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POTENTIAL ESTROGENIC EFFECTS OF WASTEWATERS ON GENE EXPRESSION IN *PIMEPHALES PROMELAS* AND FISH ASSEMBLAGES IN STREAMS OF SOUTHEASTERN NEW YORK

Barry P. Baldigo,*† Scott D. George,† Patrick J. Phillips,† Jocelyn D.C. Hemming,‡ Nancy D. Denslow,§ and Kevin J. Kroll§

†New York Water Science Center, US Geological Survey, Troy, New York, USA
‡Wisconsin State Laboratory of Hygiene, Madison, Wisconsin, USA
§University of Florida, Gainesville, Florida, USA

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Abstract: Direct linkages between endocrine-disrupting compounds (EDCs) from municipal and industrial wastewaters and impacts on wild fish assemblages are rare. The levels of plasma vitellogenin (Vtg) and Vtg messenger ribonucleic acid (mRNA) in male fathead minnows (*Pimephales promelas*) exposed to wastewater effluents and dilutions of 17α-ethinylestradiol (EE2), estrogen activity, and fish assemblages in 10 receiving streams were assessed to improve understanding of important interrelations. Results from 4-d laboratory assays indicate that EE2, plasma Vtg concentration, and Vtg gene expression in fathead minnows, and 17β-estradiol equivalents (E2Eq values) were highly related to each other ($R^2 = 0.98$–1.00). Concentrations of E2Eq in most effluents did not exceed 2.0 ng/L, which was possibly a short-term exposure threshold for Vtg gene expression in male fathead minnows. Plasma Vtg in fathead minnows only increased significantly (up to 1136 μg/mL) in 2 wastewater effluents. Fish assemblages were generally unaffected at 8 of 10 study sites, yet the density and biomass of 79% to 89% of species populations were reduced (63–68% were reduced significantly) in the downstream reach of 1 receiving stream. These results, and moderate to high E2Eq concentrations (up to 16.1 ng/L) observed in effluents during a companion study, suggest that estrogenic wastewaters can potentially affect individual fish, their populations, and entire fish communities in comparable systems across New York, USA. *Environ Toxicol Chem* 2015;34:2803–2815. © 2015 The Authors. *Environmental Toxicology and Chemistry* Published by Wiley Periodicals, Inc. on behalf of SETAC.

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INTRODUCTION

Our understanding of endocrine-disrupting chemicals (EDCs) and the effects that they have on aquatic biota has improved over the past 2 decades with increased precision of analytical methods and innovative assays designed to detect and quantify estrogenic responses. Effluents from industrial and municipal wastewater treatment plants (WWTPs) frequently contain trace amounts of human and veterinary drugs, natural and synthetic hormones, detergents, disinfectants, plasticizers, fire retardants, insecticides, and antioxidants, some of which can behave as endocrine disruptors [1–3]. The total estrogen activity (estrogenicity) of WWTP effluents can be relatively high because many of these EDCs can pass largely unaltered through treatment processes and be discharged into receiving streams [4]. At environmentally relevant concentrations, some pervasive EDCs have been found to induce masculinization or feminization in wild fish [5–8], reduce fecundity and egg size [9–11], increase Vtg gene expression [8,12], and impair the density or biomass of fish populations under experimental situations [13,14]. In addition, altered steroid hormone levels and secondary sexual characteristics, as well as decreased fecundity and egg size, have been observed in fish exposed to effluents containing very low to moderate EDC concentrations [9,15]. Estrogenic substances often increase vitellogenin (Vtg) synthesis and Vtg protein in the plasma of male fish, but these changes should be preceded by increased Vtg messenger ribonucleic acid (mRNA) production at the cellular level [8,12,16]. Attempts to link EDCs in effluents to adverse impacts on wild fish populations and communities, however, have only rarely [13] conformed to laboratory-based predictions.

Almost 1900 public, private, and commercial WWTPs are permitted to discharge effluents into streams, lakes, and groundwaters across New York (USA) [17]. Discharges of EDCs into New York's waters have the potential to adversely affect water quality, fish health, the structure and function of aquatic ecosystems, and human health. Many effluents discharge into surface waters directly upstream from drinking water intakes or into tributaries to water-supply reservoirs [17]. Although hormone data are not available for most effluents in New York, an assessment of compounds at 4 WWTPs confirmed that their effluents contain a variety of pharmaceuticals [18], hormones, and EDCs [19–22]. The distribution of EDCs in streams of New York and their effects on natural fish assemblages remain largely unknown.

Fortunately, the effects that estrogenic contaminants have on resident aquatic biota and natural ecosystems can be evaluated effectively, because methods that quantify estrogen activity and very low concentrations of hormones and nonhormone EDCs have advanced over the last 20 yr. E-screen assay methods [23,24] are now commonly used to quantify total estrogen activity or estrogenicity (as 17β-estradiol equivalents [E2Eq

All Supplemental Data may be found in the online version of this article.

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* Address correspondence to bbaldigo@usgs.gov

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values]) accurately in complex mixtures of contaminants that are often found in wastewater effluents [25,26]. Improved methods to quantify genomic changes also provide a means to assess the potential physiologic effects of stressors and (or) contaminants at the molecular level (in advance of morphometric changes or toxicity) [8,27,28]. Recently refined analytical methods can also quantify very low concentrations of hormones, pharmaceuticals, personal care products (PPCPs), and other known EDCs in effluent and stream waters [4,29]. These new procedures provide the tools needed to accurately characterize common organic contaminants, variations in levels of EDCs, the total estrogenic effects that effluents potentially have on biota, and the effects that various WWTP processes have on the transport and fate of emerging contaminants in surface waters. Although estrogenicity is a primary indicator of potential reproductive effects, the inclusion of additional endpoints can improve the assessment, definition, and understanding of the effects that EDCs have on individual fish, their natural populations, and entire fish communities.

In 2008, the US Geological Survey and several collaborators began an extensive study to quantify the potential effects of EDCs in effluents on resident biota in 33 receiving streams located mainly across southeastern New York. The primary objectives of the investigation were to improve our understanding of EDCs and to improve our ability to protect aquatic ecosystems from adverse impacts as a result of known, suspected, and unknown EDCs. To meet these objectives, multiple lines of evidence were used to characterize the potential and the actual impacts of EDCs on stream fish assemblages in 3 study phases. In phase 1, estrogenicity levels in 33 effluents and several streams were measured and used to estimate the probability for adverse effects within stream ecosystems [17]. For phase 2, estrogenicity levels, Vtg mRNA (to quantify Vtg gene expression), and Vtg protein levels in male fathead minnows (*Pimephalas promelas*) were used to characterize the potential adverse impacts of EDCs on the endocrine systems of fish in receiving streams. In phase 3, fish assemblages were quantified both upstream and downstream of effluent-discharge points at 10 WWTPs to ascertain whether any tangible impacts of estrogenic effluents could be detected at the species population or fish-community levels. The present study evaluated results from the second and third phases, especially the relations among estrogenicity levels in effluents and in 17α-ethinylestradiol (EE2) experiments, Vtg mRNA fold increases in livers and Vtg protein levels in plasma of male fathead minnows exposed to whole effluents and to EE2 dilutions, potential impacts on fish populations and communities in receiving streams, and some important implications.

METHODS

The present study summarizes the findings from the second and third phases of a regional study of potentially estrogenic effluents conducted from 2008 to 2011. During 2008 to 2010, the levels of estrogenicity and selected hormones, nonhormone EDCs, and PPCPs were characterized from grab samples collected 1 to 6 times from effluents at 33 municipal or commercial WWTPs located across New York. The resulting estrogenicity and chemistry data from this first study phase were used to document spatial and temporal variability in estrogenicity and to assess important relations between estrogenicity and EDCs in effluents, influents, and receiving streams [17]. The present investigation was completed at 15 of the same study sites (Table 1; Supplemental Data, Figure S1) during 2011 to assess the potential and tangible effects of estrogenic effluents on fish populations and communities. For phase 2, fathead minnows were exposed to whole-effluent samples from 9 WWTPs and to several EE2 dilutions to assess Vtg gene expression and interrelations with E2Eq and Vtg levels in plasma. For phase 3, quantitative fish surveys were conducted at sites located upstream and downstream of 10 WWTPs.

Effluent sampling

A total of 225 L of effluent water from each of 9 WWTPs (Table 1) was collected using a peristaltic pump over 1 h and then shipped (overnight) in Teflon-lined barrels to the University of Florida (Gainesville, FL, USA), where fathead minnow exposures and Vtg gene-expression assays were immediately performed during winter 2011. Aliquots (grab samples) for e-screen analysis were collected from each EE2 dilution or whole effluent at the start and end of each test in glass bottles, stored on ice, and shipped (overnight) to the Wisconsin State Laboratory of Hygiene in Madison (WI, USA) for estrogenicity analysis.

Fish assemblages

Fish assemblages were surveyed once from upstream and downstream reaches at each WWTP under base flow conditions during June to August 2011 to quantify population and community metrics and to assess potential impacts of effluents. Reaches were from 50 m to 100 m long, depending on stream width, and sample boundaries were blocked with seines (7-mm mesh). At each site, fish were collected using 3 or 4 passes with a battery-powered backpack electroshocker and 2 to 3 fish netters. All specimens were identified to the species level, and the lengths and weights of all individual fish longer than 150 mm were recorded. The lengths and weights of species that were shorter than 150 mm and (or) collected in large numbers were only recorded from 40 to 50 individuals to avoid excessive holding times, stress, and mortality. The total weights and counts for additional small fish were recorded (by species) in batches of 10 to 50 specimens. All fish were kept alive during processing and subsequently returned to the stream.

Information needed to estimate total sample area and general habitat conditions was collected at each study site generally following methods described in Mulvihill et al. [30]. The total length of each study reach was recorded along with wetted channel width at 10 equally spaced transects along the thalweg. Water depth, velocity, and the dominant substrate category were recorded at 3 equally spaced points (channel center and midway to each bank) across each of the 10 transects.

The number and weight of fish captured during each pass were used to estimate the population size and biomass, and corresponding 95% confidence intervals (CIs), for each species and for the total fish community by the Moran–Zippin method of proportional reduction [31] and Microfish (Ver 1) software [32]. Estimates of density and biomass for each species' population and the total community were standardized by the area sampled at each reach. Estimates of species diversity (the number of fish species, or richness) and Shannon–Wiener diversity index (species equitability) were calculated through standard methods [33]. The overlap of 95% CIs was used to assess absolute differences ($p \leq 0.05$) in indices of community density and biomass between upstream and downstream reaches. These assessments are analogous to 2-tailed Student's t tests.

Fathead minnow assays

All fathead minnow bioassays followed standard procedures developed by the US Environmental Protection Agency to

Table 1. Study site code, US Geological Survey (USGS) station ID, plant type, drainage area, permitted discharge, mean annual stream discharge, sample dates, and the estimated dilution ratio for 15 wastewater treatment plants where fish-community surveys (Supplemental Data, Table S2) were completed and effluent samples were collected for fathead minnow assays (Table 2) during 2011[a]

Site code	USGS station ID	Plant type	Fish survey date	FHM-assay sample date	Drainage area (mi^2)	No. of estrogenicity samples	Mean estrogen equivalents (ng/L)	Minimum estrogen equivalents (ng/L)	Maximum estrogen equivalents (ng/L)	Permitted effluent discharge (ft^3/s)	Mean stream discharge (ft^3/s)	Mean estimated dilution ratio
ny1	411700073452001	TF	8/3/11	4/14/11	9.9	6	2.20	1.35	3.48	2.321	18.9	0.1230:1
ny2	412450073400901	AS	8/5/11	3/17/11	2.3	5	0.45	0.08	1.72	0.542	4.3	0.1269:1
ny3	422212074405501	AS	8/15/11	8/28/11	32.5	8	1.53	0.24	3.58	0.248	59.4	0.0042:1
ny4	421054074473001	SBR	6/22/11		10.2	1	0.21	0.21	0.21	0.096	18.9	0.0051:1
ny8	420936075081801	AS		4/14/11	333.0	5	0.18	0.05	0.50	1.810	603.5	0.0030:1
ny9	412118073441801	AS	6/17/11		0.1	1	0.10	0.10	0.10	0.073	0.1	0.6171:1
ny10	412301073473301	RBC	7/28/11	8/28/11	2.6	2	0.73	0.71	0.75	0.309	5.0	0.0617:1
ny11	412332073370901	RBC		3/17/11	82.3	3	6.14	1.08	16.08	0.170	149.8	0.0011:1
ny15	413034073362801	AS		4/14/11	4.7	3	0.62	0.09	1.59	0.015	8.3	0.0019:1
ny23	420821074391601	RBC		8/28/11	165.0	2	1.11	0.64	1.59	0.619	335.5	0.0018:1
ny24	420721074274901	RBC	7/13/11		12.4	1	0.69	0.69	0.69	0.774	28.1	0.0275:1
ny25	421908074261101	AS		3/17/11	237.0	3	0.89	0.75	1.14	0.133	481.9	0.0003:1
ny26	422410074374001	AS	6/2/11		7.7	1	0.08	0.08	0.08	0.774	13.7	0.0563:1
ny27	421113074090301	RBC	7/7/11		9.4	1	0.23	0.23	0.23	1.238	20.8	0.0596:1
ny28	411812073394501	AS	6/16/11		3.2	1	0.06	0.06	0.06	0.093	6.2	0.0151:1

[a]The mean, minimum, and maximum estrogen activity levels are from 1 to 8 samples collected at each study site from 2008 to 2010 [16]. Site locations are shown in Supplemental Data, Figure S1.
AS = activated sludge; RBC = rotating biological contactor; SBR = sequential batch reactor; TF = trickling filter; FHM = fathead minnow.

determine chronic toxicity of effluents and receiving water to freshwater organisms [34]. These 96-h static-renewal tests exposed mature male fathead minnows to whole effluents from 3 WWTPs on 3 occasions (total of 9 effluent exposures), to at least 1 reference water (dechlorinated tap) exposure per set of effluent tests (total of 3 controls), and to a separate positive-control experiment using a reference water (0 ng/L) and 4 dilutions (1 ng/L, 2.5 ng/L, 5 ng/L, and 10 ng/L) of EE2. Each treatment consisted of four 12-L glass aquariums, each containing 3 adult male fathead minnows and 4 L of exposure solution per effluent. The effluents were used immediately after receipt, and 50% changes in the water were made every 24 h over the 4-d exposure. Effluents were sampled and tested in 3 different exposure groups. At the end of each test, at least 4 fish were sacrificed from each effluent, control, or EE2-spiked water. Blood and liver were collected from each fish to quantify Vtg concentrations in blood and induction of Vtg mRNA (Vtg gene expression) in liver using methods described by Gordon et al. [6] and Wang et al. [35]. The estrogenicity (quantified as estradiol equivalents [23,24] of each whole-effluent), control, and EE2-assay dilution were estimated from samples collected at the start and end of each exposure using the e-screen assay.

Plasma Vtg analysis

Concentrations of plasma Vtg were determined by indirect enzyme-linked immunosorbent assay (ELISA) using the monoclonal antibody (mAb) 2D3, which is specific for carp but also cross-reacts well with fathead minnow Vtg, following methods provided in Denslow et al. [36] and Hemming et al. [37]. In general, samples and standards were loaded onto 96-well ELISA plates; stored overnight at 4 °C (in a humidified container); washed 4 times with 10 mM phosphate, pH 7.6, 150 mM NaCl, 0.02% azide, 10 KIU/mL Aprotinin (PBSZ-AP); and then blocked with 1% bovine serum albumin in 10 mM Tris, 150 mM NaCl, 0.05% Tween, 0.02% azide, 10 KIU/mL Aprotinin, pH 7.6 (1% BSA/TBSTZ-AP) for 2 h at room temperature. All plates were then rewashed 4 times with PBSZ; the monoclonal antibody was loaded into wells on each plate, and the lowest dilution (1:100) was probed with 1 μg/mL of the mAb and the higher dilution of 1:10 000 was probed with 0.1 μg/mL. After addition of the mAb, the plates were again stored at 4 °C overnight and washed again. The biotinylated secondary antibody (goat anti-mouse IgG-biotin) was added to each well at 1:1000 dilution in 1% BSA/TBSTZ-AP, and then the plates were incubated at room temperature for 2 h. The plates were washed again, streptavidin–alkaline phosphatase was added at 1:1000 dilution in 1% BSA/TBSTZ-AP, and the plates were incubated for 2 h at room temperature. After a final wash, the color was developed by adding 1 mg/mL *p*-nitrophenyl phosphate in carbonate buffer (0.03 M carbonate, 2 mM $MgCl_2$, pH 9.6) and measured using an ELISA plate reader (SpectraMax Plus384; Applied Biosystems) at 405 nm. Concentrations of all unknowns were determined from standard curves. The lowest observable detection concentration for fathead minnow plasma Vtg was 0.5 μg/mL. All assays were performed in triplicate and reported as the mean of the 3 measurements. The coefficient of variation was less than 10% for all samples analyzed. Inter- and intra-assay variability, assessed from spiked controls on several plates and different runs, was less than 10% and less than 5%, respectively.

Vtg gene expression

Expression of the Vtg gene, as Vtg mRNA, was quantified in 3 steps using methods described by Garcia-Reyero et al. [28]

and Garcia-Reyero et al. [8]. First, total RNA was extracted using the RNA STAT-60 reagent (Tel-Test) and reconstituted in RNAsecure (Ambion). The RNA quantity and purity for quantitative polymerase chain reaction (qPCR) analysis was measured using the NanoDrop ND-1000, and RNA quality was evaluated using the Agilent 2100 BioAnalyzer with the RNA 6000 Nanochip. The RNA integrity values were greater than 8.0 for all samples used in the analysis. Vitellogenin was evaluated by reverse transcription–PCR (RT-PCR). The primers used for these transcripts [8,28] are summarized in Supplemental Data, Table S1. Each total RNA sample was DNase treated using the TURBO DNA-free kit (Ambion), and complementary DNA-(cDNA) was synthesized using Super-ScriptTM II Reverse Transcriptase (Invitrogen). Reverse transcriptase PCR analysis was carried out in a volume of 25 μL using 1× iQ SYBR Green Supermix (Bio-Rad), 1 μL of each gene-specific primer (10 mM), and 100 ng cDNA. The 2-step thermal cycling parameters were as follows: initial 1-cycle Taq activation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s, and 58 °C for 1 min. After 40 cycles, a dissociation curve was produced starting at 55 °C (+1 °C/30 s) to 95 °C. Transcripts were assayed on an iCycler Thermal Cycler (Bio-Rad). We used 18S ribosomal RNA as the reference gene to normalize expression data. Standards and experimental samples were run in duplicate, along with 2 negative controls. The 2 negative controls were a no-reverse transcriptase (–RT) control, in which DNase-treated RNA samples were pooled and water was used in place of reverse transcriptase during the reverse transcription reaction, and a no-template control (NTC), in which water was used in place of template cDNA during the RT-PCR reaction. Melting curves for each gene indicated a single product being formed. Significant differences ($p \leq 0.05$) between controls of exposure to sewage effluents for the Vtg gene were determined by Student's *t* test utilizing JMP software (Ver 5.1.2, SAS Institute).

E-screen assays

Estrogenicity was determined for all effluent samples and EE2-experiment dilutions using the biological e-screen assay, which quantifies estrogenicity as estradiol equivalents for extracts of whole-water [23,24], and a chemical model that estimates estrogenicity by summing the products of all hormones and nonhormone EDCs concentrations times their estrogen equivalency factors [38] as described in Baldigo et al. [17]. The e-screen assay quantifies biological estrogenicity using a proliferation test with human estrogen receptor–positive Michigan Cancer Foundation-7 (MCF-7) breast cancer cells [24,39,40]. These breast cancer cells respond to compounds that bind to and activate the estrogen receptor by proliferating. Thus, the more estrogenic a water sample extract is, the more cells (that are exposed to such water extract) will proliferate. The e-screen method detailed by Drewes et al. [23] is briefly described here. Water sample extracts were mixed with cell media at ≤1%, and a range of dilutions was tested by transferring the media to plates containing the MCF-7 cells. Each plate contained 1 column of negative controls and 1 row of 17β-estradiol (E2)-spiked samples for every dilution of each sample tested to assess possible interferences such as cytotoxicity. Each batch was run with a 15-point E2 calibration standard curve. The plates were incubated for 5 d and then fixed, stained, and read. Estrogenic activity was calculated from the standard curve, corrected for the concentration of the extract by the corresponding dilution factor, and reported in E2Eq values. A mean value was calculated from the 3 replicates for each sample. The effective detection limit varied, but was generally 0.027 ng/L.

Analyses of estrogenic effects

The Vtg gene expression and Vtg plasma results from calibration EE2 assays were summarized and compared with each other and with E2Eq measures to identify and characterize important interrelations and possible estrogenic-effect thresholds. The gene-expression data were compared with estrogenicity data from effluents previously sampled across New York and the mean estimated effluent-to-stream dilution ratios to evaluate the potential effects of estrogenic effluents on local or region-wide fish assemblages. Any actual estrogenic effects on fisheries in receiving streams were assessed by testing hypotheses that the condition (relative health) of fish assemblages at downstream reaches was impaired by effluents and that any impairment was reflected by significant differences in species richness, diversity, density, and biomass between assemblages at downstream and upstream reaches. For these analyses, key community metrics and estimated density and biomass for selected species populations at each upstream reach were compared with the same metrics from the corresponding downstream reach to ascertain gross differences that could be attributed to individual WWTPs. The significance of any differences in community (total richness, diversity, density, and biomass) and population (estimated species density and biomass) metrics between upstream and downstream reaches were determined by comparing overlap in the 95% CIs [41] and either parametric 1- or 2-factor analyses of variance (ANOVAs) or nonparametric Kruskal–Wallis tests, where applicable. In addition, spatial patterns in the composition and classification of fish communities (groupings of sites with similar species assemblages) were assessed via nonmetric multidimensional scaling (MDS) of taxa relative-abundance (square-root–transformed) data [42,43]. The MDS ordination generates an arrangement of samples (study reaches) in species-space according to the nonparametric ranks of Bray–Curtis similarities [44]. Significant site groupings were determined through hierarchical cluster (group-average linking) analysis and permutation tests of similarity profiles ($p \leq 0.05$) [44]. Sites and reaches with similar or significantly different assemblages are displayed graphically.

RESULTS

With some important exceptions, measures of Vtg levels in plasma and Vtg gene expression in livers of male fathead minnows, estrogenicity in waters from most WWTP-effluent and EE2-spiked assays, and the results of the resident fish-community surveys indicate that most effluents should have relatively minor to modest effects on fish reproduction and species populations in receiving streams. Several fathead minnows in the EE2 and WWTP effluent assays appeared to be females (they had ovaries) and thus were excluded from all analyses. Differences for all statistical analyses were considered significant at $p \leq 0.05$ unless otherwise indicated.

Fathead minnow assays

Positive-control EE2 assays. The estrogenic response of male fathead minnows to various EE2-spiked dilutions was generally proportional and, consequently, could be used to calibrate the EE2 doses to the estrogenicity levels (as E2Eq values) and to assess Vtg gene expression and Vtg plasma responses for the range of E2Eq levels observed in prior surveys

of WWTP effluents [17]. The enzyme immunoassay [36,37] was used to measure EE2 concentrations for the EE2 assays of 0 ng EE2/L, 1.9 ng EE2/L, 4.1 ng EE2/L, 8.0 ng EE2/L, and 14.0 ng EE2/L for the control (0) and the 1 ng EE2/L, 2.5 ng EE2/L, 5 ng EE2/L, and 10 ng EE2/L target treatments, respectively (Table 2). The estrogenicity levels measured with the MCF7 test at the start and end of each set of exposures averaged 0.1 ng/L, 0.9 ng/L, 2.3 ng/L, 5.1 ng/L, and 7.4 ng/L E2Eq for the control and 1 ng/L, 2.5 ng/L, 5 ng/L, and 10 ng/L EE2-target treatments, respectively (Table 2). The relation between the measured EE2 concentrations and E2Eq was very strong ($R^2 = 0.99$; Figure 1).

The plasma Vtg concentrations in male fathead minnows increased in a dose-responsive manner with at least 3 of the 4 E2Eq dilutions (EE2 assays; Figure 2A). All E2Eq dilutions had significantly higher levels of plasma Vtg than did the controls. Similarly, Vtg mRNA (gene expression) in fathead minnows also increased in all E2Eq dilutions in a dose-response manner and differed significantly from that measured in control fish (Figure 2B). The relation between estrogenicity (expressed as E2Eq) and Vtg mRNA was very strong (Figure 3) and indicates that gene expression was only affected appreciably after E2Eq concentrations exceeded 2.0 ng/L. The mean change in Vtg mRNA (compared with controls) in the EE2 bioassays ranged from 27-fold for the lowest dose (0.9 ng/L E2Eq) to roughly 76 000-fold for the highest dose (7.4 ng/L E2Eq; Table 2).

WWTP effluent bioassays. Mean plasma Vtg concentrations for male fathead minnows exposed to effluents from sites ny10 and ny3 were 552.6 μg/mL to 1136 μg/mL, respectively (Table 2), and were significantly higher than that in corresponding control fish (Figure 4A). Mean plasma Vtg concentrations from fathead minnows exposed to effluents from the other 8 WWTPs were much lower than at these 2 sites (i.e., ranging from 0 μg/mL to 44.6 μg/mL), and either were not significantly different from or were slightly lower than the levels observed in corresponding controls (Figure 4A). The mean change in hepatic Vtg mRNA (compared with controls) for male fathead minnows exposed to effluents from 6 WWTPs ranged from less than 1-fold times that of the control at ny8, ny11, and ny23 to as much as 2 or 3 times that of controls at ny1, ny2, ny3, and ny25 (Table 2). Although small fold increases in Vtg mRNA were evident in fathead minnows exposed to effluents from ny1 and ny2, noted increases were significantly higher than that measured in control fish only at ny3 (Figure 4B).

Fish assemblages

Fish communities. Community biodiversity metrics (richness and diversity) indicate that fish assemblages were largely alike at most paired reaches (upstream and downstream from effluents), yet both metrics varied considerably among the 10 study sites (Figure 5A and B). Richness ranged from 1 species at the upstream ny9 reach to 19 species at the upstream ny3 reach (Figure 5A; Supplemental Data, Table S2). Across all sites, richness averaged 8.6 species at upstream reaches and 9.1 species at downstream reaches, and site-to-site differences were consistently less than 2 species, except at ny9, where 4 species were observed downstream and only 1 species was collected upstream (Figure 5A). Species diversity ranged from 0 at the upstream ny9 reach to 0.88 at the upstream ny3 reach (Figure 5B; Supplemental Data, Table S2). Across all sites, diversity averaged 0.60 at upstream reaches and 0.66 at downstream reaches. Relatively large differences in diversity were only noted between upstream and downstream reaches at sites ny9 and ny24 (Figure 5B). Like richness, diversity at most downstream reaches was generally comparable or higher than that in corresponding upstream reaches.

Estimates of community density and biomass were more variable across study sites and less similar between upstream and downstream reaches within study sites than were measures of diversity. Total fish density ranged from 36 fish/0.1 ha at the upstream ny9 reach to 3581 fish/0.1 ha in the upstream reach at ny3 (Figure 5C; Supplemental Data, Table S2). Across all sites, density averaged 1658 fish/0.1 ha at upstream reaches and 1561 fish/0.1 ha at downstream reaches. A comparison of means and 95% CIs indicated that total density was significantly lower in downstream reaches at sites ny3, ny24, and ny10, but either no different or significantly higher in downstream reaches at other study sites (Figure 5C). Total fish biomass ranged from

Table 2. Mean E2Eq and EE2 levels in effluent, Vtg plasma concentrations in blood, and mean increases in Vtg mRNA gene expression (fold increase over controls) for male fathead minnows exposed to effluents from wastewater treatment plants and EE2 positive control assays[a]

Site ID	Mean E2Eq in effluent (ng/L)	Mean EE2 in effluent (ng/L)	Mean Vtg in plasma (μg/mL)	SE of Vtg in plasma	Mean Vtg mRNA gene fold increase over controls	SE of gene fold increase
Control	0.029[b]	na	4.9	2.2	1.00	0.35
ny11	0.58	na	1.8	1.8	0.83	0.25
ny2	1.0	na	0.0	0.0	2.19	0.73
ny25	0.83	na	3.5	2.5	2.13	4.81
Control	0.029[b]	na	38.5	18.3	1.00	0.11
ny15	0.20	na	28.6	9.9	1.27	0.22
ny8	0.02	na	44.6	12.6	0.53	0.05
ny1	1.49	na	7.4	2.9	2.15	0.96
Control	0.029[b]	na	14.8	12.0	1.00	0.30
ny10	0.61	na	552.6	527.7	1.29	0.09
ny3	0.57	na	1136.0	940.2	3.23	1.16
ny23	0.53	na	5.3	3.5	0.48	0.10
EE2-cont	0.1	0.0	0	na	1.00	0.1
EE2-1	0.9	1.9	2	8.8	27	15.6
EE2-2.5	2.3	4.1	1100	649.8	2756	2176.6
EE2-5	5.1	8.0	2800	906.0	22 324	8659.9
EE2-10	7.4	14.0	900	261.5	76 051	57 022.9

[a]Site locations are provided in Table 1 and Supplemental Data, Figure S1.
[b]Value equals one-half of the lowest detectable concentration (0.057 ng/L).
E2Eq = estrogenicity; EE2 = ethinylestradiol; Vtg = vitellogenin; SE = standard error; mRNA = messenger ribonucleic acid; na = not applicable.

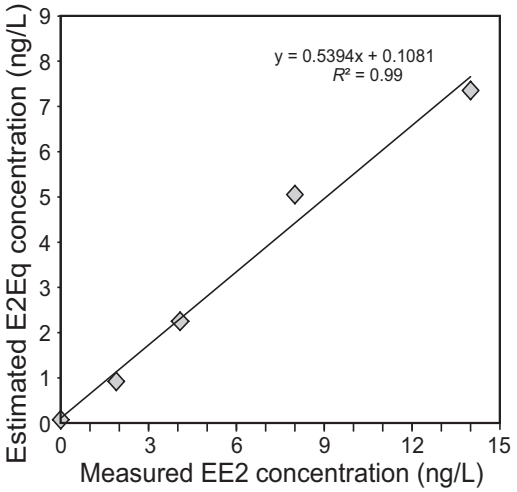

Figure 1. The relation between measured ethinylestradiol (EE2) doses and estrogenicity (E2Eq) levels as determined from EE2 assays.

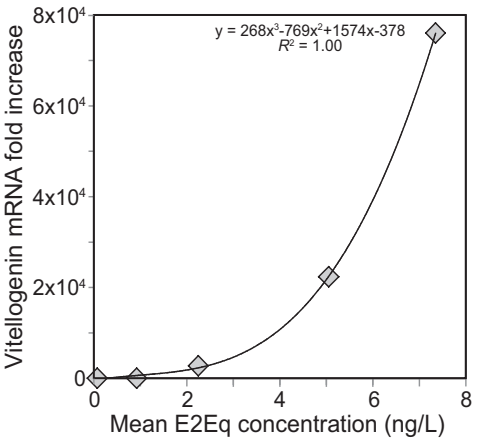

Figure 3. The relation between mean estrogenicity (E2Eq) concentrations and mean vitellogenin messenger ribonucleic acid (mRNA) gene expression plotted as fold increase over control in male fathead minnows from ethinylestradiol (EE2) assays.

114 g/0.1 ha at the upstream ny9 reach to 34 696 g/0.1 ha at the upstream ny10 reach (Figure 5D; Supplemental Data, Table S2). Across all sites, fish biomass averaged 12 422 g/0.1 ha at upstream reaches and 9208 g/0.1 ha at downstream reaches. The means and 95% CIs demonstrate that total biomass was significantly lower in downstream reaches at sites ny3 and ny10, but biomass at most downstream reaches was either no different from or significantly higher than that in upstream reaches at 8 of the 10 study sites (Figure 5D).

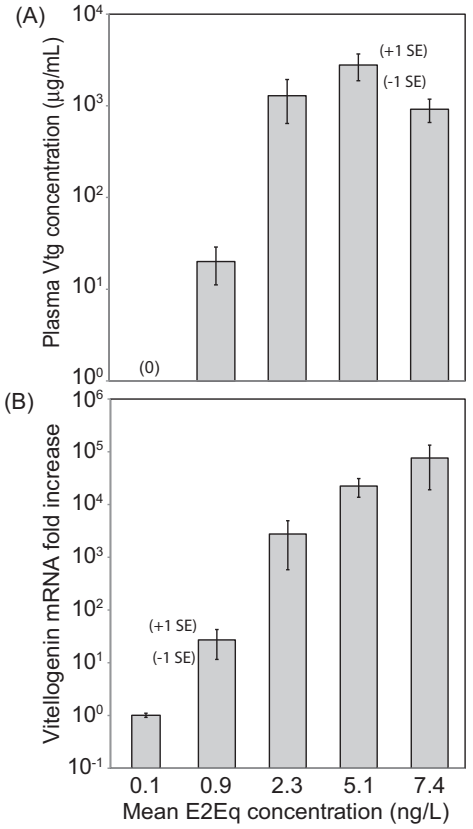

Figure 2. The relations between mean estrogenicity (E2Eq) level and (**A**) mean plasma vitellogenin (Vtg) concentrations and (**B**) mean Vtg messenger ribonucleic acid (mRNA) gene expression plotted as fold increase over control in male fathead minnows from ethinylestradiol (EE2) assays. The bars depict ± 1 standard error (SE).

Species populations. Estimates of density and biomass for each of the 27 fish species found in upstream and downstream reaches at the 10 study sites (Table 3) illustrate the direction and significance of potential population responses to WWTP effluents. Density estimates were significantly lower at upstream reaches than at downstream reaches for 4 or more species populations at ny1, ny2, ny27, ny28, and ny26 (gray-highlighted data pairs, Table 3). Density estimates were significantly higher at upstream reaches than at downstream reaches for 4 or more species populations at ny3, ny27, and ny10 (white-boxed data pairs, Table 3). When density differed significantly between paired reaches, it was usually higher for species populations at downstream reaches. The most obvious exception to this tendency was at ny3, where the densities for 12 of 19 species were significantly lower (and 1 species was significantly higher) at the downstream reach (Table 3;

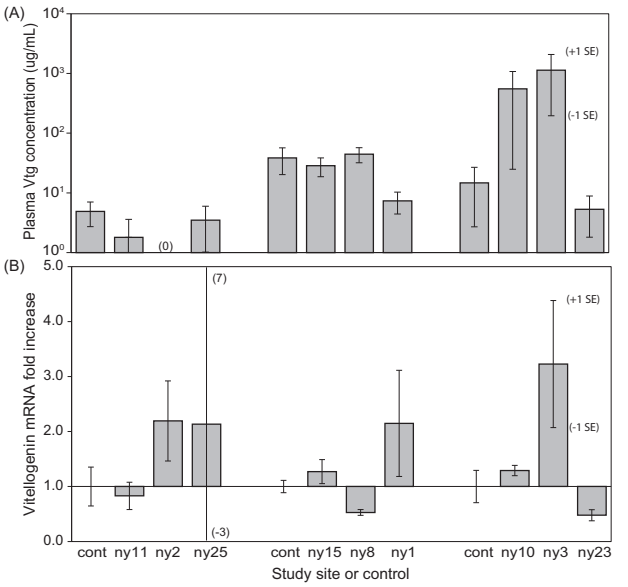

Figure 4. Estimates of mean (**A**) concentrations of plasma vitellogenin (Vtg) and (**B**) Vtg messenger ribonucleic acid (mRNA) gene expression plotted as fold increase over control in male fathead minnows exposed for 96 h to effluents from 9 wastewater treatment plants. The bars depict ± 1 standard error (SE). Study site locations are shown in Supplemental Data, Figure S1.

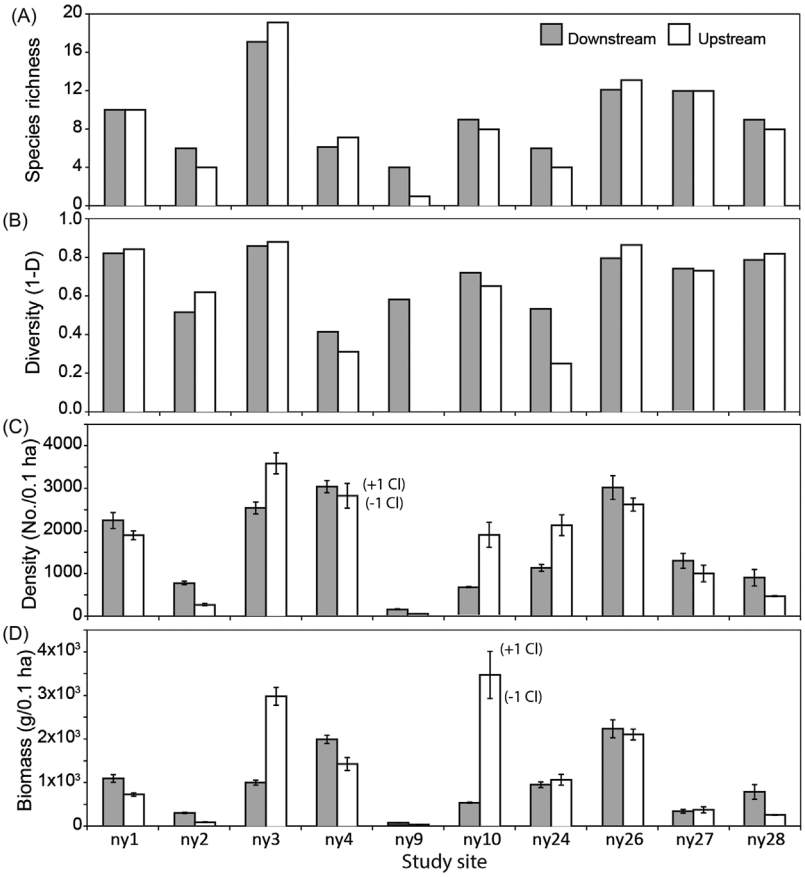

Figure 5. Estimates of community (**A**) richness, (**B**) diversity, (**C**) density, and (**D**) biomass from quantitative fish-community surveys conducted at stream reaches upstream and downstream of effluents at 10 wastewater treatment plants in southeastern New York, USA, 2011. The bars represent ± 1 95% confidence interval (CI). Study site locations are shown in Supplemental Data, Figure S1.

Supplemental Data, Table S2). Biomass estimates were significantly higher at downstream reaches than at upstream reaches for 4 or more species populations at ny1, ny2, ny9, ny27, ny28, and ny26 (Table 3). Biomass estimates were significantly higher at upstream reaches than at downstream reaches for 4 or more species populations at ny3, ny27, ny10, and ny26 (Table 3). Like density, when biomass differed significantly between reaches, it was generally higher for species populations at downstream reaches; site ny3 was the major exception (Table 3; Supplemental Data, Figure S2). The upstream–downstream differences in density and biomass of most species populations at ny3 (Figure 6) deviated considerably from the nondirectional tendencies observed at all other study sites. Estimates of density and biomass for 12 or 13 of the 19 species at ny3 were significantly lower in the downstream reach than in the upstream reach (Table 3). Except for ny3 and possibly 1 other site (ny10), reach-to-reach differences in density or biomass were not significant for more than a few species, and most of the nonsignificant differences were not directional (Table 3; Supplemental Data, Figure S2). The density of common shiner, fallfish, central stoneroller, cutlips minnow, and white sucker populations were seriously reduced in downstream reaches compared with upstream reaches at ny3 (Figure 6A). The biomass of common shiner, fallfish, central stoneroller, cutlips minnow, white sucker, brown trout, largemouth bass, and common carp populations also declined radically in the downstream reach at ny3 (Figure 6B). Differences in carp, largemouth bass, and trout biomass could have been exaggerated at ny3 (Figure 6B) because of the capture of 1 or 2 large individuals only at the upstream reach. There were no species populations for which density or biomass decreased consistently at all or most downstream reaches; however, the density and biomass of eastern blacknose dace populations were significantly higher in downstream reaches at 6 and 5 sites, respectively, out of the 9 where they were collected (Table 3).

Habitat. Habitat conditions differed widely across study sites but were generally comparable between upstream and downstream reaches at most study sites (Table 4). Although mean habitat measures did not differ significantly between upstream and downstream reaches at any of the 10 study sites (Student's *t* test), the depths and velocities at upstream and downstream reaches at ny2 and ny10 differed more widely than they did at all other sites.

Species ordinations. The MDS ordination of square-root–transformed fish-density data shows that there were generally no significant differences in the proportional abundance of the various fish species that constitute communities at reaches upstream and downstream from effluent discharge points at the 10 study sites (Supplemental Data, Figure S3). This is especially noteworthy at ny3, where large decreases in density and biomass of most species at the downstream reach did not eliminate common resident species or greatly alter their proportions in the community. The similarities for fish assemblages, in study reaches that were not significantly different from one another, varied from approximately 39% to 82%. Significant site-to-site differences, however, were evident between fish communities observed among individual sites and groups of sites, including ny1; ny9; ny4, ny24, and ny27;

Table 3. Estimates of population density and biomass for common fish species inventoried from stream reaches upstream (US) and downstream (DS) of effluent discharges at 10 wastewater treatment plants in eastern New York, USA, 2011[a]

	ny1		ny2		ny3		ny4		ny9		ny10		ny24		ny26		ny27		ny28	
Common name	DS	US	DS	US	DS	US	DS	US	DS	US	DS	US	DS	US	DS	US	DS	US	DS	US
Density (no./0.1 ha)																				
Creek chub	563	249	238	39	496	145	4	4	–	–	10	42	–	–	878	610	2	5	127	135
Common shiner	–	–	–	–	82	312	–	–	–	–	–	–	–	–	33	208	2	0	–	–
Golden shiner	1	0	–	–	–	–	–	–	81	0	15	223	–	–	–	–	–	–	4	0
Fallfish	–	–	–	–	54	255	–	–	–	–	–	–	–	–	75	250	2	0	–	–
Common carp	–	–	–	–	0	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pumpkinseed	0	64	–	–	2	11	–	–	–	–	0	112	–	–	0	3	4	3	95	68
Bluegill sunfish	7	204	–	–	–	–	–	–	7	0	70	1186	–	–	–	–	–	–	296	34
Redbreast sunfish	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	2	–	–
White sucker	379	151	–	–	252	527	12	2	–	–	155	872	–	–	283	224	59	100	74	89
Longnose sucker	–	–	–	–	2	2	–	–	–	–	–	–	–	–	12	51	–	–	–	–
Central stoneroller	–	–	–	–	37	375	–	–	–	–	–	–	–	–	154	373	–	–	–	–
Cutlips minnow	266	292	–	–	425	829	–	–	–	–	–	–	10	0	150	69	4	14	–	–
Margined madtom	–	–	–	–	129	149	–	–	–	–	–	–	–	–	21	5	–	–	–	–
Fathead minnow	35	3	–	–	2	14	–	–	–	–	–	–	–	–	–	–	26	12	–	–
Mummichog	–	–	–	–	–	–	–	–	37	36	–	–	–	–	–	–	–	–	–	–
Brown trout	–	–	–	–	13	18	320	201	–	–	–	–	34	84	–	–	2	2	0	5
Brook trout	–	–	–	–	–	–	0	6	–	–	–	–	–	–	42	40	–	–	–	–
Rainbow trout	–	–	–	–	–	–	–	–	–	–	–	–	52	107	–	–	0	2	–	–
Blacknose dace	491	475	460	142	526	473	357	233	–	–	285	0	114	0	1182	535	433	221	21	49
Longnose dace	331	361	–	–	430	612	83	85	–	–	–	–	205	104	491	248	279	366	–	–
Tesselated darter	182	133	24	39	105	79	–	–	–	–	5	0	–	–	279	202	–	–	218	39
Shield darter	–	–	–	–	84	154	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Largemouth bass	1	5	8	0	13	50	–	–	15	0	5	7	–	–	–	–	84	21	35	21
Redfin pickerel	–	–	8	32	–	–	–	–	–	–	85	70	–	–	–	–	–	–	–	–
Brown bullhead	–	–	8	0	0	2	–	–	–	–	15	7	–	–	–	–	–	–	–	–
Yellow bullhead	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4	0
Slimy sculpin	–	–	–	–	2	23	2318	2480	–	–	–	–	779	1846	–	–	450	124	–	–
Biomass (g/0.1 ha)																				
Creek chub	3931	1809	1443	174	1080	677	5	9	–	–	160	1498	–	–	5740	4925	7	10	1047	1033
Common shiner	–	–	–	–	540	1524	–	–	–	–	–	–	–	–	119	380	3	0	–	–
Golden shiner	8	0	–	–	–	–	–	–	361	0	146	2977	–	–	–	–	–	–	11	0
Fallfish	–	–	–	–	526	2448	–	–	–	–	–	–	–	–	131	811	7	0	–	–
Common carp	–	–	–	–	0	2051	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pumpkinseed	0	251	–	–	12	58	–	–	–	–	0	951	–	–	0	10	11	6	412	144
Bluegill sunfish	29	784	–	–	–	–	–	–	7	0	372	7784	–	–	–	–	–	–	2779	100
Redbreast sunfish	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	253	–	–
White sucker	2992	928	–	–	1939	5776	5731	16	–	–	3110	20 704	–	–	6278	5027	101	181	1078	399
Longnose sucker	–	–	–	–	49	65	–	–	–	–	–	–	–	–	639	1961	–	–	–	–
Central stoneroller	–	–	–	–	346	4332	–	–	–	–	–	–	–	–	1013	2118	–	–	–	–
Cutlips minnow	1255	1201	–	–	1225	3192	–	–	–	–	–	–	166	0	1489	852	46	109	–	–
Margined madtom	–	–	–	–	868	1257	–	–	–	–	–	–	–	–	195	81	–	–	–	–
Fathead minnow	112	6	–	–	7	31	–	–	–	–	–	–	–	–	–	–	40	12	–	–
Mummichog	–	–	–	–	–	–	–	–	140	114	–	–	–	–	–	–	–	–	–	–
Brown trout	–	–	–	–	986	3882	6027	5695	–	–	–	–	3534	2262	–	–	262	421	0	363
Brook trout	–	–	–	–	–	–	0	72	–	–	–	–	–	–	2091	2539	–	–	–	–
Rainbow trout	–	–	–	–	–	–	–	–	–	–	–	–	1429	2022	–	–	0	158	–	–
Blacknose dace	751	550	1217	315	637	785	608	327	–	–	962	0	149	0	2924	1125	544	348	24	147
Longnose dace	1383	1065	–	–	1291	2031	560	474	–	–	–	–	1798	1161	1179	733	876	1590	–	–
Tesselated darter	312	220	42	9	231	146	–	–	–	–	10	0	–	–	378	295	–	–	419	80
Shield darter	–	–	–	–	245	411	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Largemouth bass	4	269	47	0	62	1033	–	–	9	0	9	24	–	–	–	–	44	10	1704	80
Redfin pickerel	–	–	45	215	–	–	–	–	–	–	558	747	–	–	–	–	–	–	–	–
Brown bullhead	–	–	38	0	0	32	–	–	–	–	13	11	–	–	–	–	–	–	–	–
Yellow bullhead	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	171	0
Slimy sculpin	–	–	–	–	7	139	7045	7689	–	–	–	–	2513	5284	–	–	1241	431	–	–

[a]Boxed values denote that estimates were significantly lower at downstream reaches, and gray highlighted values indicate that estimates were significantly lower at upstream reaches ($p \leq 0.05$). Site locations are provided in Table 1 and Supplemental Data, Figure S1.

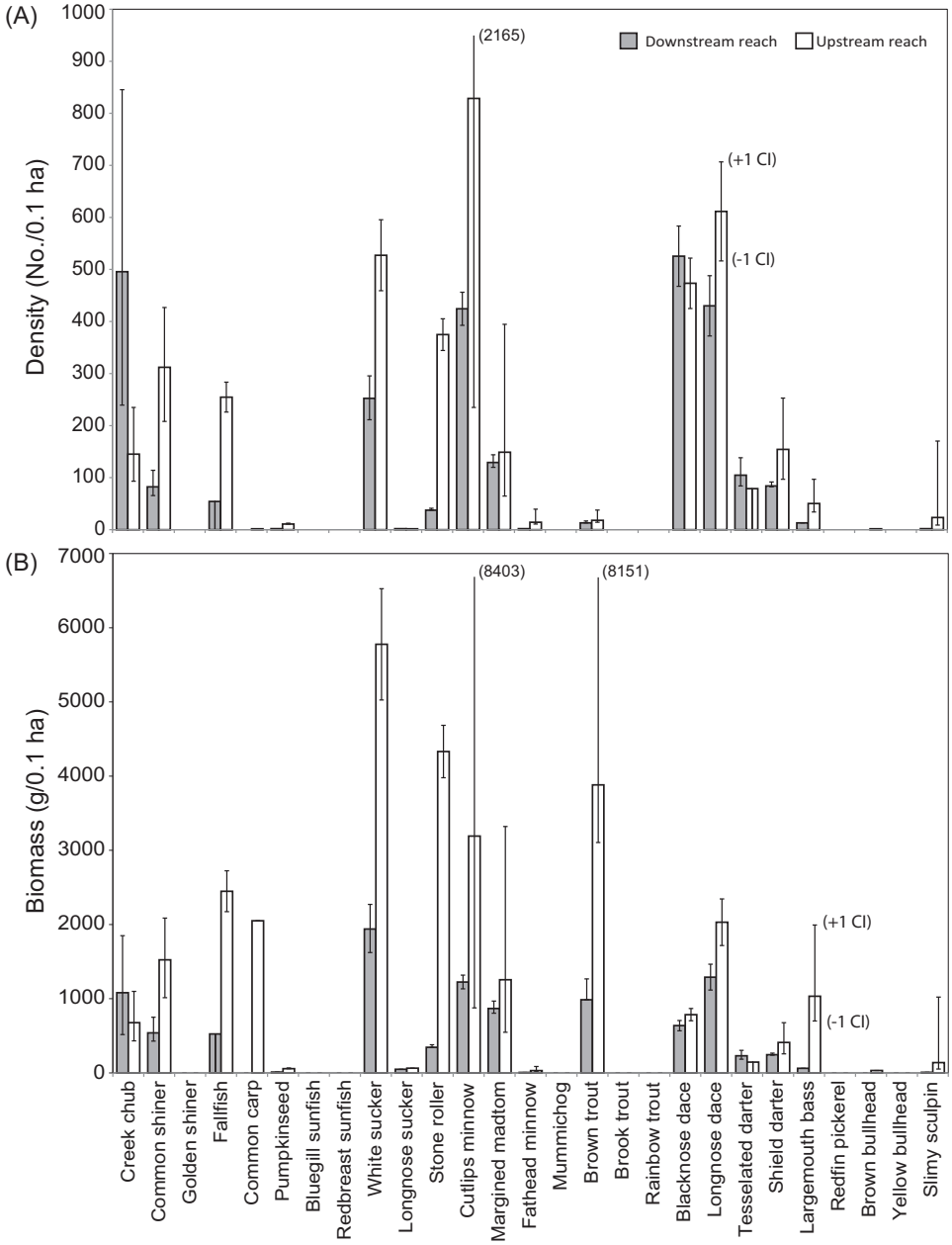

Figure 6. Estimates of mean (**A**) density and (**B**) biomass for all fish species collected from upstream (white columns) and downstream (shaded columns) reaches at the ny3 wastewater treatment plant during the 2011 survey. The bars depict ±1 95% confidence interval (CI). Study site locations are shown in Supplemental Data, Figure S1.

ny3 and ny26; and ny2, ny10, and ny28 (Supplemental Data, Figure S3).

DISCUSSION

The results summarized in the present study provide information that may be used to refine estimates of estrogenic activity in effluents and contaminant mixtures, to identify physiologic and genomic effect thresholds for EE2 and E2Eq, and to evaluate the potential effects that estrogenic effluents could have on resident fish assemblages in receiving streams. For example, the relation between E2Eq and EE2 offers information relevant to estimating the total estrogenicity of municipal and industrial wastewater effluents. Vajda et al. [38] found that E2Eq weights for EE2 ranged from 1.2 to 33. The slope of the equation describing the relation between E2Eq and EE2 in the present study (Figure 1) suggests that the E2Eq weight for EE2 may be near 0.54 in the 0 ng/L to 14 ng/L EE2 range. This weight is considerably less than those reported by Vajda et al. [38]. The revised E2Eq weight may be used to increase the accuracy of calculations that estimate total estrogenicity for simple and complex mixtures of contaminants commonly found in wastewater effluents.

The fact that hepatic Vtg mRNA only increased prominently above levels observed in control fish when E2Eq concentrations exceeded 2.0 ng/L in the EE2 assay is important. If further substantiated, 2.0 ng/L E2Eq could be an appropriate short-term (96-h) effect threshold, below which few or no molecular changes may be anticipated in male fathead minnows (and other fish species). The lack of significant increases in Vtg mRNA in fathead minnows exposed for 96 h to effluents (grab samples) from all 9 WWTPs, with E2Eq concentrations consistently

Table 4. Habitat characteristics for all stream reaches, upstream (US) and downstream (DS) of effluent discharges for 10 wastewater treatment plants where quantitative fish-community surveys were done during 2011[a]

	ny1		ny2		ny3		ny4		ny9		ny10		ny24		ny26		ny27		ny28	
Reach character	DS	US	DS	US	DS	US	DS	US	DS	US	DS	US	DS	US	DS	US	DS	US	DS	US
Velocity mean (m/s)	0.19	0.10	0.31	0.29	0.08	0.10	0.54	0.48	0.36	0.48	0.29	0.28	0.90	0.77	0.23	0.22	0.08	0.11	0.35	0.53
SD	0.16	0.17	0.16	0.22	0.11	0.09	0.36	0.34	0.28	0.25	0.24	0.25	0.44	0.55	0.13	0.16	0.08	0.12	0.27	0.33
Median (m/s)	0.17	0.05	0.30	0.24	0.03	0.09	0.46	0.40	0.34	0.43	0.21	0.26	0.91	0.64	0.21	0.24	0.06	0.09	0.32	0.47
No.	30	30	36	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30
Depth mean (m)	0.16	0.06	0.30	0.30	0.15	0.12	0.09	0.10	0.18	0.22	0.25	0.27	0.23	0.23	0.18	0.13	0.09	0.14	0.27	0.23
SD	0.08	0.05	0.11	0.18	0.10	0.06	0.04	0.05	0.09	0.11	0.12	0.12	0.11	0.12	0.09	0.07	0.04	0.11	0.12	0.11
Median (m)	0.15	0.05	0.29	0.24	0.13	0.11	0.08	0.09	0.15	0.20	0.25	0.27	0.21	0.21	0.16	0.12	0.09	0.11	0.28	0.22
No.	30	30	36	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30
Width mean (m)	2.7	3.2	6.4	6.4	8.4	4.7	2.2	1.8	5.2	6.6	10.4	6.5	3.9	4.5	2.8	4.2	2.9	2.4	7.6	6.9
SD	0.8	0.9	1.0	1.6	2.1	1.4	0.4	0.6	1.2	0.9	2.3	0.8	0.8	0.6	0.5	1.8	0.8	0.6	1.4	1.0
Median (m)	2.7	3.1	6.5	6.2	8.1	4.2	2.2	1.6	5.3	6.5	10.9	6.4	3.8	4.5	2.7	3.8	3.0	2.5	7.7	6.9
No.	10	10	12	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Length total (m)	46.0	39.6	82.9	87.8	81.1	80.5	63.5	45.0	100.0	81.1	91.0	90.2	62.0	84.0	100.0	91.0	68.6	59.1	80.5	50.0
Area total (m^2)	126.0	126.7	534.7	557.6	680.6	377.0	136.6	83.1	518.2	531.9	950.7	582.4	240.2	375.6	283.8	383.9	199.8	143.3	613.7	347.2

[a]Site locations are provided in Table 1 and Supplemental Data, Figure S1.

below 2.0 ng/L, appears to corroborate the threshold. Assuming that the response of fathead minnows represents potential genomic effects of WWTP effluents on many resident fish species, then few changes in fish reproduction (and populations) would be anticipated for short-term exposures to environmental E2Eq concentrations less than 2.0 ng/L. Unfortunately, a lower long-term E2Eq effect threshold may be warranted for wild fish populations because EE2 can bioaccumulate and other investigators have reported significant decreases in fathead minnow egg fertilization, sex ratio (reduced proportion of males), and demasculinization of males during long (150-d) life-cycle exposure to EE2 concentrations of 0.32 ng/L and 0.96 ng/L [15]. Although E2Eq and Vtg mRNA were not assessed during the study [15], 1.0 ng/L EE2 corresponds roughly to 0.65 ng/L E2Eq in our EE2 assays (Figure 1) and suggests that the long-term effect threshold may be closer to 0.3 ng E2Eq/L or 1.0 ng E2Eq/L.

The lack of serious effects in most fathead minnow tests and in 8 of the 10 fish assemblages is surprising considering that a prior phase of the present study (phase 1) [17] found that estrogenicity levels were elevated in effluents from a large fraction of samples from 33 WWTP effluents. Several circumstances may help explain why E2Eq levels for grabs from effluents at many WWTPs sampled during the present study (phases 2 and 3) were unusually low or did not represent estrogenicity levels measured during the first phase of the present study [17]. Results from that study showed that E2Eq concentrations in effluents at 33 WWTPs (some sampled multiple times) approached or exceeded 1.0 ng/L in 30% of all samples, 2.0 ng/L or more in 10% of all samples, and 16.1 ng/L in 1 effluent. Estrogenicity levels in effluents from WWTPs generally vary widely over time [17,45]; thus grab samples from any study phase may have been collected under atypical conditions. In addition, the volume of the effluent in relation to the flow of the receiving stream is a primary factor that determines where, or when, estrogenic effluents occur and whether resident biota are at risk. Large-volume effluents that discharge EDCs into low-order streams with low average (annual) or base (summer) flows may not be highly diluted and thus could expose fish populations to high levels of estrogenicity. For instance, 11 of the 33 WWTPs sampled by Baldigo et al. [17] potentially contribute 10% or more (on average) to the total flow in their receiving streams, and 2 WWTPs are permitted to discharge effluents that could contribute as much as 62% of the total flow in receiving streams. Small dilution ratios, combined with WWTPs that discharge effluent with low to moderate levels of E2Eq, could create short-term (episodic) or long-term (chronic) estrogenicity (near or exceeding 2.0 ng/L) in receiving streams. Normal variations in receiving stream and effluent discharge could easily produce long periods of low or undetectable estrogenicity during multiple sampling efforts. Wide variations in stream and effluent discharge, effluent estrogenicity, and the susceptibility of fish species (and life stages) likely affect their populations and communities differently over the short and long term and are important reasons why strong linkages between estrogenicity and impacts in aquatic ecosystems are difficult to quantify and generally lacking in the literature.

Whether EDCs from WWTP effluents can directly (and adversely) affect the abundance, biomass, and sustainability of wild fish populations (and other organisms) is debatable. To date, many studies have identified estrogenic effects at the molecular, organ, and organism level under natural (field) conditions [16,46–48], and 1 study even linked the collapse of

fish populations to additions of EE2 within an experimental lake [13]; yet none has strongly associated population degradation with elevated EDCs in the wild. Consequently, the decreases in density and biomass of 79% to 89% of the 19 species at ny3 and 60% to 70% of the 10 species at ny10 (Table 3) are particularly noteworthy. The impacts at both reaches appear to be linked directly to estrogenicity because plasma Vtg concentrations in male fathead minnows increased to 500 μg/mL to 1000 μg/mL after exposure to effluents from ny3 and ny10 (Figure 4A) and because prior surveys noted E2Eq concentrations between 0.24 ng/L and 3.58 ng/L at ny3 and between 0.71 ng/L and 0.75 ng/L at ny10 [17]. Although habitat does not differ significantly at upstream and downstream reaches, habitat differences between reach pairs are nominal at ny3, and slightly more substantial at ny10 (Table 4). Thus, habitat variations ostensibly could contribute to upstream–downstream differences in species assemblages at ny10. Although the WWTPs surveyed by Baldigo et al. [17] were not selected randomly, the high E2Eq concentrations (1–16.1 ng/L) observed in several effluents in the region suggest that a large number of the 1900 WWTPs permitted in New York State could have estrogenic effluents that could potentially affect fish assemblages.

The response of plasma Vtg to increasing levels of E2Eq was more informative than was the response of the Vtg mRNA gene in 2 of the 4-d effluent tests. This was unexpected because Vtg mRNA is generally considered to be a more sensitive indicator of estrogen exposure than plasma Vtg [49]. Although the responses of both biomarkers were comparable (and muted for the 0.9 ng/L E2Eq dilution) in the EE2 assays (Figures 2A and 3), the large and significant increases in plasma Vtg—552.6 μm/mL and 1136 μg/mL at ny3 and ny10, respectively (Table 2 and Figure 4A)—suggest that the plasma Vtg biomarker may be more sensitive to low concentrations of E2Eq (means of 0.57–0.61 ng/L in the 2 effluents) than Vtg mRNA expression. Plasma Vtg is the product in the liver from translation of the Vtg mRNA into protein, but each mRNA can be translated many times by polysomes, and the final amount made does not necessarily depend on a 1:1 relationship with the mRNA. Hepatic Vtg mRNA has a half-life of approximately 2 d [50] and has been shown to peak within 48 h of exposure after a single intraperitoneal injection [51]. Plasma Vtg has been shown to reach maximum concentrations between 3 d and 4 d post injection [51] and its half-life is more than 1 mo [8]. Both biomarkers can also exhibit a large and variable range in responses. Plasma Vtg concentrations in males that are not exposed to estrogens are typically less than 1 μg/mL (in the 50–500 ng/mL range), and they can be as high as 12 mg/mL in reproductively active females. Plasma Vtg concentrations in males exposed to high levels of estrogens, however, can be as high as 100 mg/mL. A 1000-fold increase in Vtg mRNA levels has been observed in male fish exposed to EDCs.

An estrogenic WWTP effluent appears to be responsible for the significantly lower density and biomass of most species populations in downstream reaches at ny3, based on recent water-quality data, results of the fathead minnow tests, historic flow data, and several logical inferences. First, E2Eq concentrations near 1.0 ng/L and as high as 3.58 ng/L were occasionally quantified in the effluent from ny3 [17], and results reported in the present study indicate that Vtg gene expression and Vtg levels in plasma of male fathead minnows generally increased significantly once E2Eq concentrations exceeded 2.0 ng/L in short-term exposures. This means that E2Eq concentrations in downstream reaches at ny3 could conceivably have surpassed 2.0 ng/L occasionally (or frequently) during summer low flows because of the small flow of the receiving stream. Although the estimated effluent:stream dilution ratio based on mean annual flow of 59 ft^3/s and maximum permitted discharge for the WWTP (0.248 ft^3/s) is 0.0042:1 (0.4%; Table 1), much lower summer flows [52] indicate that periods of low dilution were not uncommon. For example, several annual minimum 7-d low flows ranged near 3.0 ft^3/s and were as low as 0.11 ft^3/s between 2001 and 2013 at a US Geological Survey discharge station 1.3 km upstream from ny3 [52]. The effective effluent:stream dilution ratios in reaches downstream from ny3 under these conditions could have ranged from 0.04:1 to 1.12:1. Second, EE2 concentrations much lower than 1.0 ng/L (E2Eq concentrations < 0.65 ng/L) have been shown to adversely affect the reproductive systems of fathead minnows during prolonged exposures [15]. Therefore, long-term exposure—even to very low or moderate E2Eq levels from the effluent of ny3—could have had cumulative effects on the reproductive success of resident fish species in the receiving stream. Third, except for pharmaceuticals, effluent concentrations for a wide range of wastewater-associated compounds (including, but not limited to, personal care and domestic use compounds, plasticizers, and other compounds such as cholesterol and coprostanol) at ny3 were either similar to or lower than concentrations at many other WWTPs in the region [17]. Lastly, an analysis of 8 pharmaceuticals in the effluent of ny1, ny2, and ny3 showed that their concentrations at ny3 were 1 to 3 orders of magnitude higher than at ny1 and ny2 (and at other WWTPs) [53]. The investigators attributed high concentrations of pharmaceuticals in the effluent of ny3 to the contributions of a pharmaceutical manufacturing facility that discharges industrial wastewater to the WWTP (site ny3). Phillips et al. [53] noted, however, that a complete list of pharmaceuticals formulated at the pharmaceutical manufacturing facility discharging to ny3 was unavailable. Although this is not concrete evidence for EDCs in the effluent from ny3, a pharmaceutical manufacturing facility in France was recognized as the primary source for a number of known and unknown pharmaceuticals that function as agonists and (or) antagonists for different steroid receptors [54]. More evidence for estrogenic pharmaceuticals at ny3 was generated from analyses of several split effluent samples [17]. Estimates of biological estrogenicity (E2Eq determined via e-screen analysis) were often several orders of magnitude higher than estimates of chemical estrogenicity (calculated as the E2Eq sum for measured hormones and known-estrogenic compounds) at ny3 and suggested that some known or unknown pharmaceuticals, or their byproducts, were highly estrogenic. Although the discharge of estrogenic effluents appeared to be partly or mostly responsible for the diminished density and biomass of most fish species populations in the receiving stream at ny3, more information on the full array of pharmaceuticals, their concentrations, and their estrogenicity is needed before wastes from the pharmaceutical manufacturing facility can be established as the source for observed impacts in the stream ecosystem.

With 1 or 2 notable exceptions (sites ny3 and ny10), the upstream–downstream differences in fish population and community metrics in the present study indicate that effluents commonly have either random or positive effects on local species populations. Increased abundance and biomass of resident fish at downstream reaches could be indicative of slight to moderate nutrient enrichment and corresponding effects on aquatic ecosystems. This scenario seems plausible considering that increasing concentrations of nutrients (up to a point) can

enhance productivity and primary producers, which in turn could alter periphyton and macroinvertebrate assemblages and subsequently affect fish assemblages [55]. Such linkages are illustrated by the rapid increases in plankton abundance, drawn-out increases in macroinvertebrate biomass, and large increases in growth rates and biomass of brown trout following long-term additions of nitrogen and phosphorus to an alpine lake [56]. Although increases in community richness and the density and biomass of species populations often suggest improvements in biological integrity (associated with a reduction in contaminants), similar changes can sometimes occur in highly polluted reaches. Thus, increases and decreases in fishery metrics at downstream reaches do not necessarily mean that the health of local fish assemblages is better or worse than that at upstream reaches.

The findings of the present study have several local (individual site) and broad (regional or statewide) implications. The lack of major changes in Vtg gene expression in male fathead minnows exposed to whole WWTP effluents and the scarcity of population- and community-level impacts suggest that EDCs may not be a ubiquitous problem for fish assemblages in streams across the region. The increases in plasma Vtg concentrations in male fathead minnows, high levels of E2Eq in effluents of 2 WWTPs, and significant declines in density and biomass of most species from 1 or 2 study sites, however, indicate that estrogenic discharges can cause harm to individual fish as well as their populations in streams. Moderately estrogenic effluents from WWTPs evidently can negatively affect aquatic organisms and ecosystems under low stream flows and high effluent discharges. Medical-based industries (e.g., pharmaceutical plants, nursing homes, and hospitals) may be sources of untreated wastewater (influents) at WWTPs that are not well treated and may potentially be responsible for estrogenic effluents and altered fish species assemblages in receiving streams in this region and probably elsewhere. Additional efforts clearly are needed to better characterize estrogenicity of WWTP discharges across a wide range of point (and nonpoint) contaminant sources, to expand research and monitoring programs to include WWTPs with specific influent characteristics (e.g., hospitals, nursing homes, pharmaceutical plants, and etc.), to characterize the susceptibility of receiving streams, to better define the relations among EDCs and fish assemblages at sites with highly estrogenic effluents, and to reassess the sustainability of fish populations and communities in highly susceptible surface waters across New York.

SUPPLEMENTAL DATA

Tables S1–S2.
Figures S1–S3. (1.8 MB DOCX).

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Data availability—Hydrologic information for USGS site number 01421610 are available online at http://wdr.water.usgs.gov/wy2013/pdfs/01421610.2013.pdf; standard wastewater compounds, hormones, and PPCPs data for samples collected from the effluent at study site ny3 (USGS Site number 422212074405501) from 12/10/2008 to 2/5/2013 are available in the USGS report [17] and online at http://waterdata.usgs.gov/nwis/; estrogenicity data for prior surveys at all sites are available in the USGS report [17]; raw estrogenicity, gene expression, EE2, and fishery data from laboratory bioassays and recent fish surveys are available from the primary author (bbaldigo@usgs.gov).

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