Science of the Total Environment 828 (2022) 154176

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Comprehensive assessment of chemical residues in surface and wastewater using passive sampling, chemical, biological, and fish behavioral assays

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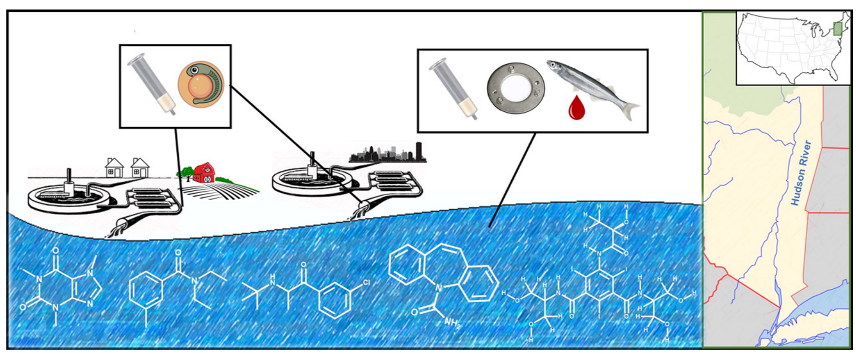
HIGHLIGHTS

- Assessment of chemical residues using targeted and non-targeted analyses.
- Biological assays to assess effects of high effluent input on aquatic environment.
- Detections include; 63 pharmaceuticals, 10 industrial chemicals, and 21 pesticides.
- Higher frequency of detection for vitellogenin in fish from the Hudson River
- Exposure to wastewater effluents linked to hyperactivity in zebrafish embryos

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 16 November 2021
Received in revised form 31 January 2022
Accepted 23 February 2022
Available online 1 March 2022

Editor: Damià Barceló

Keywords:
Non-targeted analysis
Liquid chromatography tandem mass spectrometry
Contaminants of emerging concern
Pharmaceuticals and personal care products
Hudson River

ABSTRACT

Effluents from ten full-scale municipal wastewater treatment plants (WWTPs) that discharge into the Hudson River, surface waters, and wild-caught fish samples were analyzed using liquid chromatography with tandem mass spectrometry (LC/MS/MS) to examine the influence of wastewater discharge on the concentrations of contaminants of emerging concern (CECs) and their ecological impacts on fish. Analysis was based on targeted detection of 41 pharmaceuticals, and non-targeted analysis (suspect screening) of CECs. Biological effects of treated WWTP effluents were assessed using a larval zebrafish (*Danio rerio*) swimming behavior assay. Concentrations of residues in surface waters were determined in grab samples and polar organic chemical integrative samplers (POCIS). In addition, vitellogenin peptides, used as biomarkers of endocrine disruption, were quantified using LC/MS/MS in the wild-caught fish plasma samples. Overall, 94 chemical residues were identified, including 63 pharmaceuticals, 10 industrial chemicals, and 21 pesticides. Eight targeted pharmaceuticals were detected in 100% of effluent samples with median detections of: bupropion (194 ng/L), carbamazepine (91 ng/L), ciprofloxacin (190 ng/L), citalopram (172 ng/L), desvenlafaxine (667 ng/L), iopamidol (3790 ng/L), primidone (86 ng/L), and venlafaxine (231 ng/L). Over 30 chemical residues were detected in wild-caught fish tissues. Notably, zebrafish larvae exposed to chemical extracts of effluents from 9 of 10 WWTPs, in at least one season, were significantly hyperactive. Vitellogenin expression in male or immature fish occurred 2.8 times more frequently in fish collected from the Hudson River as compared to a reference site receiving no direct effluent input. Due to the low concentrations of pharmaceuticals detected in effluents, it is likely that chemicals other than pharmaceuticals measured are responsible for the behavioral changes observed. The combined use of POCIS and non-target analysis demonstrated significant increase in the chemical coverage for CEC detection, providing a better insight on the impacts of WWTP effluents and agricultural practices on surface water quality.

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http://dx.doi.org/10.1016/j.scitotenv.2022.154176

1. Introduction

Pharmaceutical residues enter the aquatic system through municipal, industrial, agricultural, and hospital wastewater discharges. While some pharmaceutical compounds are removed in conventional wastewater treatment plants (WWTPs), many remain unchanged and are discharged in the receiving surface waters (Kolpin et al., 2002; Kostich et al., 2014). Removal efficiencies and degradation of pharmaceuticals are dependent on the design and operation of the WWTP; while some pharmaceuticals are biodegraded completely, others either remain intact or undergo only partial transformations during the treatment processes (Borova et al., 2014; Evgenidou et al., 2015; Li et al., 2018; Subedi and Kannan, 2015). As a consequence of the continuous release into surface waters (Kostich et al., 2014; Subedi and Kannan, 2015; Cantwell et al., 2018; Carpenter and Helbling, 2018; Puckowski et al., 2016) some pharmaceuticals have been shown to accumulate in fish (He et al., 2019; Ramirez et al., 2009; Ramirez et al., 2007; Subedi et al., 2012) and other aquatic organisms at concentrations ranging from low ng/Kg to μg/Kg range.

Most environmental monitoring studies involving chemical analysis of wastewater and surface waters have primarily used solid phase extraction (SPE) for sample concentration, and targeted analysis by the liquid chromatography with tandem mass spectrometry (LC/MS/MS) for detection and quantification (Kostich et al., 2014; Subedi and Kannan, 2015; Cantwell et al., 2018; Carpenter and Helbling, 2018; Angeles et al., 2020a). The high selectivity and sensitivity achieved using selected reaction monitoring (SRM) have made LC/MS/MS a popular choice for many environmental laboratories when analyzing micropollutants, such as pharmaceuticals (Angeles and Aga, 2018). However, targeted analysis include only a limited number of compounds and do not provide a comprehensive chemical analysis. Therefore, targeted analytical approaches may fail to measure important contaminants in the samples and may not target the relevant compounds among the hundreds or thousands of contaminants present in the environment.

A rapidly growing approach in environmental analysis and toxicology research involves the use of non-target analysis (NTA) with high-resolution mass spectrometry (HRMS), where data on accurate masses of molecular and fragment ions are collected without a priori information on the chemicals being analyzed. Non-target suspect screenings, where a list of suspect compounds of interest are searched against all detected peaks from NTA, are commonly used to increase the chemical coverage, while having more manageable data analysis times than full NTA. This approach can aid in screening and analysis of the vast universe of contaminants, and provides an opportunity to comprehensively examine the occurrence, fate, bioaccumulation, and transformations of chemical of emerging concern (CECs) in the aquatic systems. In the recent years, NTA has been used for the identification of unknown chemicals and transformation products, for metabolomic studies, and for retrospective screening to discover contaminants that were not previously included in the target analysis (Angeles et al., 2020b; Chavez Soria et al., 2019; Guardian et al., 2020). Data from NTA using HRMS can be compared with databases that include exact masses of molecular and fragment ions, expanding the number of chemical residues identified in the samples. However, to fully confirm the identity of a contaminant with a “level 1 confidence” (Schymanski et al., 2014), the compound must be confirmed with a reference standard, with high resolution mass, MS^2 fragmentation, and retention time match (Schymanski et al., 2014). Therefore, NTA is typically qualitative, with semi-quantitation possible when a standard is available for use based on either standard addition, external calibration, or a matrix-matched calibration curve.

Another limitation of target analysis is the use of sample preparation procedure that is optimized to extract the target analytes. The hydrophilic-lipophilic-balanced (HLB) Oasis™ SPE sorbent is the most commonly used for water extractions (Kostich et al., 2014; Subedi and Kannan, 2015; Cantwell et al., 2018; Angeles et al., 2020a; Angeles and Aga, 2018; Angeles et al., 2020b; Angeles and Aga, 2020) because of its ability to retain chemicals with a wide range of polarity. Despite the efficiency of many SPE sorbents, highly polar compounds may still be lost during the extraction process due to slow sorption kinetics and the limited capacity of the sorbent, introducing bias in the analysis. Several NTA applications in surface water monitoring have employed passive sampling using polar organic chemical integrative samplers (POCIS) based on similar HLB sorbent material housed in a disk for field deployment (Alvarez, 2010; Alvarez et al., 2007; Criquet et al., 2017; Guibal et al., 2018; Martinez Bueno et al., 2016). Passive samplers have been used for the collection of pharmaceuticals and pesticides in the aquatic environment (Criquet et al., 2017; Martinez Bueno et al., 2016; Wille et al., 2011), offering the advantages of long-term deployment, time-dependent measurements, pre-concentration of trace level chemical residues, and ease of sample transport. This study is the first analysis of the Hudson River using POCIS samplers and NTA suspect screening approach. Previous studies using POCIS samplers for pharmaceuticals were limited to targeted analytes (Alvarez et al., 2021; Bartelt-Hunt et al., 2009; Vrana et al., 2021).

This study combined targeted and NTA, using both SPE and POCIS for sample pre-concentration, to analyze synthetic chemical residues in the Hudson River (New York, USA) that receives point and nonpoint sources of pollutants. The Hudson River is an aquatic system that contains a complex assemblage of resident and migratory fish species, pelagic and benthic invertebrates, and receives large inputs of effluents from major WWTPs. To date, there has been no study that performed comprehensive analysis of CECs entering the Hudson River system directly from surrounding WWTPs. In this regard, a targeted analytical method was used first to quantify selected pharmaceuticals and personal care products (PPCPs) primarily in WWTP effluents, but also with limited sampling of receiving waters, and fish samples from the same areas where water samples were collected. Psychoactive drugs are of particular interest due to their high frequency of prescription in the U.S. resulting in their widespread occurrence and persistence in the environment (Kostich et al., 2014; Calisto and Esteves, 2009; Metcalfe et al., 2010). Some psychoactive drugs, such as antidepressants have been shown to accumulate in fish tissues, including brains (Arnnok et al., 2017; Brooks et al., 2005; Grabicova et al., 2014; Schultz et al., 2010). and cause behavioral changes and endocrine disruption in a number of fish species (Brooks et al., 2003; Painter et al., 2009; Park et al., 2012).

The combination of chemical and biological analysis of environmental samples provides an important approach linking exposure of aquatic biota to understanding biological responses (Farré and Barceló, 2003; Muller et al., 2008; Prasse et al., 2015; Schoenfuss et al., 2016). Due to a lack of government regulations on the release of many chemical residues from WWTPs, knowledge on the occurrence of contaminants and the ecological responses they may elicit in the aquatic environment is needed. Animal behavior studies provide a sensitive approach for examining toxicity of chemicals and for identifying those with greater potential to impair physiological functions (Scott and Sloman, 2004). Therefore, combining behavioral studies with chemical analysis provides a valuable and robust approach to identifying contaminants in water that may present a risk to ecosystem health. A few studies have examined the impact of WWTP effluents on larval fish behavior, reporting on single WWTP (Schoenfuss et al., 2016; Melvin, 2016; McCallum et al., 2017a; McCallum et al., 2017b), or comparing multiple sites at a single time period (Angeles et al., 2020a). Mixed results from fish exposure to WWTP effluent have been reported, ranging from no significant behavioral effects (Angeles et al., 2020a; Schoenfuss et al., 2016; McCallum et al., 2017a), to alterations in swimming behavior (Scott and Sloman, 2004), and reduction in aggression (McCallum et al., 2017b). Additionally, vitellogenin (VTG) expression was analyzed in wild-caught fish as a biomarker for endocrine disruption by LC/MS/MS analysis for peptide sequences (ALHPELR and FIELIQLLR) (He et al., 2019) unique to VTG and applicable to fish species (as determined by Protein BLAST®). VTG is a precursor to egg-yolk protein, and its presence in mature male or juvenile fish has been seen to increase after exposure to estrogen and estrogenic compounds, making it a common biomarker for endocrine disruption (He et al., 2019; Denslow et al., 1999; Hansen et al., 1998). The analysis of VTG through use of specific peptides requires that the targeted peptides (ALHPELR and FIELIQLLR) are present in the genome of the selected fish species.

This study reports targeted PPCP data from 10 major WWTPs that discharge directly into the Hudson River (Fig. S1) over three seasons, as well as NTA suspect screening (including PPCPs, pesticides, hormones and industrial chemicals) for selected effluent samples. In addition, samples from receiving surface waters, as well as tissue and plasma from fish samples collected from a Hudson River site, and a reference site on Long Island (New York, USA), were analyzed. This study combines biological and chemical analyses to assess potential impacts of WWTP effluents on receiving waters. This knowledge will enhance our understanding of the fate and ecological impacts of chemical residues in the Hudson River watershed, and inform similar studies on other bodies of fresh water experiencing high volumes of wastewater influx.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals and reagents used for all analyses and details on origin are included in supplementary information.

2.2. Sampling

2.2.1. Wastewater treatment plant sampling

Effluent samples were collected from 10 participating WWTPs that discharge treated wastewater into the Hudson River. Detailed information on specific plants has been withheld to maintain the confidentiality of the data specific to each WWTP. All plants provided secondary treatment with activated sludge, differing primarily only in the type of disinfection procedure utilized (hypochlorite versus ozonation). The rated size of the plants varied from 4 to over 100 million gallons per day (mgd), all discharging into the Hudson River and serving sewersheds from Westchester County up to north of Albany. Measured flow rates during each sampling period were all within the capacity of the plants and ranged from 2 to 60 mgd. Average removal efficiencies for all plants averaged 97.3, 96.9, and 93.6% of biochemical oxygen demand for the July, September and December sampling periods respectively. Many of the plants serviced hospitals with some servicing more than one. A 24-h time proportional composite samples of effluent (cooled with ice) were obtained from all plants over the same 24-h period in July, September, and December 2019 to provide information on seasonal distribution of targeted compounds. Effluent samples were collected into amber 1-L glass bottles preloaded with 2 mL of 85% phosphoric acid to prevent bacterial degradation within 4 h of sampling completion. Samples were shipped overnight on blue ice packs to the University at Buffalo (UB) for chemical analysis.

2.2.2. Surface water sampling

Grab samples of surface water were collected in 0.5 L amber glass bottles and acidified with 2 mL of 85% phosphoric acid. Grab water samples were collected at each fishing location, approximately one foot below the surface. In addition, triplicate POCIS samplers were secured with rope at each fish sampling location and deployed for 6 weeks. A triplicate group of POCIS was used as field blank exposed to the field during deployment, retrieval, and transport. After deployment, samplers were shipped, separately wrapped in aluminum foil to UB for analysis. Upon retrieval, samplers were rinsed with NANOpure™ water to remove sediment, dried, and stored in a − 20 °C freezer until analysis.

2.2.3. Fish sample collection

Fish samples were collected in August and September 2019 from Piermont fishing pier on the Hudson River, and from a reference site, Flax Pond, located near Stony Brook University (SBU) respectively (Fig. S1). Piermont Pier is located downstream from 9 of 10 sampled WWTPs, and adjacent to a wastewater effluent outfall, not included in the sampled plants. The reference site has no direct input from a WWTP effluent and is located in a bird sanctuary adjacent to a very low density (minimum 2 acre) residential area. Silversides (*Menidia mendia*), juvenile striped bass (*Morone saxatilis*) and killifish (*Fundulus* spp.) were collected by seine netting or trapping, with silverside and killifish samples obtained from both locations. Fish were anesthetized in 50–100 mg/L buffered tricaine methanesulfonate before standard length (mm) and wet weight (g) was measured. Blood samples were collected in 100 μL heparinized microhematocrit tubes. Blood samples were immediately centrifuged for 10 min at 1400 *x*g to collect plasma, which was stored in microcentrifuge tubes. Fish tissue and blood plasma samples were stored at −20 °C until analysis. The presumptive sex of each fish sample was determined by visual inspection of gonads. Plasma and fish samples were transported to UB overnight on dry ice, and fish tissues were immediately freeze-dried and homogenized by mortar and pestle upon arrival. General information on the fish samples such as length, weight, and sex, are listed in the supplementary information (Tables S1, S2).

2.3. Extraction

2.3.1. Water sample extraction

Sample extraction method for wastewater and surface water was adopted from a previously published method (Angeles et al., 2020a). Briefly, two 500 mL aliquots of water from each sample were extracted by using 500 mg HLB Oasis™ SPE cartridges. One aliquot was spiked with 50 μL of 1000 μg/L with a surrogate mix of 15 labeled pharmaceuticals for chemical analysis, while the second aliquot was not spiked and reserved for fish behavior bioassays. Cartridges were conditioned with methanol (6 mL) and NANOpure™ water (6 mL), loaded with water samples at approximately 6 mL/min. (Angeles et al., 2020a), and dried under vacuum for about 1 h. The SPE cartridges used for samples without surrogates were sent to SBU for elution and fish behavioral studies. The SPE cartridges used for samples spiked with surrogates were eluted with two 4 mL portions of methanol, and the combined extract were dried slowly under nitrogen gas. Dried samples were reconstituted to 0.5 mL with the LC/MS/MS starting mobile phase, spiked with 25 μL of 1000 μg/L d3-diphenhydramine internal standard, and transferred into a 2-mL vial for LC/MS/MS analysis (Angeles et al., 2020a).

2.3.2. Fish sample extraction

Freeze-dried fish tissue samples were extracted using a previously published method (Arnnok et al., 2017). Briefly, a 500 mg aliquot of whole-body fish homogenate was fortified with 20 μL of 1000 μg/L surrogate mix and allowed to equilibrate overnight. Samples were extracted with 5 mL of 50:50 acetonitrile/ isopropanol with 5% formic acid by bath sonication for 5 min (Arnnok et al., 2017). The samples were centrifuged and the supernatant from each sample was decanted into a 50 mL centrifuge tube. The extraction process was repeated and extracts from each sample were pooled and combined with 5 mL of 5 mM ammonium acetate and 2 g of alumina (used for lipid removal) (Arnnok et al., 2017). After lipid removal, each extract was decanted and diluted with 300 mL of NANOpure™ water, and were concentrated using HLB Oasis™ SPE cartridges (500 mg, 6 cc), as described above. Samples were reconstituted in 200 μL of starting mobile phase spiked with 25 μL of 1000 μg/L of internal standard, d3-diphenylhydramine. Samples were held for at least 20 min at −40 °C before centrifuging (12,000 xg, 20 min) to prevent trace amounts of lipids. Clear supernatant was transferred into a clean insert and analyzed by LC/MS/MS.

2.3.3. Plasma digestion and vitellogenin peptide extraction

Fish plasma were analyzed using previously optimized LC/MS/MS method for vitellogenin (VTG) peptide biomarkers (ALHPELR and FIELIQLLR); these peptides have been shown occur in VTG across multiple fish species (He et al., 2019). Plasma samples were prepared via on-pellet trypsin digestion (He et al., 2019; Duan et al., 2009; Ouyang et al., 2012). After digestion, samples were centrifuged (12,000 x*g*, 5 min), and the clear supernatant was transferred to a 2 mL amber vial. Samples were dried under nitrogen gas, and then reconstituted in 1 mL of 0.1% formic acid, followed by sonication for 10 min. Samples were cleaned up using HLB Oasis™ SPE cartridges (30 mg, 1 cc). First, cartridges were conditioned with methanol (1 mL) and NANOpure™ water (1 mL) before gravity loading the 1 mL reconstituted digested sample. Then, cartridges were dried under vacuum, followed by elution with 3 mL of 50:50 (v:v) NANOpure™ water: acetonitrile with 0.1% formic acid. Eluates were collected and dried under nitrogen to near completeness. Samples were spiked with 10 μL of 500 μg/L internal standard (angiotensin II), and reconstituted to 100 μL in 0.1% formic acid (He et al., 2019). The samples were analyzed for the presence of VTG peptide biomarkers ALHPELR and FIELIQLLR using an adaptation of a previously published LC/MS/MS method (He et al., 2019).

2.3.4. Polar organic chemical integrative sampler (POCIS) extraction

Sorbent from each POCIS sampler was transferred into 15-mL polypropylene tubes, using NANOpure™ water to rinse membranes. Samples were fortified with 25 μL of 1000 μg/L surrogates and shaken at 4 °C overnight with 10 mL NANOpure™ water. POCIS sorbent was transferred with NANOpure™ water into pre-weighed glass SPE cartridges with glass wool stoppers in order to retain sorbent. Once transferred, POCIS sorbents were dried under vacuum, followed by elution with three aliquots of 4 mL methanol (Alvarez, 2010; Alvarez et al., 2007). Cartridges were then dried under vacuum for about 2 h and the weight of POCIS sorbent was recorded to normalize to sorbent weight. Eluate was dried under N_2 and samples were reconstituted in 250 μL using the starting LC/MS/MS mobile phase (95:5, 0.1% formic acid in water/0.1% formic acid in ACN). Samples were spiked with 25 μL of 1000 μg/L d3-diphenhydramine as internal standard.

2.4. Analysis by liquid-chromatography- mass spectrometry

Analyses for pharmaceuticals and VTG peptides were performed using an Agilent 1200 LC system (Palo Alto, CA) and a Thermo TSQ Quantum™ Ultra (Waltham, MA) triple quadrupole MS. The LC/MS/MS instrument was equipped with a heated electrospray ionization (HESI) probe, and was operated under positive electrospray ionization mode (+ESI). Quantification for targeted compounds was performed using isotope dilution, and were positively identified using quality assurance parameters for retention time (±1 min) and quantifier/qualifier ion ratio (±30%) compared to surrogate standards (Angeles and Aga, 2018). All NTA was performed using a Dionex UltiMate™ 3000 UHPLC with a Thermo Q-Exactive™ Focus Orbitrap™-MS under +ESI mode. To compare analyte concentrations obtained by target analysis with those obtained by NTA, semi-quantitation of identified compounds in NTA were estimated using a 1-pt external calibration; this represents a "back-of the envelope" calculation to estimate concentration of pharmaceuticals from NTA without accounting for matrix effects and potential losses from sample preparation. A 1-pt external calibration is often used in NTA when confirming standard identification, since a known concentration of standard is injected for confirmation, this injection can also be used to estimate the concentration. This 1-pt semi-quantitation method was compared to the isotope dilution targeted analysis results. Additionally, semi-quantitation of identified pesticides by NTA was completed using a 7-pt external calibration curve. For each pesticide, a calibration curve was used to semi-quantify the concentrations of the pesticide in NTA samples.

2.4.1. Targeted analysis of pharmaceuticals

Wastewater effluent, surface water and extracted fish samples were analyzed for 41 target pharmaceuticals by LC/MS/MS using a Waters Cortecs™ C18$^+$ column (2.1 mm × 150 mm and 2.7 μm) (Angeles and Aga, 2018). Timed SRM mode was used; SRM transitions, tube lens, and retention times for the targeted PPCPs are shown in supplementary information (Table S3) (Angeles and Aga, 2018). Separation was achieved with a 45-min run using 0.3% formic acid in NANOpure™ water [A], and 75% methanol and 25% acetonitrile [B] with a 0.2 mL per min flow rate as previously reported (Angeles and Aga, 2018).

2.4.2. Analysis of VTG peptides

The VTG peptides (ALHPELR and FIELIQLLR) (He et al., 2019) were analyzed by LC/MS/MS using an Xselect CSH™ C18 HPLC column (4.6 mm × 150 mm,130 Å pore size, 3.5 μm particle size). Structures, SRM transitions, tube lens, and retention times for the VTG peptides and the internal standard (Angiotensin II) are shown in supplementary information (Tables S4, S5). Separation was achieved using gradient elution with a total run time of 17 min, using mobile phases 0.1% formic acid in NANOpure™ water [A] and 0.1% formic acid in methanol [B] with a 0.5 mL per min flow rate. The gradient was held for 3 min at 95% A and 5% B before ramping to 30% A and 70% B at 5 min. The gradient was held at 30% A and 70% B for 4 min before returning to staring conditions, and held at 95% A and 5% B to equilibrate for 8 min. Samples that showed the presence of m/z corresponding to target peptides, but fell outside the tolerance limit for the quantifier/qualifier ratios, were re-analyzed using a Thermo Scientific™ QExactive Orbitrap™ high resolution MS for verification. Gradient and mobile phases were transferred to the Orbitrap™ LC/MS, which was ran in full scan mode with an MS^2 inclusion list for fragmentation confirmation of peptides.

2.4.3. Non-targeted (suspect screening) analysis

Extracts for three effluent samples, three wild-caught fish samples from the Hudson River, and POCIS samples were analyzed using a NTA LC/MS method. For NTA, the following samples were chosen: (a) 3 effluent samples from fall, 2019 with selected effluent samples from the WWTPs with the highest concentrations of targeted analytes in summer, 2019; and (b) 3 wild caught fish samples from the Hudson River. These fish samples required confirmation of VTG peptides in HRMS because the ion ratio was outside of the acceptable tolerance limit.

Separation was performed using a Waters Cortecs™ C18$^+$ column (2.1 mm × 150 mm and 2.7 μm). The mobile phases were: [A] 0.1% formic acid in water and [B] 0.1% formic acid in acetonitrile as previously reported (Guardian et al., 2021). Analysis was done in full scan mode with data dependent MS^2 using an inclusion list. This inclusion list consisted of the compounds included in the suspect screening, and when an accurate mass listed in the inclusion list was detected, MS/MS fragmentation was triggered. Samples were analyzed using Compound Discoverer 3.1™ with an included suspect screening mass list (Guardian et al., 2021). This list consisted of 1048 previously optimized compounds, including pharmaceuticals, pesticides, hormones, and industrial chemicals (Guardian et al., 2021). All reported identifications are "level 1" confirmations based confirmation to high resolution mass, MS^2 fragmentation, and retention time match to a reference standard (Schymanski et al., 2014).

2.5. Larval zebrafish behavior screening

Behavioral studies on larval zebrafish were performed as previously reported (Angeles et al., 2020a). SPE cartridges containing the extracts (non-spiked with surrogates) were shipped to SBU, where they were eluted with 8 mL of HPLC grade methanol. Eluates were evaporated to dryness under nitrogen gas. Samples were resuspended in HPLC grade methanol and stored at −20 °C until ready for biological testing. Extracts were diluted in zebrafish embryo media (0.3 g/L Instant Ocean, 7.5 mg/L $HCO3-$, 1 mL/L methylene blue) to equivalent concentrations to wastewater samples and adjusted to a pH of 7.0–7.2 using 0.1 M HEPES buffer to avoid toxic effects due to the acid added prior to SPE concentration.

All animal husbandry and experimental manipulation of embryos and larvae were approved by SBU Institutional Animal Care and Use Committee (IACUC). Six to eight pairs of breeding adult wild type zebrafish were set up to collect embryos for experimentation. A mix of embryos from multiple pairs of zebrafish were used for each experiment. Embryos were individually exposed in a 48-well plate, with one fish per well containing 1 mL of their respective treatment solutions starting at 24 h post fertilization. Fish larvae were continuously exposed (static exposure with no renewal) until 6 days post fertilization (dpf). Sample sizes ranged from 14 to 16 replicate fish per treatment. Behavior was observed in a Zebrabox imaging system

(Viewpoint Life Sciences, FR) at 6 dpf following protocols previously developed to screen individual neuroactive pharmaceuticals (Huang et al., 2019). Total distance swam (mm) per min were monitored during a 20 min acclimation period (acclimation), 15 min spontaneous swimming period (pre-stimulus), and 15 min post-stimulus period where photic responses were evoked by a shift to dark illumination (post-stimulus). See Fig. S2 for a graphic representation of the behavior paradigm.

Larval swimming behavior was summarized by calculating the area under the swimming curve for each individual fish. A two-sided *t*-test was used to identify statistically significant differences in the swimming activities between control and effluent-exposed larvae. General trends in activity changes are summarized by a relative activity measurement, which is a ratio of the average area under the curve of effluent-exposed fish divided by the average area under the curve of control siblings. A relative activity measure of >1 denotes a general hyperactive effect of effluent treatment and a relative activity measure of <1 describes a general hypoactive effect.

3. Results and discussion

3.1. Wastewater treatment plant effluents

Of the 41 targeted PPCPs in WWTP effluents (Table 1) the highest concentrations observed were from caffeine (up to 33,200 ng/L) and iopamidol (up to 42,900 ng/L), with detection frequencies of 93.3% and 100%, respectively. However, the concentrations of caffeine and iopamidol were highly variable across the treatment plants and sampling dates (Fig. S3). Caffeine has been previously reported to have large variations in removal efficiencies depending on WWTP technology being implemented, which can range from 32% to 100% removal (Angeles et al., 2020a). All sampled plants had similar secondary treatment methodologies, with major variations seen only in the mode of disinfection used, varying between hypochlorite and ozonation. Caffeine concentrations varied widely, and no clear trends between concentration and mode of disinfection or plant size was observed. Variations in caffeine could be influenced by other factors such as, population size, and composition of influent water (ex. hospital, agricultural, household), and temperature (Comber et al., 2020). Further information would be required to determine cause of variations between plants and seasons; to avoid disclosing plant identities, further investigation into plant technologies, and populations are not discussed. Iodinated contrast media (e.g. iopamidol) enter wastewater mainly from hospital wastes, often with procedures occurring on certain days each week causing high variability in the wastewater effluents across the week (Weissbrodt et al., 2009). Iopamidol is excreted more than 90% unchanged from the human body (Kormos et al., 2011), and has been shown to have a relatively low elimination efficiency (35% ± 14%) in the conventional activated sludge treatment process (Weissbrodt et al., 2009). Hence, it is not surprising that iopamidol concentrations in WWTP effluent are high and variable.

Psychoactive pharmaceuticals and antibiotics were detected in all WWTP effluent samples (Table 1) with total concentrations ranging from 646 ng/L to 3250 ng/L, and 307 ng/L to 1810 ng/L, respectively. Psychoactive pharmaceuticals such as bupropion, carbamazepine, citalopram, desvenlafaxine, lamotrigine, primidone, sertraline, and venlafaxine were detected in ≥90% of the samples. Psychoactive medications include antidepressants, which are prescribed to about 16.7% of adult Americans (Moore and Mattison, 2017). Antibiotics azithromycin, ciprofloxacin, and trimethoprim were also detected in the WWTP effluents with ≥90% frequency. Given the consistent presence of antibiotics in the effluent samples evaluated in this study, efforts to enhance removal of pharmaceuticals in WWTPs is warranted to reduce the emergence of antibiotic resistance in aquatic microbes.

Seasonal comparisons of effluents (Fig. 1) show a general decrease in trends from summer to winter in the concentrations of both psychoactive drugs (1A), antibiotics (1B), and all detected the PPCPs (1C). Iopamidol and caffeine were graphed separately (Fig. S3) due to their relatively high

Table 1
Average concentrations (ng/L), maximum concentrations (ng/L) and frequency of detection of targeted pharmaceuticals in wastewater effluent from 10 WWTPs (HR1-HR10) that release treated effluents into the Hudson River. Sampling occurred over three seasons: Summer (July), Fall (September) and Winter (December) of 2019.

Compounds	Use	Wastewater Effluent from Plants HR1-HR10 (ng/L)									
		Median	Summer			Fall			Winter		
			Average	Max	Frequency	Average	Max	Frequency	Average	Max	Frequency
acetaminophen	analgesic	60.8	75.4	187	30%	87.3	240	30%	1560	6000	50%
acetyl-sulfamethoxazole	antibiotic	174	335	1070	60%	176	462	90%	197	539	100%
amitriptyline	antidepressant	17.6	18.4	22.6	30%	18.7	36.7	100%	14.9	23.8	100%
anhydro-erythromycin	antibiotic	10.9	N·D.	27.3	10%	15	34.8	100%	6.6	8	20%
azithromycin	antibiotic	187	341	1190	100%	218	351	100%	95	274	70%
bupropion	antidepressant	194	250	399	100%	267	572	100%	132	231	100%
caffeine	stimulant	496	2970	16,200	90%	902	4330	100%	4990	33,200	90%
carbamazepine	anticonvulsant	91.4	175	434	100%	107	194	100%	77	170	100%
ciprofloxacin	antibiotic	190	174	290	100%	146	306	100%	271	422	100%
citalopram	antidepressant	172	203	289	100%	205	275	100%	123	195	100%
clarithromycin	antibiotic	9.5	11.7	38.3	80%	12.5	28	100%	41.4	92.7	30%
desvenlafaxine	antidepressant	667	739	1160	100%	782	1060	100%	376	819	100%
diclofenac	NSAID	1.8	N·D.	11	10%	1.2	2.7	90%	2.5	3.9	80%
haloperidol	antipsychotic	1.4	N·D.	N·D.	0%	1.5	2.9	40%	1.4	2	30%
iopamidol	iodinated contrast agent	3790	11,000	42,900	100%	4900	16,800	100%	6740	18,100	100%
lamotrigine	anticonvulsant	316	658	964	90%	134	316	90%	269	437	90%
norfloxacin	antibiotic	16.4	N·D.	N·D.	0%	N·D.	16.4	10%	N·D.	N.D.	0%
norfluoxetine	antidepressant	7.1	N.D.	N.D.	0%	4.2	7	40%	11.5	22.1	80%
paroxetine	antidepressant	4.9	N.D.	N.D.	0%	6.3	7.7	20%	N.D.	4.9	10%
primidone	anticonvulsant	85.6	111	204	100%	170	392	100%	52.8	75.4	10%
risperidone	antipsychotic	2.5	N.D.	N.D.	0%	N.D.	2.5	10%	N.D.	N.D.	0%
sertraline	antidepressant	35	21.5	40.5	90%	36.7	79.1	100%	53.5	86.9	100%
sulfamethazine	antibiotic	0.8	N.D.	N.D.	0%	1.2	1.5	20%	N.D.	0.8	10%
sulfamethoxazole	antibiotic	26.6	N.D.	95.2	10%	39.6	130	40%	51.6	107	50%
trimethoprim	antibiotic	232	308	415	100%	230	400	90%	155	324	100%
venlafaxine	antidepressant	231	360	585	100%	240	332	100%	157	257	100%

Note: Non-detects are shown as N.D. Individual WWTP concentrations are shown in Table S6. Compounds without detections were not included in the table (anhydro-erythromycin, amitriptyline, ciprofloxacin, diclofenac, enrofloxacin, enrofloxacin, erythromycin, norfluoxetine, norfloxacin, oxolinic acid, primidone, paroxetine, roxithromycin, sarafloxacin, sulfachloropyrazidine, sulfadimethoxine, sulfamethoxydiazine, sulfamethizole, sulfamerazine, sulfamethoxazole, sulfamethazine, sulfadiazine, spiramycin, sulfathiazole, tylosin).

concentrations and lack of seasonal trend. It should be noted that data are not normalized to plant flow and decreases in the concentrations in the final effluent is potentially related to changes in volume of effluent. Flow data provided by the plants during the period of collection, indicate all but one of the plants experienced elevated flow rates during the December sampling period, which was on average two times the average flow rates for the July and September sampling period when flow rates were similar to each other. To our knowledge, none of the plants sampled receive influent from combined sewer overflows, therefore rain events are unlikely to have a large impact on the volume of sewage being treated. Analysis of average precipitation data obtained from National Oceanic and Atmospheric Administration (NOAA) for the region sampled indicated precipitation in the days immediately preceding collection from 0.3 to 1.2 in./day in July, negligible precipitation in September, and from 0.3 to 1.8 in./day in December, when most areas also had several inches to a foot of snow on the ground. Studies have found increased rates of infiltration during wet months, and with the aging infrastructures, the increased flow during these months is likely due to increased groundwater infiltration (Cahoon and Hanke, 2017; Dirckx et al., 2016). When concentrations of targeted PPCPs are normalized to the plant flow, there is no longer a significant difference between seasons, suggesting increase in plant flows during winter months result in dilution, and lower detected concentrations.

Although a general decreasing trend is observed based on overall concentrations, single analytes have varying trends. For compounds detected in all seasons, a trend was determined if present in more than half of the WWTPs. Multiple compounds (amitriptyline, acetyl-sulfamethoxazole, azithromycin, clarithromycin, and diclofenac) were seen to be relatively stable across the seasons, or no consistent trend across the WWTPs. Of these compounds, amitriptyline, acetyl-sulfamethoxazole, azithromycin, and clarithromycin are classified as antibiotics, and as shown in Fig. 1B, do not have a significant trend (ANOVA p-value 0.12). Seasonal decreases were seen for bupropion, carbamazepine, citalopram, desvenlafaxine, lamotrigine, trimethoprim, and venlafaxine; all these, except trimethoprim, are classified as psychoactive pharmaceuticals (Fig. 1A) and were seen to have a significant decreasing trend (ANOVA p-value 9.24e-6). In contrast, ciprofloxacin and sertraline had increasing trend, with highest concentrations in winter samples, while primidone was consistently the highest observed in the fall samples. With limited previous studies reporting on seasonality of pharmaceutical concentrations in WWTP effluents, no clear overarching total trend emerges. Alternatively, seasonal compound-specific trends have been reported for limited analytes (Vieno et al., 2005; Yu et al., 2013), such as previously seen decreases in carbamazepine (Yu et al., 2013) from summer to winter samples.

The NTA of selected WWTP effluent samples resulted in the "level 1" detections of 50 pharmaceuticals, 9 industrial chemicals and 8 pesticides. The pharmaceuticals detected included 14 psychoactive drugs (including targeted analytes bupropion, carbamazepine, citalopram, desvenlafaxine, haloperidol, lamotrigine, paroxetine, primidone, risperidone, sertraline, venlafaxine), 8 antibiotics (including targeted analytes acetyl-sulfamethoxazole, azithromycin, ciprofloxacin, sulfamethoxazole, trimethoprim), 5 stimulants (including caffeine), 4 beta-blockers, 4 anti-hypertensive, 3 antiarrhythmic and 3 opioids. A full list of detected compounds can be found in Table S9.

Combined results from targeted analysis and NTA of WWTP effluent samples show 74 total compounds (Fig. 2A). Of these detections, 15 compounds were detected in both targeted and NTA, as shown in the shared area of the Venn Diagram in Fig. 3A, and an additional 52 compounds were detected only in the NTA. Surprisingly, 7 pharmaceuticals (amitriptyline, anhydro-erythromycin, diclofenac, norfluoxetine, sulfamethoxazole, clarithromycin, and iopamidol) detected in the targeted analysis (Fig. 2A) were not detected in NTA. Iopamidol was not included in the NTA because of its elution at the void volume, leading to exclusion from the suspect list. After manual inspection of the MS data, three compounds (amitriptyline, anhydro-erythromycin, sulfamethoxazole) were seen when the m/z was extracted in Xcalibur™ data processing software. Similar observations have been reported previously, with false negative results occurring in NTA due to peak picking software, even if the mass error is within tolerance limits (Ng et al., 2020). Three of the compounds (diclofenac, norfluoxetine, sulfamethoxazole) that were only detected in the targeted LC/MS/MS were present at concentrations close to the limit of detection (LOD), which may have lead to their non-detection by NTA due to the low abundances of their fragment ions needed to trigger ddMS^2 fragmentation.

Of the 52 compounds detected exclusively in NTA, 3 analytes (acetaminophen, paroxetine, and risperidone) were included in the targeted analytes, but their concentrations were below or near the LOD of the targeted method. Based on semi-quantitation of the compounds detected in NTA (Table 2), the estimated concentrations of acetaminophen ranged from 6.9 to 12.1 ng/L (LOD in targeted analysis is 7.2 ng/L), and the paroxetine concentrations were below 1.5 ng/L (LOD in targeted analysis is 0.4 ng/L). Peaks were observed for acetaminophen and paroxetine during targeted analysis, but were not reported because their concentrations are near LOD and the quantifier/qualifier ratios were highly variable (Angeles and Aga, 2018). The concentrations of acetaminophen, paroxetine, and risperidone detected in the NTA were estimated using a 1-pt external calibration, which does not correct for matrix effects or sample losses.

Concentrations of 15 analytes that were detected in effluent samples by both target analysis and NTA are shown in Table 2. Percent differences between the concentration determined using the isotope dilution method (targeted), and 1-point external calibration (NTA) were calculated. For all compounds detected in target analysis, the concentrations calculated with the 1-point calibration standard were always lower than those calculated using isotope dilutions. Additionally, semi-quantitation of compounds revealed by NTA resulted in high percent differences ranging from (32% to 170%) based on an external calibration when compared to the targeted results based on isotope dilution calculations. Lower concentrations detected using external calibration compared to isotope dilution method can be expected because quantification by external calibration does not correct for losses during sample extraction or for signal suppression due to matrix effects. Hence, it must be noted that the estimated concentrations from NTA using an external calibration will likely under-report actual

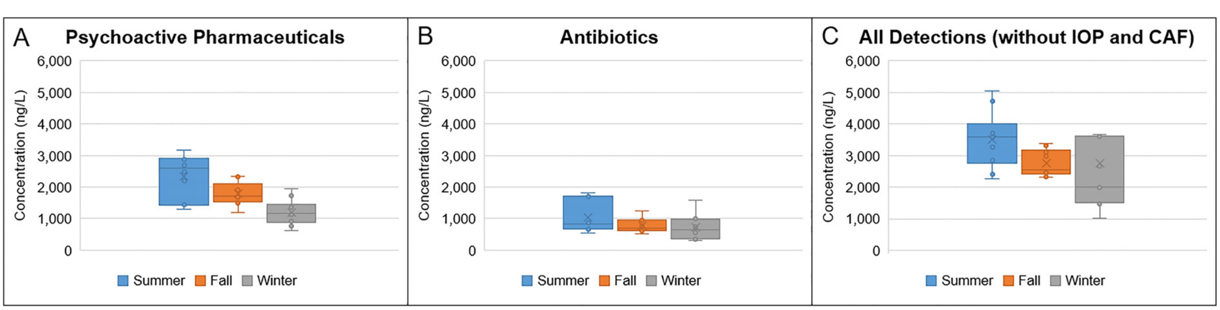

Fig. 1. Seasonal comparison of wastewater effluent for psychoactive pharmaceuticals (A), antibiotics (B) and all detected PPCPs (C), are shown with box and whisker plots. Iopamidol (IOP) and caffeine (CAF) were removed from (C) due to high abundances and lack of trends over time (Fig. S3). The whiskers (vertical lines) extend from the minimum value to the maximum value (excluding outliers). The box ranges from the 1st to 3rd quartile with the solid center line at the median, and the mode is depicted by an X. Total concentrations (ng/L) for each plant are depicted by circles.

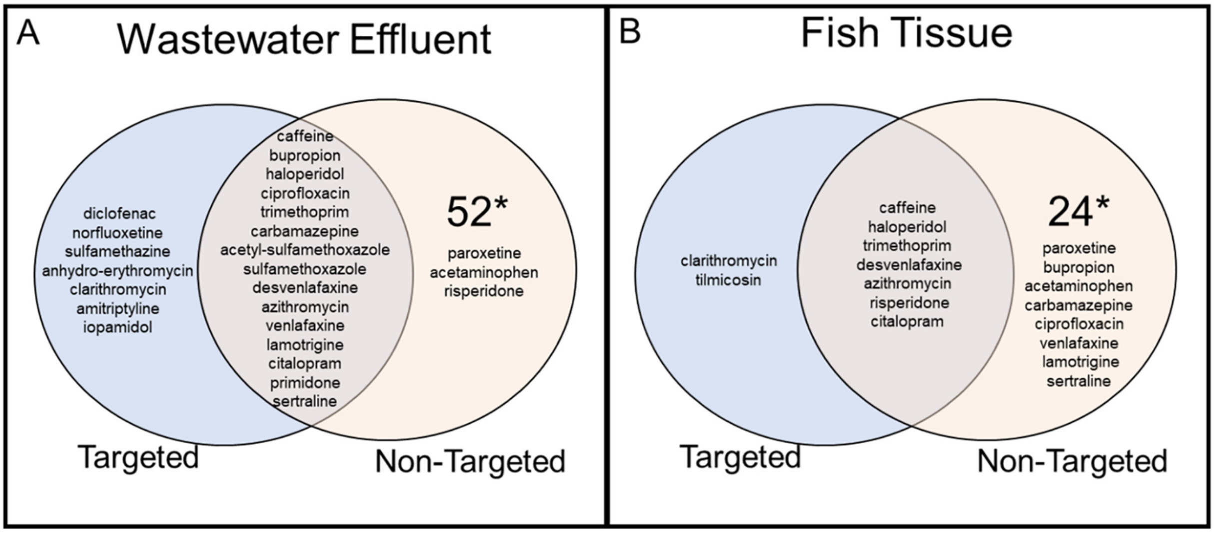

Fig. 2. Detections in targeted (blue) and non-targeted analysis (pink) methods in samples processed by SPE: wastewater effluent (A), and wild-caught fish (B). Analytes detected in NTA that were included in the targeted method are listed. Total number of detections for non-targeted compounds are shown (52 in effluent and 24 in fish tissue); a list of all detected compounds can be found in Tables S9 and S10.

concentrations of environmental contaminants. Nevertheless, new contaminants revealed by NTA can provide insights on what reference standards are important to include in the target analysis, should accurate quantification of these contaminants become important in future environmental studies.

3.2. Surface water

Analysis of grab samples of surface water, as well as samples collected by POCIS deployed in the Hudson River and at the reference site, were performed using target and NTA. Because the sorbents for POCIS and SPE were both based on HLB material, it is expected that contaminants will have the same sorption behavior on these two sample concentration approaches. POCIS samplers were deployed at three fish sampling sites; however, only the POCIS from the reference site and Piermont Pier marsh were recovered.

Targeted analysis of surface water samples (Table 3) collected by grab sampling showed highest detections of caffeine (up to 156 ng/L) and iopamidol (up to 96.1 ng/L). Notably, the reference site only had detections of caffeine and carbamazepine. In contrast, the Hudson River surface water samples (Piermont Pier) had detectable concentrations of eleven compounds, including seven psychoactive drugs (bupropion, carbamazepine, citalopram, desvenlafaxine, lamotrigine, sertraline, venlafaxine) and two antibiotics (acetyl-sulfamethoxazole, sulfamethoxazole, trimethoprim), as shown in Table 3. The concentrations of carbamazepine in the Flax Pond (reference site) sample were much lower than that observed in the Piermont Pier samples (1 ng/L as compared to 3.5 and 4.8 ng/L).

Concentrations of pharmaceuticals detected in the Hudson River surface water in this study were compared to earlier studies in the same river system (Cantwell et al., 2018; Carpenter and Helbling, 2018; Pochodylo and Helbing, 2015). Previous studies of Hudson River surface water have reported detections of 15 analytes that were included in this method (acetaminophen, amitriptyline, caffeine, carbamazepine, ciprofloxacin, citalopram, clarithromycin, desvenlafaxine, diclofenac, lamotrigine, norfloxacin, primidone, sertraline, trimethoprim, venlafaxine); acetyl-

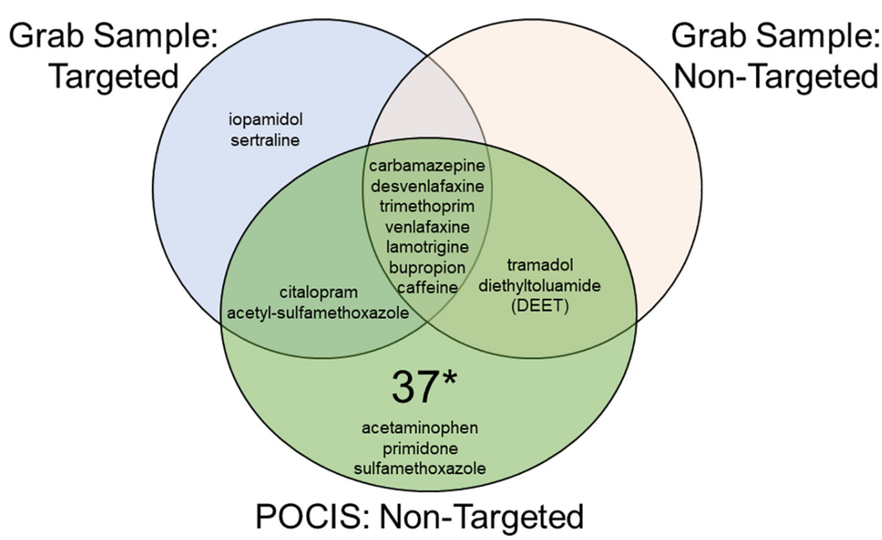

Fig. 3. Venn diagram showing detections in targeted (blue) and non-targeted analysis (pink) methods of SPE sample extracts for surface water grab samples, in addition to non-targeted analysis of POCIS extracts (green). Analytes included in the targeted analysis are listed. Targeted analysis detected 11 compounds; NTA of grab samples detected 9 compounds, including 7 targeted analytes; NTA of POCIS samplers (Piermont Pier Marsh) detected 47 compounds, including 12 targeted analytes; NTA of POCIS samplers (reference site) detected 44 compounds, including 12 targeted analytes. A list of all detected compounds can be found in Table S8.

Table 2
Comparison of selected targeted analytes determined by targeted SRM method, quantified by isotope dilution (A), and using NTA, quantified with a with 1-point external calibration (B) for three effluent samples (Fall, 2019 HR2, HR7 and HR8). Concentrations are shown in ng/L and percent differences between the two methods are shown where applicable.

Compounds	Fall HR2			Fall HR7			Fall HR8		
	Targeted	NTA	Percent Difference	Targeted	NTA	Percent Difference	Targeted	NTA	Percent Difference
	Isotope Dilution (ng/L)	1-pt Calibration Standard (ng/L)		Isotope Dilution (ng/L)	1-pt Calibration Standard (ng/L)		Isotope Dilution (ng/L)	1-pt Calibration Standard (ng/L)	
acetaminophen	N.D	12.1	–	N.D	6.9	–	N.D	11.1	–
acetyl-sulfamethoxazole	462	50.3	161%	235	45.3	135%	N.D	<1	–
azithromycin	185	25.9	151%	188	15.1	170%	351	37.6	161%
bupropion	239	95.5	86%	232	72.7	105%	295	123	82%
caffeine	324	141	79%	47.3	17.1	93%	46.4	18.8	85%
carbamazepine	133	28.7	129%	127	18.6	149%	80.4	20.8	118%
ciprofloxacin	306	117	89%	15.8	9.7	48%	116	73.1	46%
citalopram	270	55	132%	137	26.0	136%	247	75.3	107%
desvenlafaxine	880	194	128%	660	109	143%	1060	221	131%
lamotrigine	N.D	12.7	–	117	41.0	96%	266	136	64%
paroxetine	N.D	<1	–	N.D	<1	–	N.D	1.5	–
primidone	222	50.4	126%	81.9	31.9	88%	96.3	69.7	32%
sertraline	22.4	4.7	130%	55.0	6.5	158%	79.1	21.8	114%
trimethoprim	235	43.4	138%	206	52.8	118%	188	63.3	99%
venlafaxine	332	128	88%	216	64.2	108%	282	115	84%

Note: Non-detects are shown as N.D.

sulfamethoxazole, bupropion, and iopamidol have not been included in previous studies. Of the previously detected compounds, acetaminophen, amitriptyline, ciprofloxacin, clarithromycin, diclofenac, norfloxacin, and primidone were not detected in the grab samples in this study. This is not surprising considering that these compounds had lower frequency of detections in earlier studies, ranging from 5 (primidone) to 36 (amitriptyline) detections out of 127 grab samples.

Caffeine, carbamazepine, citalopram, desvenlafaxine, lamotrigine, sertraline, trimethoprim, and venlafaxine were detected in both the current study, as well as in previous studies in surface water (Cantwell et al., 2018; Carpenter and Helbling, 2018; Pochodylo and Helbling, 2015). Caffeine was detected in 104 out of 127 grab surface water samples (26 to 2339 ng/L), carbamazepine in 108 out of 127 (1 to 262 ng/L), citalopram in 35 out of 127 (1 to 56 ng/L), desvenlafaxine in 122 out of 127 (2 to 1636 ng/L), lamotrigine in 124 out of 127 (1 to 1699 ng/L), sertraline in 3 out of 127 (26 to 88 ng/L), trimethoprim in 106 out of 127 (1 to 531 ng/L), and venlafaxine in 121 out of 127 (2 to 519 ng/L) (Carpenter and Helbling, 2018). Compared to detected concentrations in this study, caffeine, carbamazepine, citalopram, desvenlafaxine, lamotrigine, and trimethoprim were measured within the previous range, while venlafaxine and sertraline were below previous study ranges, due to lower LOD in the current study, with detections in the previous study often reported as below LOD (Carpenter and Helbling, 2018). With the exception of acetyl-sulfamethoxazole, anhyrdo-erythromycin, haloperidol, iopamidol, norfluoxetine, paroxetine, and risperidone which were not included in the previous study, all pharmaceuticals detected in the targeted analysis

Table 3
Concentrations (ng/L) of detected targeted analytes are shown for surface water grab samples, as well as average concentrations, maximum concentrations and frequency of detection of targeted pharmaceuticals in fish tissues (ng/Kg) are shown. Wild-caught fish and surface waters were collected from the Hudson River at Piermont Pier, as well as a reference site (Flax Pond).

Compounds	Surface Waters (ng/L)			Wild-Caught Fish (ng/Kg)								
	Reference Site	Hudson River: Open River	Hudson River: Marsh	Reference Site			Hudson River: Open River			Hudson River: Marsh		
				Average	Max	Frequency	Average	Max	Frequency	Average	Max	Frequency
acetaminophen	N.D.	N.D.	N.D.	N.D.	N.D.	–	N.D.	N.D.	–	N.D.	2.7	1 out of 14
acetyl-sulfamethoxazole	N.D.	4.0	3.9	N.D.	N.D.	–	N.D.	N.D.	–	N.D.	N.D.	–
azithromycin	N.D.	N.D.	N.D.	N.D.	N.D.	–	21.5	36.8	3 out of 15	18.5	32.6	2 out of 14
bupropion	N.D.	2.2	0.6	N.D.	N.D.	–	N.D.	N.D.	–	N.D.	N.D.	–
caffeine	56.8	156	50.8	18.5	41.2	5 out of 34	12.9	29.1	13 out of 15	13.9	36.8	11 out of 14
carbamazepine	1.0	4.8	3.5	N.D.	N.D.	–	N.D.	N.D.	–	N.D.	N.D.	–
citalopram	N.D.	3.8	1.5	N.D.	N.D.	–	N.D.	0.8	1 out of 15	N.D.	N.D.	–
clarithromycin	N.D.	N.D.	N.D.	N.D.	N.D.	–	N.D.	N.D.	–	N.D.	0.6	1 out of 14
desvenlafaxine	N.D.	11.8	3.5	N.D.	N.D.	–	N.D.	N.D.	–	N.D.	1.0	1 out of 14
haloperidol	N.D.	N.D.	N.D.	N.D.	N.D.	–	1.1	2.4	7 out of 15	N.D.	1.3	1 out of 14
iopamidol	N.D.	96.1	37.7	N.D.	N.D.	–	N.D.	N.D.	–	N.D.	N.D.	–
lamotrigine	N.D.	23.0	38.1	N.D.	N.D.	–	N.D.	N.D.	–	N.D.	N.D.	–
risperidone	N.D.	N.D.	N.D.	N.D.	N.D.	–	N.D.	1.2	1 out of 15	N.D.	4.95	1 out of 14
sertraline	N.D.	0.7	0.3	N.D.	N.D.	–	N.D.	N.D.	–	N.D.	N.D.	–
tilmicosin	N.D.	N.D.	N.D.	N.D.	N.D.	–	N.D.	8.5	1 out of 15	N.D.	9.7	1 out of 14
trimethoprim	N.D.	3.4	N.D.	N.D.	N.D.	–	N.D.	N.D.	–	N.D.	12.4	1 out of 14
venlafaxine	N.D.	5.2	1.8	N.D.	N.D.	–	N.D.	N.D.	–	N.D.	N.D.	–

Note: Non-detects are shown as N.D. Individual fish concentrations are shown in Table S7. Compounds without detections were not included in the table (anhydro-erythromycin, amitriptyline, ciprofloxacin, diclofenac, enrofloxacin, enrofloxacin, erythromycin, norfluoxetine, norfloxacin, oxolinic acid, primidone, paroxetine, roxithromycin, sarafloxacin, sulfachloropyrazidine, sulfadimethoxine, sulfamethoxydiazine, sulfamethizole, sulfamerazine, sulfamethoxazole, sulfamethazine, sulfadiazine, spiramycin, sulfathiazole, tylosin).

of effluents were observed in surface water samples in earlier publications (Carpenter and Helbling, 2018).

Analysis of surface water grab samples by NTA resulted in detections of 8 pharmaceuticals and 1 pesticide. The detected pharmaceuticals included 5 psychoactive drugs, 1 antibiotic, 1 opioid, and 1 stimulant (Fig. 3). While targeted analysis of surface water grab samples from the reference site (Flax Pond) showed only detections of caffeine (56.8 ng/L) and carbamazepine (1.0 ng/L), NTA of the same grab samples had detections of 1 additional compound (diethyltoluamide, DEET) at the reference site and 2 additional compounds (tramadol and DEET) at the Hudson River site. Diethyltoluamide (DEET) is a commonly used pesticide and the most common active ingredient in insect repellants worldwide.

Analysis of deployed POCIS in Hudson River showed 46 overall detections from targeted and NTA, including 19 pharmaceuticals, 9 industrial chemicals, and 17 pesticides. Pharmaceuticals detected include 7 psychoactive drugs, 4 antibiotics and 3 stimulants (Fig. 3). All POCIS detections can be found in Table S8. NTA of POCIS sample from the reference site showed detectable concentrations of 42 total compounds, which included caffeine and carbamazepine (Fig. 3, Table S8), and 9 of the compounds included in the targeted analysis (acetaminophen, acetyl-sulfamethoxazole, bupropion, citalopram, desvenlafaxine, lamotrigine, primidone, sulfamethoxazole, trimethoprim). However, the latter 9 compounds were not detected in the grab samples, suggesting that POCIS provides a bigger chemical coverage as a sampling approach. Additionally, 14 pesticides (Table 4), 9 industrial chemicals and 7 additional pharmaceuticals, not included in the targeted analysis, were detected in the POCIS from the reference site.

Analysis of the Hudson River (Piermont Pier marsh) POCIS samples had detections of 46 total compounds by NTA (Fig. 3, Tables S8, S11). Two compounds (iopamidol, sertraline) were detected in the targeted method, but were not detected in the NTA; this is because iopamidol was not included in the suspect screening in-house database (Guardian et al., 2021) and sertraline is more readily biodegradable (Singh et al., 2019). In a previous study, sertraline was determined to only be stable for 0–7 days when stored at room temperature or when stored at -4 °C (Singh et al., 2019).

NTA of grab surface water samples resulted in limited detections, including seven analytes (carbamazepine, desvenlafaxine, trimethoprim, venlafaxine, lamotrigine, bupropion, and caffeine) that were also detected in the targeted analysis. Citalopram and acetyl-sulfamethoxazole were detected in the targeted method, but not detected in the NTA of grab samples, likely due to low concentrations (<4 ng/L). However, POCIS samples by NTA detected 39 additional compounds that were not detected in the extracted grab samples by either targeted analysis or by NTA (excluding tramadol and DEET). These included 13 pharmaceuticals, 3 of which were analytes (acetaminophen, primidone, sulfamethoxazole) included in the target analysis method, 16 additional pesticides (Table 4), and 9 industrial chemicals. Acetaminophen, primidone, and sulfamethoxazole were not detected by either targeted analysis or NTA of the grab samples due to their low concentrations, but were detected in POCIS extracts because of long-term pre-concentration in the passive samplers.

Semi-quantitation of pesticides detected by NTA in POCIS samplers, surface grab water and/or effluent samples was completed using 7-pt external calibration curves (Table 4). Concentrations of pesticides in effluent samples were low (<1 to 22.4 ng/L), with mostly below 1 ng/L. Only carbendazim, DEET, diuron, imazapyr, picaridin and spiromesifen enol had semi-quantitative detections over 1 ng/L in effluent samples. Comparison of effluent concentrations with those in the surface water collected by POCIS show only two overlapping detections over 1 ng/L (carbendazim and DEET), suggesting other environmental inputs of pesticides, likely from point and non-point sources, such as, edge-of-field agricultural run-offs and stormwater run-offs.

Analysis of POCIS extracts was beneficial in gaining insight on the types of pesticides present at trace levels in the Hudson River that escaped detection by grab sampling. However, passive sampling has limitations because accurate quantification of analytes is not possible without additional information on experimental sampling rates. Sampling rates are dependent on field conditions (pH, temperature, and water flow) and experimentally derived results are required for the conversion from amount of chemical accumulated by the sampler (ng/Kg POCIS sorbent) to chemical concentration in water (ng/L) (Alvarez, 2010; Alvarez et al., 2007). As a result, passive samplers are more often used as a monitoring tool for qualitative analysis (Alvarez, 2010; Martinez Bueno et al., 2016).

Generally, the Hudson River site had higher concentrations of pesticides than the reference site. The Hudson River showed highest detections of DEET (192 ng/Kg POCIS sorbent), carbendazim (37.3 ng/Kg POCIS sorbent), imidacloprid (33 ng/Kg POCIS sorbent) and atrazine (27.3 ng/Kg

Table 4
Semi-quantitation of detected pesticides by NTA in wastewater effluent, surface water grab samples and POCIS samplers are shown. Semi-quantitation was performed using external calibration curves. Concentrations for water samples are shown in ng/L. Concentration for POCIS samplers are shown in ng/Kg POCIS.

Compounds	Fall Effluent (ng/L)			Surface Water Grab Samples (ng/L)			POCIS Samplers õ(ng/Kg POCIS)	
	HR2	HR7	HR8	Reference Site: Flax Pond	Hudson River: Open River	Hudson River: Marsh	Reference Site: Flax Pond	Hudson River: Marsh
Atrazine	< 1	< 1	< 1	N.D.	N.D.	N.D.	3.6	27.3
Atrazine desethyl	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.3	4.9
Bensulide oxon	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.6
Benzoguanamine	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.4	3.3
Carbendazim	11.3	6.2	10.3	N.D.	N.D.	N.D.	5.4	37.3
Cybutryne	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	< 1	< 1
Diethyltoluamide (DEET)	22.4	< 1	1.9	8.8	30.0	9.5	76.4	192
Dimethenamid	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.3
Diphenamid	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	< 1	N.D.
Diuron	7.8	1.3	5.0	N.D.	N.D.	N.D.	N.D.	N.D.
Imazapyr	2.3	16.4	0.7	N.D.	N.D.	N.D.	N.D.	N.D.
Imidacloprid	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	< 1	33.0
Imidacloprid,desnitro	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	6.9
Isonoruron	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	< 1	< 1
Metolachlor	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	< 1	9.6
Picaridin	2.0	< 1	3.7	N.D.	N.D.	N.D.	N.D.	N.D.
Prometryn	< 1	< 1	< 1	N.D.	N.D.	N.D.	N.D.	N.D.
Simazine	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	< 1	1.9
Spiromesifen enol	< 1	< 1	4.4	N.D.	N.D.	N.D.	< 1	< 1
Triadimefon	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	< 1	< 1
Trifloxystrobin acid (E,E)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	< 1	< 1

Note: Non-detects are shown as N.D.

POCIS sorbent) with all other detections below 10 ng/Kg POCIS sorbent. Comparison of NTA results to previous grab sampling studies of Hudson River surface waters showed previous detections of 25 compounds detected in our study, including 7 pesticides, 15 pharmaceuticals, and 3 industrial chemicals. Of the compounds detected in NTA that were not included in the targeted analytes, 18 were not included in previous studies (Cantwell et al., 2018; Carpenter and Helbling, 2018; Pochodylo and Helbing, 2015). To our knowledge, this is the first reported detections of acetyl-sulfamethoxazole, androstenedione, bensulide oxon, benzisothiazolone, clindamycin, cybutryne, dimethenamid, desnitro-imidacloprid, isonoruron, methamphetamine, sipromesifen enol, triadimefon, tributyl phosphate, triethyl phosphate, trifloxystrobin acid, triphenyl phosphate, triphenylphosphine oxide, tris(1-Chloro-2-propyl) phosphate, and tris(2-Butoxyethyl) phosphate in the Hudson River.

3.3. Wild caught fish and biological assays

3.3.1. Chemical analysis

Analysis of wild-caught fish from the Hudson River indicated extensive exposure to PPCPs as compared to similar sized and species of fish from the reference site. Caffeine was the most frequently detected pharmaceutical in fish tissues with concentrations up to 41 ng/Kg dry weight (Table 3 and S7). Of the fish samples collected from the Hudson River, 24 out of 29 had detections of caffeine, while only 5 out of 34 from the reference site had detections of caffeine, in the same concentration range (Table 3). Samples from the Hudson River showed detections of ten targeted compounds (acetaminophen, azithromycin, caffeine, citalopram, clarithromycin, desvenlafaxine, haloperidol, risperidone, tilmicosin, trimethoprim) in at least one sample. Fish collected in the Hudson River had more frequent targeted analyte detections, with 93% of fish samples having at least one compound detected, compared to only 15% of fish with detections (caffeine only) at the reference site. No fish samples from the reference site had detections of psychoactive pharmaceuticals or antibiotics in the targeted analysis.

Using target analysis, psychoactive pharmaceuticals citalopram, desvenlafaxine, haloperidol, and risperidone were detected in wild-caught fish from the Hudson River with total concentrations up to 7.3 ng/Kg dry weight (Tables 3, S7). Haloperidol was detected in 8 of 29 fish collected from the Hudson River, while citalopram and desvenlafaxine were detected in a single fish sample and risperidone was detected in 2 of 29 samples. Antibiotics (azithromycin, clarithromycin, tilmicosin, trimethoprim) were detected in fish species with total concentrations up to 45.3 ng/Kg dry weight in wild-caught fish (Table 3). Clarithromycin and trimethoprim were detected in 1 of 29 fish, and tilmicosin was detected in 2 of 29 fish samples. Azithromycin was the most frequently detected antibiotic, with detections in 5 of the 29 Hudson River caught fish.

The NTA of selected wild-caught fish samples from the Hudson River (Fig. 2B) showed detections of 31 compounds, including 22 pharmaceuticals (15 targeted analytes, 8 only detected in NTA), 8 industrial chemicals, and 1 pesticide. Pharmaceutical detections included 10 psychoactive drugs, 3 antibiotics, and 2 stimulants. A full list of detected compounds can be found in Tables S7 and S10. A pesticide, DEET was detected in all three fish samples from the Hudson River ranging from 1.5 to 2.4 ng/Kg dry weight. Semi-quantitation of DEET was performed via external calibration. As DEET was the only pesticide detection in fish (up to 2.4 ng/Kg dry weight), it was not included in Table 4.

Fish samples from the Hudson River had 39 compounds detected in targeted (2 detections) and NTA (37 detections) (Fig. 2B); 7 were detected in both targeted and NTA, 24 in only the NTA, and 2 detected only in the targeted analysis. Of the 24 compounds detected by NTA (Fig. 2B), 8 analytes (acetaminophen, bupropion, carbamazepine, ciprofloxacin, lamotrigine, paroxetine, sertraline, venlafaxine) included in the target list were detected in the NTA but were not detected in the targeted analysis. Semi-quantitation by 1-pt standard addition revealed that these analytes were all below 3 ng/L, which is near or below the LOD. Two compounds, clarithromycin and tilmicosin, were only detected in the targeted method. Careful inspection of the NTA raw data revealed that the abundance of the molecular ion of clarithromycin was very low such that ddMS^2 fragmentation was not triggered. Tilmicosin was not discovered in NTA workflow, however, MS and MS/MS data were present when manual integration with Xcalibur™ software was performed, suggesting a false negative due to the processing software.

Due to alterations in brain functions, and effects on the neuroendocrine system, some psychoactive drugs have been shown to cause endocrine disruption and behavioral changes in fish species (Painter et al., 2009; Park et al., 2012; Schultz et al., 2011). No compounds detected in grab surface waters or fish tissues were above the no observed effect concentration (NOEC), or the predicted no-effect concentration (PNEC) using EPA ToxCAST screening library (https://comptox.epa.gov/) and Pubchem (pubchem.ncbi.nlm.nih.gov/).

A comparison to other investigations measuring pharmaceuticals in fish tissues showed previous studies were conducted on larger fish species, allowing for the analysis of organs and muscle tissue separately (Ramirez et al., 2009; Arnnok et al., 2017; Huerta et al., 2018). Our previous study reported bioaccumulation of acetaminophen, bupropion, caffeine, carbamazepine, citalopram, erythromycin, iopamidol, norfluoxetine, sertraline, trimethoprim, and venlafaxine in muscle tissues of fish samples collected from the Niagara River (Arnnok et al., 2017). In contrast, the fish collected from the Hudson River had low or no detections for acetaminophen, bupropion, iopamidol, and sertraline (Arnnok et al., 2017). Similar, low concentration and infrequent detections for citalopram, venlafaxine, and trimethoprim (Arnnok et al., 2017; Huerta et al., 2018), as well as high frequency detections for caffeine (Arnnok et al., 2017). Additionally, carbamazepine, erythromycin, and norfluoxetine were detected in larger adult fish in the Niagara River, but not seen in fish collected from the Hudson River (Ramirez et al., 2009; Arnnok et al., 2017). This is the first reporting of pharmaceuticals body-burdens in small wild-caught fish species (silversides and killifish).

3.3.2. Vitellogenin peptide analysis in fish plasma

To study the occurrence of endocrine disruption in male and immature fish, the presence of VTG peptides ALHPELR and FIELIQLLR in plasma was determined, and the results are presented in Tables S12 and S13. The VTG levels in the plasma of collected striped bass and killifish samples were evaluated, but VTG levels in silversides could not be assessed due to lack selected peptide sequences in this species. A summary of the frequency of VTG detections from each collection site compared to the reference site is shown in Table S14.

Both the reference site and the Hudson River sites had VTG peptide detections in male or immature fish. VTG peptides were detected in 29% of the fish from the reference site, 80% of fish collected from the Hudson River site 1 (open river), and 50% of fish from Hudson River site 2 (marsh) (Tables S12–14). Fish collected from the Hudson River had 2.8 times the frequency of detection, compared to fish collected from the reference site, suggesting increased exposure to endocrine-disrupting chemicals. It should be noted that assignment of sex could not be done histologically and therefore was only presumptive based on visual presence/absence of gonads. The striped bass collected could be more obviously classified as juvenile, but the spawning killifish were sampled long after their traditional spawning season in this area, so males were less obvious than they would have been during active spawning. This component of the study should be repeated with larger number of sexually mature fish where sex can be accurately assessed. Despite these limitations, results of this study indicate enhanced exposure to endocrine disrupting chemicals in the Hudson River fish as compared to those collected from the Flax Pond reference site.

Compounds detected in the targeted results alone cannot account for endocrine disruption. The combination of NTA and POCIS results offer additional knowledge towards the cause of endocrine disruption. The NTA of passive samplers deployed in the receiving surface water showed the presence of multiple industrial chemicals including plasticizers and flame retardants (tributyl phosphate, triethyl phosphate, triphenyl phosphate, triphenylphosphine oxide, tris(1-Chloro-2-propyl) phosphate, tris(2-Butoxyethyl) phosphate, tris(2-Chloroethyl) phosphate) that are known endocrine disruptors (Bai et al., 2019; Gorga et al., 2013; Kojima et al., 2013). The contribution of psychoactive drugs cannot be discounted as some of these compounds have been shown to cause endocrine disrupting effects (Brooks et al., 2003; Park et al., 2012; Airhart et al., 2007; Calcagno et al., 2016). However, in a complex mixture such as wastewater effluent, additional chemical residues, not included in this study, could attribute to these effects, such as legacy contaminants commonly seen in the Hudson River (polychlorinated biphenyls and polycyclic aromatic hydrocarbons). Presence of complex chemical mixtures in the environment pose difficulties in the assessment of effects on endocrine disruption, these including determination of the compounds that cause endocrine disruption, possibility of synergistic effects, and effects from transformed compounds. It should be noted that both the grab samples and POCIS were HLB sorbents, and extracts were analyzed by LC/MS/MS, leaving out more nonpolar and volatile compounds that are not amenable to detection by electrospray LC/MS/MS. It is likely that many of these undetected compounds in LC/MS/MS (e.g. halogenated industrial chemicals) contribute to endocrine disruption in fish (Bai et al., 2019; Gorga et al., 2013; Kojima et al., 2013). Due to the small sampling size of fish, additional research is required, including analysis of more adult fish to investigate endocrine disruption in the Hudson River.

3.3.3. Larval behavioral studies

Behavioral studies in larval zebrafish were completed for each WWTP effluent for all three seasons. Effluent extracts taken in summer, did not cause any significant effects in larval zebrafish swimming behavior relative to control siblings. A mix of weak (<10% difference) to moderate (>10% but less than <50% difference) hypoactive effects (HR 1–5) and hyperactive effects (HR 6–7) were observed, however these changes in activity were not robust enough to be statistically significant. Some of the WWTPs in the fall sampling caused significant changes in swimming behavior, with exposure to effluents samples from WWTP 2, 3, 8, 9, and 10 causing statistically significant moderate hyperactivity. Winter samples similarly caused statistically significant changes to swimming behavior, with fish exposed to effluent samples from WWTP 3, 4, 5, 6, 7, 8, 9, and 10 showing moderate hyperactivity relative to their control siblings (Table S15, Fig. 4). Of all ten WWTPs examined, HR1 was the only plant to not cause any effects to zebrafish larvae at any seasonal sampling points.

Rather notably, WWTP effluent samples only caused hyperactivity, with no WWTP effluent causing significant decreases in swimming activity over any of the sampling periods. This is in line with a previous WWTP study where samples taken throughout different advanced wastewater treatment processes similarly caused a significant hyperactivity effect in the same zebrafish VMR assay (Angeles et al., 2020a). This contrasts with many previous laboratory studies that frequently show hypoactive effects in fish larvae exposed to some of the same pharmaceuticals that were found in our effluent samples (such as selective serotonin reuptake inhibitors, antipsychotics, and anti-epileptic medications). Typically, these studies were completed at concentrations orders of magnitude higher than what we measured in our effluent samples (Painter et al., 2009; Airhart et al., 2007; Calcagno et al., 2016). However, the literature on pure pharmaceutical effects in laboratory fish studies are not uniform and other previous studies have shown that these pharmaceuticals can have alternating hyper- and hypoactive effects that are determined by the context of exposure (Huang et al., 2019). Nevertheless, it is unlikely that the hyperactive effects we observed in this study are caused by neuroactive pharmaceutical exposure alone, especially when decreased concentrations were detected during the winter sampling. Hyperactivity is likely the result of other chemical contaminants, and potential synergistic effects, which were not identified or quantified in this study. The NTA revealed multiple industrial chemicals including plasticizers, flame retardants, and stimulants that are known to affect fish. Since the NTA was not quantitative, and only completed in selected fall samples, it is difficult to discern effects of these chemicals.

In addition to a general hyperactivity trend, there was a notable temporal trend when comparing effluent effects on fish over the three sampling

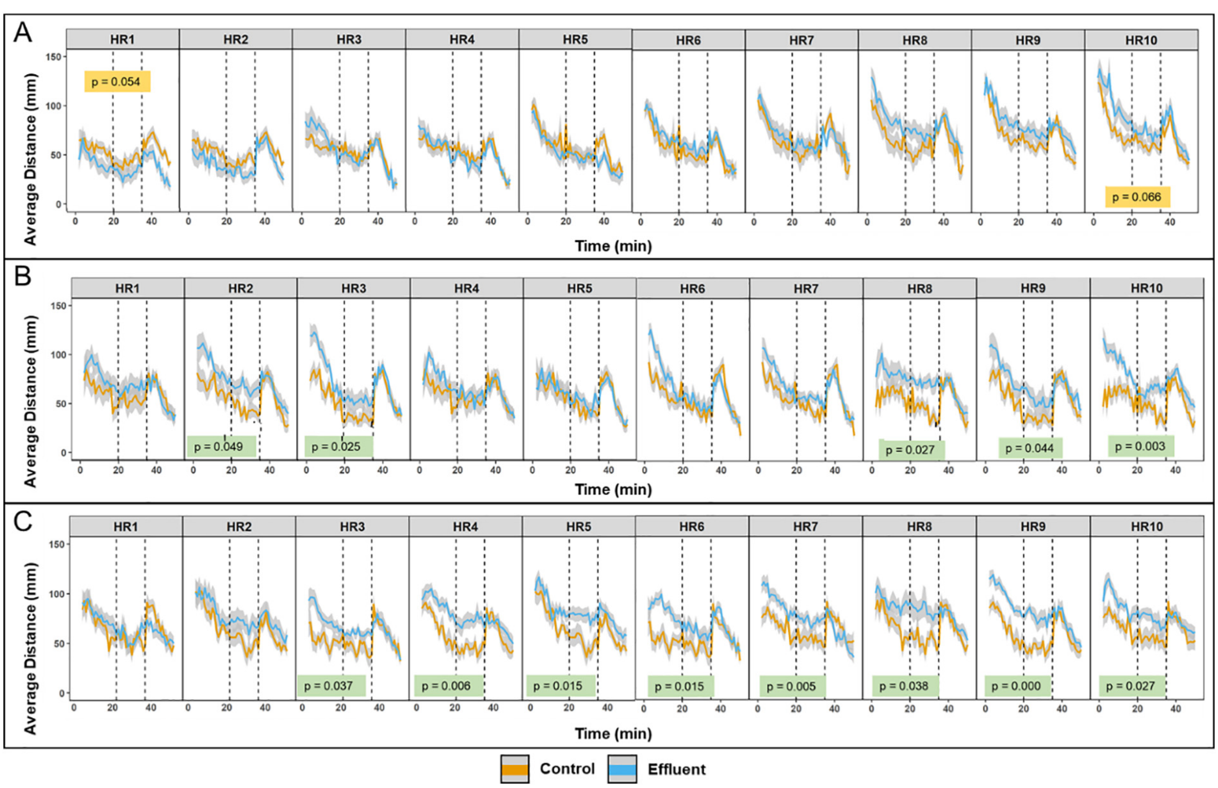

Fig. 4. Larval behavioral screening results, showing average distance (mm) traveled per minute of control (orange) versus effluent chemical extract exposed (blue) zebrafish embryos. Samples are shown for each WWTP (HR1-HR10) for Summer (A), Fall (B) and Winter (C) chemical extracts. *P*-values of significant (green) and nearly significant (yellow) result are shown.

periods. Except for WWTP HR1 and HR10, which had nearly significant hyperactive effects in summer ($p = 0.054$ and 0.066 respectively), no WWTP caused a significant effect (Fig. 4A, Table S15). In contrast, 5 out of the 10 WWTPs caused a significant hyperactive effect in the fall samples (Fig. 4B, Table S15). Extending this trend, 8 out of the 10 WWTPs caused hyperactivity in the winter samples (Fig. 4C, Table S15). While more WWTPs caused significant hyperactivity in the fall and winter, the level of hyperactivity was generally consistent, with effluent-exposed fish swimming 10–35% more than control fish. This increasing occurrence of hyperactivity with changes in sampling time could be related to a number of factors, including changes in individual pharmaceutical concentrations, presence or absence of other chemical contaminants not measured or quantified in this study, and volume of effluent treated. Although targeted pharmaceutical concentrations in total had a general decrease from summer to winter, the trends were seen to be compound-specific, and the general decrease of targeted concentrations cannot be directly correlated to the overall concentration of specific chemical contaminants in the effluent samples. The relationship between changes in swimming behavior and season, pharmaceutical mixtures, and volume of effluent being treated warrants further investigation.

4. Conclusions

The results of this study provide important information on the advantages and limitations of current practices in chemical and biological assessments of the concentrations and effects of PPCPs in surface waters that receive a high influx of wastewater effluent. A comprehensive examination of the concentrations and occurrences of PPCPs in WWTP effluents being discharged into the Hudson River indicate the types of contaminants that are likely to persist in typical river systems in the United States. This study showed that a combination of POCIS for sampling and NTA for chemical characterization should be used for developing a comprehensive list of compounds for targeted monitoring. Comparison of analytes detected in POCIS and grab water samples show advantages of passive sampling, which allows the detection of trace contaminants that are below the limits of detection when using grab samples. Moreover, large benefits were observed when the combination of NTA and POCIS samplers were used, leading to the detection of a higher number of contaminants compared to target analysis of grab samples. While the analysis of targeted PPCPs cannot account for biological effects alone, the addition of NTA assists with identification of endocrine disrupting chemicals, stimulants, and industrial chemicals that could impact aquatic life. Further studies should be conducted to fully assess long-term chronic toxicity of aquatic organisms, including analysis of adult resident fish, in surface waters receiving high volumes of WWTP effluents.

Ethical statement

All animal procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Stony Brook University and are approved by their Institutional Animal Care and Use Committee (IACUC) under protocol 269,492–15 to Howard I. Sirotkin. Collection of fish from the Hudson River was covered under an existing permit from the New York Department of Environmental Conservation to Robert Cerrato of SoMAS.

Funding sources

Hudson River Fund Research Grant 004/17A to A. McElroy. D. Aga acknowledges support from the National Science Foundation Award no.1545756 (PIRE-HEARD). Any opinions, findings, conclusions, and recommendations expressed in this publication are those of the author (s) and do not necessarily reflect the view of the NSF.

CRediT authorship contribution statement

Laura D. Brunelle: Investigation, Methodology, Writing – original draft, Writing – review & editing. **Irvin J. Huang:** Investigation, Writing – review & editing. **Luisa F. Angeles:** Investigation. **Logan S. Running:** Investigation. **Howard I. Sirotkin:** Conceptualization, Supervision, Writing – review & editing. **Anne E. McElroy:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition, Project administration. **Diana S. Aga:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition, Project administration.

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.

Acknowledgements

The authors also acknowledge Dr. Jon Wong (Research Chemist, U.S. FDA) and Dr. James Chang (Manager, Thermo Fisher Scientific) for providing the primary standards for pesticides and metabolites used in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154176.

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