4-MBC, 4-methylbenzylidene camphor; OC, octocrylene; EHMC, 2-ethylhexyl-4-methoxycinnamate; and, HMS, homosalate.

reported in Figs. S1 and S2, respectively, of the SI. During the 42-day exposure period, the average concentrations of E1, E2, EE2, BP-3, and 4-MBC in Tank 2 were about 10 times higher than the levels in Tank 1, in agreement with the experimental design.

As indicated by the log D values at pH 8.0 (see Table S1 in the SI), E1, E2, EE2, BP-3, and 4-MBC were the more hydrophilic analytes. While the concentrations measured 10 min after dosing the analytes were similar to design levels, these five chemicals exhibited significant changes ($p < 0.05$, see Table S2 in the SI) in aqueous-phase concentration during the 36-h periods between LC-MS/MS analyses. After 36 h, the average concentrations of E2 and BP-3 in Tank 2 decreased from 3400 to 260 ng L^{-1} and from 3750 to 652 ng L^{-1}, respectively. Interestingly, E1 was relatively stable for the first seven days but showed greater changes between dosing events for the remainder of the exposure period (see Fig. S1 in the SI). These data may indicate maturation of the bacterial populations in the tanks over the first two weeks (*i.e.*, one week of crayfish accumulation and one week of exposure to EDCs), but similar trends were not apparent for the other EDCs. The concentrations of the more hydrophobic UV-filters (*i.e.*, OC, EHMC, and HMS) were lower than the design levels 10 min after dosing, suggesting rapid distribution into solid phases. With the exception of OC in Tank 2 ($p = 0.003$, see Table S2 in the SI), the aqueous-phase concentrations of OC, EHMC, and HMS were comparable between the two tanks and relatively constant throughout the exposure period. For example, the average EHMC concentrations in the 10-min and 36-h samples were 39 and 40 ng L^{-1}, respectively, in Tank 1 and 78 and 37 ng L^{-1}, respectively, in Tank 2.

The partition coefficients for estrogens and UV-filters between the fecal matter and water phases are summarized in Fig. 4. The presence of EDCs in the fecal matter likely stemmed from both ingestion/egestion by crayfish and direct partitioning from the water phase. The mean K_d values ranged from 353 ± 137 L kg^{-1} for EE2 to 37,900 ± 10,000 L kg^{-1} for OC. The high standard deviation of the measured K_d values reflected the dynamic experimental design conditions corresponding to regular addition of analytes to maintain target concentration levels. With the exception of EHMC, the magnitude of K_d was positively correlated to the hydrophobicity of the target compounds (as log D). The relatively low K_d of EHMC (*i.e.*, 1740 ± 574 L kg^{-1}) was unexpected from the hydrophobicity trends and implied complex partitioning chemistry and/or a faster degradation rate (Kupper et al. 2006). As EHMC is one of the sunscreens banned in Hawaii (2018) and Florida (2019), further research to understand these complex environmental partitioning reactions is recommended.

The observed K_d values for BP-3 (487 ± 153 L kg^{-1}), 4-MBC (4670 ± 1630 L kg^{-1}), and OC (37,900 ± 10,000 L kg^{-1}) were within the previously reported range for wastewater solids (Langford et al. 2015) but higher than typical values for sediment (Tsui et al. 2015). The measured K_d for HMS (5920 ± 2080 L kg^{-1}) was several times higher than that observed in Chesapeake Bay sediment (He et al. 2019) and likely due to the higher organic carbon content of the fecal matter (not measured). Given the high levels of HMS used in personal care products (*e.g.*, up to 15% (FDA, 2007)) and the high partition coefficient reported here, future studies are recommended to assess HMS occurrence in sediment and biota. Importantly, the US EPA ECOSAR model (EPA, 2012) predicts LC_{50} and chronic values for homosalate on the order of 1–10 μg L^{-1} in fish and daphnids, reinforcing the potential for biological effects of homosalate accumulation in sediment and biota.

For estrogens, the 36-h aqueous-phase concentrations followed the trend: EE2 > E1 > E2. The observed K_d values of E1, E2, and EE2 were similar to previous reports for activated sludge (Andersen et al. 2005; Clara et al. 2004). In general, the change in aqueous-phase concentration between dosing events can be attributed to abiotic transformation (Liu and Liu 2004; Silva et al. 2012), metabolism (Ivanov et al. 2010; Welshons et al. 2003), sorption to sediment (Andersen et al. 2005), and accumulation in biota (Dussault et al. 2009a; Ricciardi et al. 2016). Because the aqueous- and fecal matter-phase concentrations followed the same trends, some combination of the above processes likely manifested; however, the degradation pathways were not specifically elucidated in this work. As the mean K_d values for E2 (355 ± 50 L kg^{-1}) and E1 (378 ± 190 L kg^{-1}) were similar, the higher aqueous-phase concentrations of E1 during the exposure period may be partially explained by biotransformation of E2 into E1 (Lai et al. 2002), although this explanation could not be experimentally confirmed in the present study since E1 and E2 were both added to the aquaria. As noted above, the experimental data indicated that the synthetic hormone EE2 was more stable than the natural estrogens, E1 and E2. This finding was further confirmed by the measurable aqueous-phase concentrations of EE2 in Tanks 1 and 2 during the elimination period (see Figs. 2 and 3, respectively). The quicker degradation of E1 and E2 compared to EE2 in Tanks 1 and 2 is in agreement with previous observations (Yu et al. 2013). After two days of elimination in Tank 1, about 73% of the dosed EE2 remained in the aqueous phase, but the E1 and E2 concentrations were both below the limits of detection (*i.e.*, less than 5 ng L^{-1}) as shown in Fig. 2b. Similar findings were observed for Tank 2 (see Fig. 3b).

The aqueous-phase concentrations of the more hydrophobic UV-filters (*i.e.*, EHMC, HMS, and OC) were relatively constant ($p > 0.05$, see Table S2 in the SI) during the elimination period, while the less hydrophobic UV-filters (*i.e.*, 4-MBC and BP-3) followed degradation trends similar to that described for EE2. Regardless of the high K_d values for the estrogens and UV-filters, the mass of fecal matter was low compared to the volume of water. Based on the overall mass balance analysis (see Fig. S3 in the SI), the maximum EDC content in the fecal matter phase was just 6% of the total mass of OC added to Tank 1. Furthermore, over

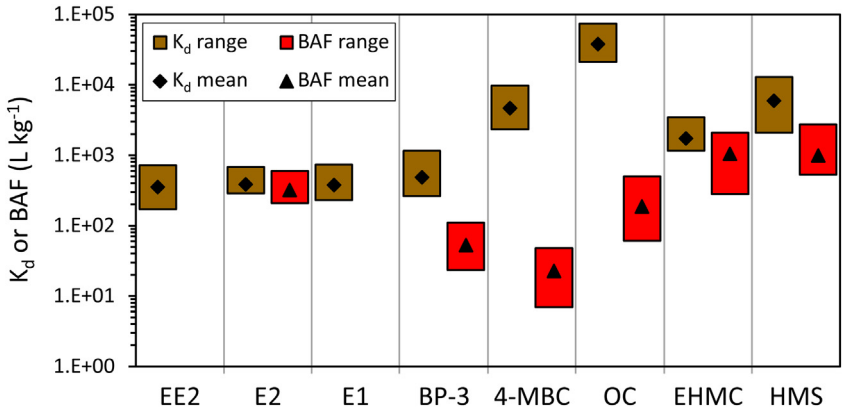

Fig. 4. Experimentally-measured ranges and mean values for the K_d (water-fecal matter system) and BAF (water-tail tissue system) parameters for estrogens and UV-filters. Note that EE2 and E1 were not measured in the crayfish tail tissue, and so BAFs are not presented for these two compounds. The EDC concentrations measured in water, fecal matter, and tail tissue samples are available in Fig. S1 of the SI, Fig. S2 of the SI, and Table 1, respectively. Chemical acronyms are as follows: EE2, 17α-ethinylestradiol; E2, 17β-estradiol; E1, estrone; BP-3, benzophenone-3; 4-MBC, 4-methylbenzylidene camphor; OC, octocrylene; EHMC, 2-ethylhexyl-4-methoxycinnamate; and, HMS, homosalate.

85% of the total mass of individual estrogens and UV-filters was lost from the system through transformation reactions.

3.2. Bioaccumulation of estrogens and UV-filters in crayfish tail tissue

The concentrations of estrogens and UV-filters in the *P. clarkii* tail tissue are summarized in Table 1. Tail tissue collected from female and male crayfish were analyzed separately, and no significant differences ($p > 0.05$, see Table S2 in the SI) were observed with respect to EDC accumulation by crayfish sex. With the exception of 4-MBC, E2, and OC, the EDC concentrations in tail tissue were similar ($p > 0.05$, see Table S2 in the SI) at Days 14, 28, and 42 of the exposure period for the 500 ng L^{-1} and 5000 ng L^{-1} scenarios. This observation suggests that the EDCs were generally present at pseudo-steady state, justifying the calculation of BAFs.

E2 was the only estrogen that was detected in the crayfish tail tissue, with concentrations of 9.9 ± 1.3 ng g^{-1} in Tank 1 and 11.1 ± 1.7 ng g^{-1} in Tank 2. Although E2 is a vertebrate steroid hormone, the occurrence of E2 has also been observed in the ovary, hepatopancreas, and hemolymph of some crustaceans (Gunamalai et al. 2006; Kirubagaran et al. 2010; Warrier et al. 2001) at concentrations as high as 7.5 ng g^{-1} (Warrier et al. 2001). In this study, E2 concentrations in the tail tissue of crayfish from the control tank were below 0.5 ng g^{-1}, and so the measured E2 in crayfish from Tanks 1 and 2 mostly stemmed from accumulation. This result was supported by the variation in tissue-phase concentrations of E2 with treatment (*i.e.*, Tank 1 *vs.* Tank 2; $p < 0.001$) and exposure time (*i.e.*, Day 42 *vs.* Day 14; $p < 0.001$), as indicated in Table S2 of the SI.

The BAFs of estrogens and UV-filters were calculated using Eq. (2). Although bioaccumulation of E1 (Gomes et al. 2004; Lai et al. 2002) and EE2 (Al-Ansari et al. 2013; Dussault et al. 2009a; Ricciardi et al. 2016) has been observed in previous laboratory exposure experiments, the reported BAFs vary over a wide range for different organisms. Lai et al. (2002) reported a BAF of 27 L kg^{-1} for E1 in a green microalgae (*Chlorella vulgaris*) over 48 h with an aqueous-phase concentration around 2 μg L^{-1}. A higher BAF for E1 (228 L kg^{-1}) was observed in *D. magna* after 24 h with aqueous concentrations of 18–343 μg L^{-1} (Gomes et al. 2004). In a 21-d exposure experiment with 20–3100 μg L^{-1} of EE2, Dussault et al. (2009b) reported BAFs in the 18–215 L kg^{-1} range for two invertebrates, a midge (*Chironomus tentans*) and a freshwater amphipod (*Hyalella azteca*). Similar BAFs for EE2 were observed in goldfish (*Carassius auratus*; 377 L kg^{-1}) (Al-Ansari et al. 2013) and blue mussels (*Mytilus edulis*; 103 L kg^{-1}) (Ricciardi et al. 2016). In the present study, the E1 and EE2 concentrations in the crayfish tail tissue were below the limits of detection and their estimated BAFs were less than 7 and 5 L kg^{-1}, respectively. Overall, these data suggest that BAFs for E1 and EE2 vary significantly between organisms and may be influenced by the aqueous-phase concentrations employed in exposure experiments. Future efforts should, therefore, be conducted at lower concentrations relevant to wastewater and wastewater-impacted surface water scenarios; furthermore, biological response endpoints should be monitored to connect bioaccumulation of EDCs to toxicity outcomes.

All five UV-filters accumulated in crayfish tail tissue during the exposure period, but 4-MBC was only detected in crayfish from Tank 2. Unlike the other EDCs, the tail tissue concentrations of 4-MBC were not constant during the exposure period: 27.0 ± 9.4 ng g^{-1} at Day 14; 6.5 ± 3.0 ng g^{-1} at Day 28; and, 14.5 ± 7.7 ng g^{-1} at Day 42. The concentration at Day 14 was significantly different than the concentrations at Day 28 ($p < 0.001$) and Day 42 ($p < 0.001$), as indicated in Table S2 of the SI. No explanation for these trends is proposed, but previous field-based observations (Fent et al. 2010) and the data from the elimination period (see Figs. 2 and 3) suggest rapid metabolism of 4-MBC, a factor that may have prevented attainment of pseudo-steady-state conditions between the water and tissue phases. Among the tested UV-filters, 4-MBC exhibited the lowest apparent BAF (*i.e.*, 23 ± 14 L kg^{-1}), potentially explaining why this analyte was not detected in crayfish from Tank 1. In this case, the aqueous-phase concentration of 4-MBC was 90 ± 21 ng L^{-1}, and the corresponding tissue concentration (calculated from the apparent BAF) was 2.1 ± 1.3 ng g^{-1}, which overlapped with the limit of detection.

The tail tissue concentrations of the other four UV-filters were relatively constant during the exposure period ($p > 0.05$, except for OC concentrations at Day 14 and Day 42) but different between the two tanks ($p < 0.001$), as indicated in Table S2 of the SI. The mean tail tissue concentrations of BP-3 in Tank 2 were about 10 times higher than those in Tank 1; however, lower concentration ratios between the two experimental tanks were observed for the more hydrophobic UV-filters. For example, the ratios of the HMS and OC tail tissue concentrations in Tanks 2 and 1 were approximately 3.1 and 1.7, respectively. Interestingly, the EHMC concentrations measured in crayfish from Tank 1 were slightly higher than those measured in crayfish from Tank 2. The

Table 1
Analyte concentrations (ng g^{-1} lyophilized tissue) in the tail tissue of male and female crayfish from Tank 1 (500 ng L^{-1} target level) and Tank 2 (5000 ng L^{-1} target level). The compounds are listed from left-to-right in order of least to most hydrophobic, according to their retention time on the LC column. Error is standard deviation (n = 3).

Tank	Crayfish sex	Exposure time (day)[a]	EE2[b]	E2	E1	BP-3	4-MBC	OC	EHMC	HMS
Tank 3 (control)	Female	0	n.d.[c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<20[d]
	Male	0	n.d.	n.d.	n.d.	n.d.	n.d.	<2.0[d]	n.d.	<20[d]
Tank 1	Female	14	n.d	10.4 ± 0.2	n.d.	5.0 ± 0.1	n.d.	2.2 ± 1.0	35.9 ± 5.6	97.4 ± 5.9
		28	n.d.	10.8 ± 1.2	n.d.	5.5 ± 0.3	n.d.	5.2 ± 2.8	38.7 ± 7.4	99.2 ± 2.2
		42	n.d.	8.7 ± 0.3	n.d.	6.6 ± 0.9	n.d.	6.7 ± 1.2	61.9 ± 13.7	84.9 ± 3.6
		56	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	29.8 ± 4.7	135 ± 18
	Male	14	n.d.	11.2 ± 0.9	n.d.	4.8 ± 0.1	n.d.	<2.0[d]	45.6 ± 9.3	84.6 ± 6.1
		28	n.d.	9.1 ± 1.0	n.d.	5.1 ± 0.2	n.d.	3.7 ± 1.5	39.9 ± 0.4	106 ± 37
		42	n.d.	9.5 ± 0.6	n.d.	5.3 ± 0.5	n.d.	12.9 ± 4.7	28.4 ± 1.9	78.2 ± 3.1
		56	n.d.	<0.5[d]	n.d.	n.d.	n.d.	n.d.	32.1 ± 2.2	107 ± 11
Tank 2	Female	14	n.d.	13.5 ± 2.0	n.d.	42.9 ± 2.0	26.5 ± 0.4	9.8 ± 2.0	27.6 ± 2.4	265 ± 25
		28	n.d.	11.1 ± 0.1	n.d.	44.7 ± 4.0	6.0 ± 1.1	10.8 ± 1.3	25.5 ± 1.6	272 ± 28
		42	n.d.	9.7 ± 0.1	n.d.	48.6 ± 5.1	10.3 ± 3.0	9.4 ± 0.8	28.3 ± 4.7	292 ± 24
		56	n.d.	<0.5[d]	n.d.	5.3 ± 0.3	n.d.	9.2 ± 1.0	28.6 ± 1.7	158 ± 44
	Male	14	n.d.	11.4 ± 0.4	n.d.	49.1 ± 0.6	27.5 ± 9.6	10.1 ± 0.8	26.5 ± 0.8	232 ± 31
		28	n.d.	11.6 ± 1.3	n.d.	44.4 ± 2.3	7.0 ± 3.0	11.7 ± 0.8	26.3 ± 0.8	288 ± 37
		42	n.d.	9.7 ± 0.1	n.d.	44.1 ± 2.2	18.6 ± 8.3	9.4 ± 0.4	25.2 ± 0.4	334 ± 41
		56	n.d.	<0.5[d]	n.d.	5.3 ± 0.2	n.d.	9.3 ± 1.4	27.1 ± 1.4	161 ± 17

[a] Days after the first addition of estrogens and UV-filters to the aquaria.
[b] EE2, 17α-ethinylestradiol; E2, 17β-estradiol; E1, estrone; BP-3, benzophenone-3; 4-MBC, 4-methylbenzylidene camphor; OC, octocrylene; EHMC, 2-ethylhexyl-4-methoxycinnamate; HMS, homosalate.
[c] Not detected, below the limit of detection.
[d] Detected, but below the limit of quantitation.

water-phase EHMC concentrations in Tanks 1 and 2 were, however, similar across the exposure period. Using those concentrations with the BAF range reported in Fig. 4, the tissue-phase concentrations of EHMC in Tanks 1 and 2 were expected to be in the 5.3–111.0 ng g^{-1} and 23.1–176.3 ng g^{-1} ranges, respectively. As these ranges have a large overlap, the observed differences in EHMC levels in crayfish tissue from Tanks 1 and 2 were not significant.

In general, higher BAFs were observed for the more hydrophobic UV-filters (Fig. 4). The mean BAFs for BP-3, EHMC, HMS, and OC were 54 ± 23, 1050 ± 457, 991 ± 569, and 187 ± 131 L kg^{-1}, respectively. No direct comparison of these BAFs is available from literature data specific to crayfish, but the high detection frequency of BP-3, EHMC, HMS, and OC in aquatic organisms (Gago-Ferrero et al. 2015; He et al. 2017; Peng et al. 2015; Picot Groz et al. 2014) confirms bioaccumulation of UV-filters in the environment. The BAFs followed a similar trend as biota-sediment accumulation factors obtained for two cephalopods, five crustaceans, and 17 fish species (Peng et al. 2017). Unlike the other EDCs, the partition coefficients for 4-MBC and OC in the water-fecal matter system were much larger (*i.e.*, approximately 200×) than the corresponding BAFs. These results suggest favorable interactions between 4-MBC and OC with chemical moieties in the fecal matter or lower absorption, distribution, and metabolism (and higher excretion) of 4-MBC and OC in crayfish. As the log D value for OC is higher than that for HMS, we posit that the surprisingly low BAF for OC also stems from lower absorption, distribution, and metabolism (and higher excretion) in crayfish. These findings may have important implications for UV-filter toxicity outcomes in aquatic invertebrates.

During the elimination period, the tail tissue concentrations of 4-MBC and BP-3 significantly decreased (see Table 1), while the more hydrophobic UV-filters (*i.e.*, EHMC and OC) were relatively stable ($p > 0.05$; see Table S2 of the SI). These results indicate that OC, EHMC, and HMS will be more persistent in the environment, raising the potential for negative ecological effects. The slow decrease in EHMC and OC concentrations observed in *P. clarkii* tissue differed from a previous observation in Mediterranean mussels (*Mytilus galloprovincialis*), wherein more than 90% of EHMC and OC degraded within 24 h (Gomez et al. 2012). This result implies significant differences in metabolic processing of UV-filters among aquatic organisms. Although HMS is one of the most widely used UV-filters (FDA, 2007), its presence in environmental samples from the United States has not been reported until recently (He et al. 2019; He et al. 2017; Tsui et al. 2014b). The high BAF for HMS calculated in this study emphasizes the need for more monitoring of its occurrence and toxicity in aquatic organisms. As mentioned above, the majority (*i.e.*, over 85%) of the total mass of individual estrogens and UV-filters was lost from the system through transformation reactions. For this reason, the residual estrogenic activity of the water samples was measured to determine whether the transformation products affected the overall estrogenicity of water in the aquaria and potentially increased pressure on the crayfish.

3.3. Estrogenic activity of the water samples

The relative estrogenic activity of the EDCs of concern was assessed using the lyticase-assisted YES assay. Fig. 5a shows the dose-response curve for E2 in the concentration range of 1 ng L^{-1} (4.14×10^{-12} M) to 200 μg L^{-1} (7.34×10^{-7} M). The EC_{50} for E2 was $(3.95 \pm 1.1) \times 10^{-10}$ M, and this value was used in Eq. (4) to determine the EEQs for other estrogens, UV-filters, and experimental samples. The calculated EEQs for EE2 and E1 were 1.1 ± 0.1 and 0.10 ± 0.02, respectively, whereas the EEQs for UV-filters were all at least four orders of magnitude lower than E2. For instance, the EEQs for 4-MBC and BP-3 were $(4.3 \pm 1.2) \times 10^{-7}$ and $(2.2 \pm 0.4) \times 10^{-4}$ (see Fig. 5a), respectively. The EC_{50} parameters for EHMC, HMS, and OC were above the solubility limits (in water), suggesting that their EEQs were negligible for the experimental conditions. These findings agree with limited information about UV-filter estrogenic activity available from previous studies (Balázs et al. 2016; Kunz and Fent 2006). Given the above findings and the higher aqueous-phase concentrations of estrogens during the exposure period, the contribution of UV-filters to the overall EEQs of experimental water samples was negligible.

The EEQs of water samples collected during the exposure period were determined by the lyticase-assisted YES assay (EEQ_{YES}) and measured aqueous-phase concentrations from LC-MS/MS analysis ($EEQ_{LC\text{-}MS/MS}$; see Eq. (5)). UV-filters were not included in Eq. (5) for the reasons described above.

$$EEQ_{LC-MS/MS} = 0.1\,[E1] + 1.0\,[E2] + 1.1\,[EE2] \quad (5)$$

The YES- and LC-MS/MS-based EEQs are plotted against each other in Fig. 6. In general, the YES assay and LC-MS/MS results were in agreement for the low EDC concentrations in Tank 1. However, differences were observed between the EEQs calculated using the YES- and LC-MS/MS-based methods for water samples collected from Tank 2.

Given the over-prediction of EEQs by LC-MS/MS in Fig. 6b, antagonistic effects were suspected during YES analysis of the samples from Tank 2. The mechanism of antagonism requires further investigation,

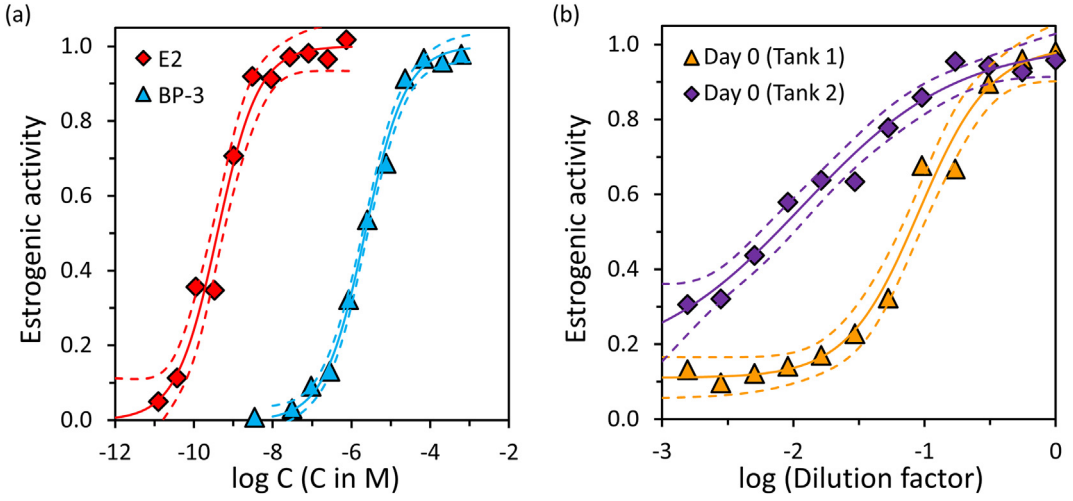

Fig. 5. Estrogenic activity measured by the lyticase-assisted YES assay for (a) E2 and BP-3 standards and (b) serially-diluted Day 0 water samples from Tanks 1 and 2. The dashed curves are 95% confidence bands. Chemical acronyms are as follows: E2, 17β-estradiol; and, BP-3, benzophenone-3.

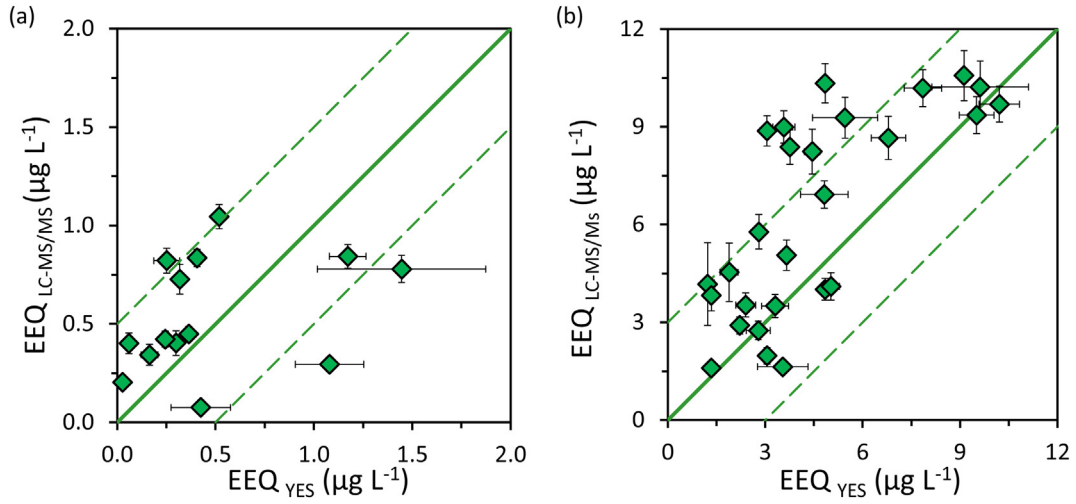

Fig. 6. Calculated E2 equivalents (EEQs) based on targeted LC-MS/MS analyses and non-targeted YES assays of water samples collected from (a) Tank 1 and (b) Tank 2. The solid line is the 1:1 line, and the dashed lines are offset by ±0.5 and ± 3.0 in (a) and (b), respectively, to help distinguish the similarity of estrogenic activity measurements by each technique. Note, less data are present in (a) because the EEQs of some water samples were below the limit of detection for the lyticase-assisted YES assay.

but possible explanations may involve the formation of inactive estrogen conjugates during the exposure period (Ihara et al. 2015) or antiestrogenic activity of UV-filters (Balázs et al. 2016; Kunz and Fent 2006). During LC-MS/MS analysis, estrogen conjugates may revert back to the parent estrogens, causing an inherent difference in the EEQs measured by YES assay (lower observed estrogenicity) and LC-MS/MS (higher observed estrogen concentrations). Similar outcomes could stem from concentration-dependent antiestrogenic activity of UV-filters. For these reasons, the data reported in Fig. 6 raise concerns about previous exposure experiments conducted at high concentrations that are not representative of environmental scenarios. Importantly, the residual estrogenicity measured in water from Tanks 1 and 2 suggested that unknown transformation products did not significantly contribute estrogenic activity. Nevertheless, the accumulation of EDCs and their degradation products (Cajthaml et al. 2009; Maniero et al. 2008) may have other toxicological consequences for crayfish, as well as other organisms that consume these ecologically-important invertebrates. Future research is recommended to evaluate potential toxicity outcomes.

4. Conclusion

All five UV-filters accumulated in crayfish tail tissue, and the corresponding BAFs ranged from 23 L kg^{-1} for 4-MBC to 1050 L kg^{-1} for EHMC. The BAF for OC (187 L kg^{-1}) was surprisingly low given the relatively high hydrophobicity of this UV-filter. The partition coefficients for 4-MBC and OC in the fecal matter-water system were approximately 200× higher than the corresponding BAFs, suggesting faster metabolic processing of these UV-filters in crayfish. Of the three estrogenic hormones, only E2 was detected in the crayfish tissue for the experimental conditions; however, the estrogenic activity of the hormones was at least four orders of magnitude higher than that of the UV-filters. Residual estrogenic activity measurements by targeted LC-MS/MS protocols and non-targeted, activity-based analysis with a lyticase-assisted YES assay demonstrated that transformation products did not significantly contribute to the total estrogenicity; however, future studies are recommended to investigate endocrine disruption and other toxicity outcomes in crayfish. Given the observed accumulation and widespread presence of crayfish in small streams throughout the world, these invertebrate organisms may serve as unique biomonitoring tools for assessing the presence of UV-filters and other EDCs in aquatic food webs and water resources.

CRediT authorship contribution statement

Ke He: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Ethan Hain:** Formal analysis, Investigation, Writing - review & editing. **Anne Timm:** Writing - review & editing, Supervision, Funding acquisition. **Lee Blaney:** Conceptualization, Methodology, Formal analysis, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.142871.

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