Mass Balance of Fipronil and Total Toxicity of Fipronil-Related Compounds in Process Streams during Conventional Wastewater and Wetland Treatment

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Supporting Information

ABSTRACT: Attenuation of the pesticide fipronil and its major degradates was determined during conventional wastewater treatment and wetland treatment. Analysis of flow-weighted composite samples by liquid and gas chromatography—tandem mass spectrometry showed fipronil occurrence at 12−31 ng/L in raw sewage, primary effluent, secondary effluent, chlorinated effluent, and wetland effluent. Mean daily loads of total fipronil related compounds in raw sewage and in plant effluent after chlorination were statistically indistinguishable (p = 0.29; n = 10), whereas fipronil itself was partially removed (25 ± 3%; p = 0.00025; n = 10); the associated loss in toxicity was balanced by the formation of toxic fipronil degradates, showing conventional treatment to be unfit for reducing overall toxicity. In contrast to these findings at the municipal wastewater treatment, both parent fipronil and the sum of fipronil-related compounds were removed in the wetland with efficiencies of 44 ± 4% and 47 ± 13%, respectively. Total fipronil concentrations in plant effluent (28 ± 6 ng/L as fipronil) were within an order of magnitude of half-maximal effective concentrations (EC50) of nontarget invertebrates. This is the first systematic assessment of the fate of fipronil and its major degradates during full-scale conventional wastewater and constructed wetland treatment.

INTRODUCTION

Fipronil is a phenylpyrazole insecticide used in a variety of pest control products, including seed coatings, roach and ant bait, flea and tick topical treatments, and various termicide formulations. Incomplete transformation of fipronil is known to yield several degradation products of similar or higher toxicity.1 Fipronil is known to undergo biotic oxidation to yield fipronil sulfone and reduction to form fipronil sulfide; these are generally the two most common environmental fipronil degradates. A pair of minor environmental transformation products are fipronil amide formed during hydrolysis and fipronil−desulfanyl produced during photolysis.1 Fipronil application to rice paddies has been directly implicated in the sharp decline in crawfish populations in southern Louisiana.2−5 Fipronil and its degradates also are toxic to nontarget vertebrates, including fish and gallinaceous birds.4 Fipronil has been implicated as a potential contributor to colony collapse disorder of honeybee populations.6−7 With lethal dosages (LD50) of 4−13 ng/bee,8−10 fipronil is extremely toxic to honeybees, which play a critical ecosystem function and also provide an added economic value to the United States crop industry estimated at $5−14 billion per year.11 Due in part to its likely role in pollinator poisoning and its effects on aquatic wildlife, China placed heavy restrictions on use of fipronil starting in 2009,12 and the European Union followed suit in 2013.13

As a result of its widespread use, fipronil has been detected in urban waterways and in rural rivers.14,15 In a survey of urban waters in Orange County, California, fipronil and fipronil sulfone exceeded aquatic toxicity benchmarks in over 70% of samples (n = 94).15 In another study of fipronil contamination in the Mermentau and Calcasieu River Basins in the United States, fipronil, fipronil sulfide, and fipronil sulfone were detected in 78.0, 90.0, and 81.7% of surveyed samples, respectively.3 These compounds were also shown to have accumulated in sediments in the same area (100% detection frequency).3 Fipronil, like other neurotoxic insecticides (e.g., the neonicotinoid compound imidacloprid), has been linked to wildlife population declines, with a notable impact on biological diversity.16 Numerous studies have investigated fipronil impacts on copepods,17 fish,18 gallinaceous birds,3 and reptiles.19 Among the suspected sources of fipronil contamination are agricultural runoff,20 urban runoff,21 and treated wastewater.22−24

Mass-spectrometry and mass-balance assessments along with flow data logging are valuable tools for studying the fate of recalcitrant anthropogenic pollutants, including pesticides,
herbicides, and biocides of agricultural and domestic use.16,17,23,25–29 Prior to the present work, only one single study employed a mass-balance approach to investigate the fate of fipronil during wastewater treatment, reporting a removal efficiency of 18 ± 22%; the large margin of error prevented any firm conclusions as to whether fipronil was removed at all, and major transformation products were not monitored in this prior work.22 It is interesting to note that partial or complete loss of fipronil during wastewater treatment does not necessarily imply a reduction of the total toxicity of the sum of fipronil-related compounds, given that transformation of the parental pesticide may give rise to equally or even more potent toxic degradates.

The primary objective of this study was, therefore, to assess the fate of parental fipronil and its major degradates (i.e., fipronil sulfate, fipronil sulfone, fipronil amide, and fipronil—desulfinyl) in a large wastewater-treatment plant (WWTP) by performing mass balances for various conventional treatment unit operations and for a constructed wetland located immediately downstream.

## MATERIALS AND METHODS

### Solvents and Standards.

Analytical grade solvents (water and acetonitrile) were obtained from Thermo Fisher Scientific (Waltham, MA) and EMD Millipore (Billerica, MA). Neat analytical standards of fipronil and fipronil—desulfinyl were purchased from Sigma-Aldrich (St. Louis, MO), and neat standards of fipronil sulfide, sulfone, and amide were produced by Bayer and BASF (Ludwigshafen, Germany). Isotopically labeled fipronil ($^{13}$C$_2$N$_2$-fipronil) was purchased from Toronto Research Chemicals, Incorporated (Toronto, Ontario Canada).

### Sampling Campaign.

The wastewater treatment plant located in the southwestern U.S. is composed of several individual conventional treatment trains operated in parallel. We systematically assessed the fipronil compound reduction capability of one representative treatment train as well as the entire treatment plant and a constructed wetland located downstream. Automatic samplers were deployed at the following locations along the treatment train to capture primary influent, primary effluent, secondary effluent, return-activated sludge, disinfection-basin effluent, wetland influent, and wetland effluent. Primary sludge was obtained by grab sampling. Sampling was carried out in mid December over five consecutive days, from 12 PM on Thursday through 12 PM the following Tuesday. The ISCO 6700 and 6712 samplers (Teledyne Technologies, Thousand Oaks, CA) were programmed for flow-weighted composite sampling. To obtain flow-weighted composites, we programmed the samplers to sample multiples of 20 mL every hour. The fraction of the total composite volume sampled any given hour was proportionate to the deviation from daily average flow into the plant (as determined by hourly flow data over a period of 21 days). More details on sampler programming can be found in the Figure S1. At 12 PM each day, the composite from the prior day was replaced with an empty 2.5 L amber bottle. Primary sludge was sampled once per day at 9 AM using a 1 L bottle. Biosolids were taken as grab samples in 40 mL glass vials, starting 21 days after the first day of the water sampling campaign, to account for the solids retention time in the anaerobic digesters.

### Solids Collection and Analysis.

Solid samples were extracted using a modified version of EPA method 1699. Aliquots (10 mL) of refrigerated, homogenized water samples were transferred to 15 mL centrifuge tubes and were subsequently centrifuged at 3500g. The supernatants were then decanted and discarded. The remaining solids were dried, weighed, spiked with 20 ng labeled fipronil, extracted with 10 mL of acetonitrile at room temperature via placement on a rotary shaker operated at 60 rpm for 24 h. The extraction mixture was centrifuged again, and the solvent was collected in a glass vial. After a second extraction with 10 mL of acetonitrile, the serial extracts were combined, evaporated under nitrogen to near dryness, and reconstituted with 6 mL of hexane. Sample cleanup was done using 1g/6 mL Sep-Pak (Waters Corporation, Milford, MA) cartridges containing Florisil. The cartridges were conditioned with 6 mL dichloromethane, 6 mL acetonitrile, and 6 mL of hexane before the samples were loaded. Once loaded, the cartridges were dried under vacuum and exhaustively eluted with dichloromethane and acetonitrile (1:1 v/v). The solvent mixture was switched to either 50% acetonitrile in water for LC–MS/MS analysis or 100% hexane for GC–MS/MS analysis. Total suspended solids (TSS) for each stream was determined by dividing the solids mass of the samples described above by the 10 mL wet volume.

### Water Extraction and Analysis.

Fipronil compounds were extracted from 500 mL aliquots of wastewater and wetland water (in duplicate for all streams except primary sludge) using automated, high-volume solid-phase extraction. Extraction was carried out using cartridges containing polystyrene divinylbenzene resin modified with pyrrolidone (500 mg/3 mL Strata X and Strata XL, Phenomenex, Torrance, CA) installed on an Autotrace 280 (Thermo Scientific Dionex, Sunnyvale, CA). Water samples were spiked with 20 ng $^{13}$C$_2$N$_2$–fipronil prior to extraction via SPE. The resin was eluted with 5% formic acid in methanol, and then aliquots of these extracts were reconstituted to either 50% methanol in water (for LC analysis) or 100% hexane (for GC analysis). Water samples with high TSS, such as waste-activated sludge (WAS) and primary sludge (PS) were centrifuged at 7500g, and 500 mL of the supernatants was decanted and extracted as described. Analyte mass on the solid fraction of those streams was determined as described in the previous section, and the weighted mass contribution of the solids was added to that of the water to determine the total mass of fipronil compounds in WAS and PS.

### Instruments and Analysis.

All analytes except fipronil—desulfinyl were separated by liquid chromatography and detected and quantified by negative electrospray ionization—tandem mass spectrometry (LC–ESI–MS/MS). Fipronil—desulfinyl displayed a significantly lower detection limit by gas chromatography electron impact—tandem mass spectrometry (GC–EI–MS/MS) and was therefore analyzed using a GC–MS/MS instead. Liquid chromatography mass spectrometric analyses were done using a Shimadzu Prominence HPLC (Shimadzu Scientific, Kyoto, Japan) controlled by Analyst 1.5 software (Applied Biosystems, Framingham, MA) coupled to an ABSciex API-4000 MS/MS (Applied Biosystems, Framingham, MA). Liquid chromatographic separation was achieved by an XBridge C$_8$-column (3.5 μm particle size, 4.6 × 150 mm; Waters Corporation, Milford, MA). The mobile phase consisted of 50% acetonitrile (ACN) and 50% water flowing at a rate of 1 mL/min with a total runtime of 10 min, and a gradient profile of 10% ACN/min to 95%. Analytes were introduced into the mass spectrometer using an electrospray ionization probe operating in negative mode, and multiple reaction monitoring (MRM) was used for qualitative analysis. Optimized conditions for the ionization and a fragmentation of the analytes are specified in the Supporting Information.
Quantitation of fipronil was done using isotope dilution and an eight-point calibration curve, with matrix spikes using $^{13}\text{C}_2^{15}\text{N}_2$—fipronil. Quantitation of other analytes was done using the standard addition method with four analysis sample spike levels. Gas chromatographic mass spectrometric analyses were performed on an Agilent 7890 GC coupled to an Agilent 7000 triple quad MS (Agilent Technologies, Santa Clara, CA) operating in positive mode, and MRM was used for qualitative analysis. More details on analytical instrument parameters and quality control, including limits of detection determination, can be found in the Supporting Information.

**Analytical Quality Control.** Method detection limits were determined by analyzing seven spiked surrogate matrix replicates and employing the USEPA’s recommended analysis for determination of limits of detection. Solid and water aliquots were spiked with authentic standards for fipronil and its major degradates prior to extraction. Spiking levels for each analyte were chosen within a ratio of 3:1 and 10:1 relative to background levels, and the detected concentrations were estimated using a six-point calibration curve. The standard deviation using six degrees of freedom was multiplied by the appropriate student’s $t$ value, providing an estimate of the lowest concentration detectable and identifiable with 99% confidence.

Because all samples of wastewater and archived sludge exhibited peaks reflective of the presence of fipronil, surrogate matrices void of fipronil compounds were obtained in the form of peat moss and peat moss slurry. This selection was made in accordance with USEPA method 1694, which recommends use of this surrogate matrix as a proxy for biosolids for quality assurance in the absence of an analyte-free reference matrix.

**Calculations.** Automatic samplers were programmed to take a number of 20 mL incremental samples within the first few minutes of a given hour. The total desired composite sample volume for 1 day was 2500 mL. The number of 20 mL increment samples taken in a given hour was calculated using eq 1.

$$N_{20\text{mL}}(t) = \frac{2500 \text{ mL}}{20 \text{ mL} \times 24} \times \frac{Q(t)}{Q} \tag{1}$$

where $N_{20\text{mL}}(t)$ is the number of 20 mL increments in the first few minutes of a given hour $t$, $Q(t)$ is the measured flow rate at hour $t$, and $Q$ is the average daily flow rate over the course of 21 days.

Mass loads for fipronil compounds in process streams were determined by multiplying determined concentrations with the flow rates for corresponding days. A combination of daily average flows (12 AM to 12 AM) and monitored hourly flows is reported (see Supporting Information).

Applying a steady-state assumption (accumulation = 0), we calculated the mass balance over the treatment train as shown in eq 2.

$$\sum_{i=1}^{n} Q_{\text{inf}}(t)C_{\text{inf}}(t)\Delta t - \sum_{i=1}^{n} Q_{\text{r}}(t)C_{\text{r}}(t)\Delta t - \sum_{i=1}^{n} Q_{\text{d}}(t)C_{\text{d}}(t)\Delta t + \sum_{i=1}^{n} Q_{\text{f}}(t)C_{\text{f}}(t)\Delta t = m_{\text{converted}}$$

$$\text{Waste activated sludge (effluent)}$$

$$\text{Reacted}$$

The bracketed terms (primary influent, etc.) represent the total mass load through each respective stream over a five day period, where $Q$ is flow rate (L/d), $C$ is concentration (ng/L), $t$ is time (day), $f$ is the mass fraction of solids in a stream ($g_{\text{solute}}/g_{\text{water}}$), and $m_{\text{converted}}$ is the mass not accounted for in all influent and effluent streams, assumed to be transformed (ng).

The notations ‘inf’ are for primary influent, ‘r’ for returned activated sludge, ‘d’ for dissolved wastewater, and ‘f’ for final effluent. The $\text{Hg}_x\text{aq}$ of the influent wastewater streams, assumed to be the same as the concentration in the plant effluent. Total analyte masses and concentrations were converted to fipronil equivalents by multiplying them by the relative molar mass of fipronil.

**Eq 5** was used to calculate the species-specific hazard quotient ($\text{Hg}_x$) of the influent and effluent wastewater streams, using methods established in literature. (Stark and Banks)

$$\text{Hg}_x = \sum_{i=1}^{3} \left( \frac{C_{\text{stream}}}{EC_{50x}} \right)$$

It should be noted that an effluent stream feeding a nearby power plant was not directly sampled, but because it was split off from the plant effluent, the concentration in that stream was assumed to be the same as the concentration in the plant effluent. Total analyte masses and concentrations were converted to fipronil equivalents by multiplying them by the relative molar mass of fipronil.

**Eq 6** was used to calculate the species-specific hazard quotient ($\text{Hg}_x$) of the influent and effluent wastewater streams, using methods established in literature. (Stark and Banks)
Toxicity indices were calculated for two arthropod species, *Hyalella azteca* and *Chironomus dilutus*, using the half-maximal effective concentrations (EC$_{50}$) for the various analytes. These species were chosen due to their relative sensitivities to fipronil; *H. azteca* is moderately sensitive, and *C. dilutus* is highly sensitive. The *C. dilutus* EC$_{50}$ values used in this calculation were 32.5 and 10 ng/L for fipronil and its degradates, respectively. The *H. azteca* EC$_{50}$ values used in this calculation were 0740, 161, and 0.5 ng/L for sulfone, and fipronil, respectively.

**RESULTS AND DISCUSSION**

**Method Performance.** Detection limits in surrogate wastewater ranged from 0.05 to 0.77 ng/L, while for surrogate biosolids, they ranged from 0.02 to 0.24 ng/g (dry weight). Relative recovery of fipronil was 116 ± 14% in water and 120 ± 13% in solids. Absolute recoveries of individual analytes (prior to normalization with the respective isotope-labeled surrogate standards) from water samples ranged from 60 ± 14% to 101 ± 195% (overall average recoveries for all analytes was 78 ± 20%), while absolute recoveries of individual analytes from solid samples ranged from 48 ± 18% to 90 ± 21% (overall average recoveries for all analytes was 73 ± 28%).

All water and solids samples were spiked with 20 ng of labeled fipronil prior to extraction, and final fipronil concentrations were quantified using the isotope-dilution method. Other analyte concentrations were assessed using standard addition with either three or four calibration points generated from sample extracts spiked just prior to instrument analysis. Method development indicated that nearly all losses were due to matrix effects, and standard addition and isotope dilution proved to mitigate the quantitative effects of these losses. All samples were quantified by background subtraction of method blank controls.

**Fipronil and Degradate Fate and Mass Balances across One Representative Conventional Treatment Train.** In the wastewater treatment train selected for extensive monitoring, fipronil was present in raw sewage at an average daily concentration of 17 to 31 ng/L and exited in disinfected treated effluent at levels of 13 to 21 ng/L. Fipronil sulfone was detected in all process streams at concentrations ranging from 0.5 to 40 ng/L. The sulfide, amide, and desulfinyl degradates were detected in most WWTP process streams at low levels: 0.1–3.8 ng/L.

A mass balance of total fipronil through the treatment train indicated that as a group, fipronil and its immediate degradates were conserved throughout. A 5 day mass load of total fipronil entering and exiting the treatment train yielded 77 ± 11 and 69 ± 6 mmol/5 days, respectively; mass loads in primary and secondary effluent were similar to those in the primary influent stream, suggesting conveyance of the contaminants through the treatment train (Figure 1). Overlapping error bars and a two-tailed t-test (95% confidence level) revealed that the mean daily influent and effluent masses of total fipronil compounds were statistically indistinguishable ($p = 0.29$, $n = 10$), implying that conventional wastewater treatment is ineffective at converting fipronil beyond the four immediate degradates studied herein (sulfone, sulfide, amide, and desulfinyl). Limited settling of fipronil compounds occurred in the primary and secondary clarifiers, despite their considerable high logarithmic n-octanol–water partitioning coefficients ($\log K_{OW} \approx 4.0–5.4$). While total fipronil compounds experienced no appreciable mass loss during passage through the treatment train, the parent compound fipronil was transformed at a rate of approximately 25% ($p = 0.00025$, $n = 10$), with about 1% being removed from water by the solids in waste activated and primary sludge. The decreased fipronil mass in the treatment train was accounted for in the total mass of the degradates, primarily fipronil sulfone (Figure 2). This result is in agreement with and refines prior estimates from a 2009 study, in which fipronil was found to be removed from a similar U.S. conventional wastewater-treatment plant at a rate of 18 ± 22%; the considerable analytical error in that study did not allow the unambiguous identification of differences between influent and effluent concentrations, and unlike in the current study, neither a detailed analysis of fipronil’s degradates nor the effectiveness of individual unit operations was undertaken.

**Mass Balance across All Parallel Treatment Trains Extant at the WWTP.** Approximately 58% of the flow and 48% of the total fipronil mass discharged by the wastewater facility was directed to an engineered wetland located immediately downstream, whereas 43% of total fipronil mass was distributed to a power plant, and 9% was sequestered in biosolids. The average daily mass loads of total fipronil in the WWTP inputs and outputs were 33.2 ± 5.6 mmol/day and 37.6 ± 7.3 mmol/day, respectively (see Figure 3, panel A). Similar to the individual treatment train, the daily mean input and output masses of the entire WWTP were not significantly
different \((n = 10, \ p = 0.14)\), indicating a complete lack of, or only insignificant removal of, total fipronil. The computed error in reported masses is cumulative, accounting for variability of calibration in flow meters used to measure flow rates, of recovery rates during extraction, of estimated solids retention time of anaerobic digesters, and of instrument response.

Relative abundance of fipronil-related compounds in input and output streams underwent little change. The input stream composition was approximately 75% fipronil, 1% fipronil sulfide, 21–22% fipronil sulfone, 0–4% fipronil amide, and 1–2% fipronil—desulfynil. However, the mass ratio of sulfone degrade to parental fipronil in waste-activated sludge was about 0.74, whereas in primary influent, the same ratio was much lower at about 0.3; this implies that fipronil sulfone was formed in either the aeration basins or in the secondary clarifiers. If the solids retention time in the clarifiers enabled the conversion of fipronil to fipronil sulfone, then this pattern should also be seen in the primary sludge, but it was not \((\text{sulfone/parent ratio} = 0.14)\). Considering that fipronil sulfone is an oxidative byproduct of fipronil, the evidence suggests that the sulfone degrade was formed during aerobic digestion. Aerobic transformation is the most likely mechanism for sulfone formation, given that the only unit operations in secondary treatment are aerobic digestors and secondary clarifiers; however, anaerobic microenvironments also may exist within this treatment unit. The primary sedimentation step did not appear to contribute to the formation of fipronil sulfone, and aerobic basins are more prone to facilitate enzymatic oxidation.

Fipronil sulfone did not appear to form at appreciable levels during chlorination, a result running counter to findings from a prior study examining the chemical oxidative removal of fipronil in water-treatment plants \(\text{(using permanganate, chlorine, etc.)}\); the latter study identified fipronil sulfone as the predominant byproduct of fipronil transformation by chlorine.32 However, the chlorination basin in the wastewater treatment train examined here had a contact time of less than a minute, with a dose of 2.5 mg/L, and a residual concentration of about 1 mg/L. Secondary-treated wastewater has a high chlorine demand, so it is not surprising that fipronil was not easily oxidized.

In biosolids, the proportions of the individual analytes were roughly as follows: 15% fipronil, 65% fipronil sulfide, 9% fipronil sulfone, 1% fipronil amide, and 9% fipronil—desulfynil. The dominant species in biosolids was the sulfide degrade, a result that is consistent with a study examining the degradation of fipronil in anaerobic sediment porewater.33 In all water streams, the primary photodegrade fipronil—desulfynil accounted for less than 3% of the total fipronil compound mass. In the wetland, fipronil—desulfynil accounts for less than 5% of the fipronil compound mass loss. This might seem surprising because fipronil has been shown to readily photodegrade, but USGS surface water screenings confirm that fipronil—desulfynil is considerably less abundant in environmental waters than the parent, the sulfone, or the sulfide transformation products.34

**Wetland Mass Balance.** The wetland downstream of the WWTP had a hydraulic retention time (HRT) of about 4.7 days, and so the mass load into the wetland on the first day of sampling should correspond with the mass load out of the
wetland 4 to 5 days later. A mass balance on the wetland (Figure 3B) using the first day’s influent mass load and the fifth day’s effluent mass load indicates that fipronil was reduced at a rate of 44 ± 4%, and total fipronil was attenuated in the wetland at a rate of 47 ± 13%. Over the five-day period, the average effluent concentrations of total fipronil were about 24% lower than the influent concentrations (n = 10, p = 2·10⁻⁶). The discrepancy between mass and concentration changes can be accounted for by evapotranspiration (the effluent flow rate is about 87% of the influent flow rate) and daily mass-load deviations over the five-day period not captured by the mass balance (the wetland mass balance only uses the first and fifth day mass loads to account for the wetland’s hydraulic retention time, while the average concentration over 5 days accounts for all 5 days of sampling, wherein concentration fluctuations occurred).

Total fipronil compound levels detected in the wetland ranged from 61–41 ng/L. These concentrations are much lower than concentrations detected in California urban waterways, where median total fipronil concentrations ranged from 204–440 ng/L and 90th percentile concentrations ranged from 340–1170 ng/L.²¹ Data sets from the United States Geological Survey in several states indicate that concentrations of fipronil-related compounds in urban and agricultural runoff are generally similar and typically less than 200 ng/L.³⁴ In Louisiana rice-field runoff, combined fipronil concentrations have been reported as high as 5290 ng/L. Losses of 24–44% of total fipronil mass during passage of water through the wetland suggests that further attenuation may take place in the effluent receiving stream; however, a deeper water column and higher turbidity may hinder the effectiveness of some loss mechanisms, such as photoysis.

Relative Toxicity. To assess whether the fipronil-related toxicity was affected by treatment, we calculated hazard quotients for process streams in the studied treatment train, including primary influent, disinfection basin effluent, wetland influent, and wetland effluent. For the moderately sensitive species, H. azteca, these values were 0.072 ± 0.014, 0.23 ± 0.14, 0.18 ± 0.07, and 0.22 ± 0.09, respectively. The mean HQ (H. azteca) of the primary influent stream was compared with the effluent from disinfection, the wetland influent, and the wetland effluent using a two-tailed t-test (n = 10) assuming equal variances; p values for these analyses were 0.002, 0.0001, and 0.00006, respectively. For the highly sensitive organism C. dilutus, the primary influent, disinfection basin effluent, wetland influent, and wetland effluent HQs were 1.4 ± 0.3, 1.3 ± 0.5, 1.3 ± 0.4, and 1.0 ± 0.3, respectively. Testing for statistical differences in the means of the HQs of disinfected effluent, wetland influent, and wetland effluent process streams relative to the primary influent stream yielded p values of 0.8, 0.5, and 0.007, respectively. Thus, conventional wastewater treatment did not significantly affect the overall toxicity posed by fipronil related compounds to C. dilutus, but the fipronil-related toxicity was increased toward H. azteca due to the formation of fipronil sulfide, to which H. azteca is highly sensitive. Passage of water through the wetland reduced the threat posed by fipronil related compounds for C. dilutus but not for H. azteca. These HQ values indicate that treated and untreated wastewater streams are probably toxic to highly sensitive organisms and potentially toxic to moderately sensitive organisms.

Study Implications and Future Research Needs. The wastewater treatment plant in this study discharges an estimated 7.9 g/day of phenylpyrazole pesticide mass (as fipronil) into the wetland, with 34–60% estimated to be attenuated there. To what extent fipronil and its degradates are taken up by plant and animal life is not well understood and likely varies by degradation and exposed species. Fipronil manufacturers recommend no more than 0.050 lb (23 g) of active ingredient to be applied annually per acre of land for varied uses such as mole cricket control. The water exiting the wetland discharges an estimated total fipronil load of 5.2 g/day. Biosolids produced by the treatment plant contribute a total fipronil-related compound load of 1.4 ± 0.7 g/day, mostly in the form of fipronil sulfide. Considering that the toxic load inherent to total fipronil compounds was left essentially unattenuated upon conventional wastewater treatment, the next best opportunity to control harmful exposures of aquatic biota and ecosystems is to limit use and loading of raw wastewater with the parent pesticide, fipronil. Although mechanisms of fiprole toxicity to ecosystems were not evaluated here, it has been demonstrated that fipronil can be taken up by angiosperms, transported through their xylem, and deposited on pollen and seedlings.³⁵–³⁷ Bees and other pollinating insects may be exposed to fipronil upon direct application via treated seeds and potentially upon application of biosolids on land used to grow flowering plants. Supportive of risks stemming from direct pesticide application, one survey in treated sunflower fields in France showed levels of total fipronil-related compounds in pollen as high as 8.3 ng/g.³⁸

It is unclear how wastewater contributes to fipronil loads detectable in angiosperm pollen, body burdens of aquatic organisms, or to toxicological effects in other nontarget organisms. Further research is needed to determine whether and to what extent fipronil related compounds contained in wastewater effluents can impact plants and nontarget organisms. Fipronil is among the most potent insecticides on the market, with a toxicity to honeybees over 6000 times greater than that of the banned pesticide DDT (27 000 versus 4.2 ng/bee).³⁹,⁴⁰ Acute lethal doses (LD₉₀) for numerous nontarget invertebrates also are in the ng range per individual organism.⁴,⁸,¹⁷,²³,³⁹ Some studies have shown that indirect exposure to certain insecticides may have adverse effects on vertebrate organisms, as well. A study in Madagascar indicated that insectivorous lizards and birds are exposed to fipronil compounds through the food chain, due to the fact that their diets consisted largely of the target organism (termites), and that they experienced sublethal effects.³¹ To fill information gaps, it would be necessary to evaluate the bioaccumulative and toxic effects of fipronil at various levels of the food chain. In addition, the plants in environments impacted by sources containing fipronil can be evaluated for uptake and xylem transport by extracting and analyzing pollen and leaves, as described by one study in France, wherein fipronil related residues were detected in 13% of randomly selected pollen-load samples in honeybee hives.³⁶

It is currently uncertain whether the levels released into the environment via wastewater effluent may cause accumulation of fipronil-related compounds in sediments and aquatic flora and elicit acute toxic effects in foragers or benthic organisms. The present study showed that fipronil and its immediate transformation products are remarkably resilient to degradation in wastewater treatment plants despite their passage through multiple unit operations that potentially could facilitate their removal (e.g., aerobic digestion, anaerobic digestion, chemical oxidation). Considering that the half-life of fipronil in water and
sediments is typically on the order of several days (more than 200 days in the case of the sulfone, sulfide, and desulfinyl degradates) and considering the paucity of knowledge about the ecological impacts of both direct and indirect discharge of fipronil into the environment, a more extensive longitudinal study of the transport of fipronil in surface waters and their fate in sediments, combined with biomonitoring studies, may help to illuminate potential associations between wildlife population changes and the presence of fipronil in the environment.

**ASSOCIATED CONTENT**

1. **Supporting Information**
   The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b04516.
   Additional details regarding the sampling campaign and statistical analyses. Figures showing diurnal flow patterns and daily fipronle concentrations by stream. (PDF)

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**Notes**
The authors declare no competing financial interest.

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**REFERENCES**

(1) Gunasekara, A. S.; Truong, T. Environmental fate of fipronil. Environmental Monitoring Branch; California Environmental Protection Agency: Sacramento, CA, 2007; http://cdpr.ca.gov/docs/emon/pubs/fatememo/fipronilev.pdf.

(2) Schlenk, D.; Huggett, D.; Allgood, J.; Bennett, E.; Rimoldi, J.; Beeler, A.; Block, D.; Holder, A.; Hovinga, R.; Bedient, P. Toxicity of fipronil and its degradation products to Procambarus sp.: Field and laboratory studies. Arch. Environ. Contam. Toxicol. 2001, 41 (3), 325–332.

(3) Bedient, P. B.; Horsak, R. D.; Schlenk, D.; Hovinga, R. M.; Pierson, J. D. Environmental impact of fipronil to the Louisiana crawfish industry. Environ. Forensics 2005, 6 (3), 289–299.

(4) Tingle, C. C.; Rother, J. A.; Dewhurst, C. F.; Lauer, S.; King, W. J. Fipronil: environmental fate, ecotoxicology, and human health concerns. In Reviews of Environmental Contamination and Toxicology; Springer: New York, 2003; pp 1–66.

(5) Vidau, C.; Diogon, M.; Aufauvre, J.; Fontbonne, R.; Vigués, B.; Brunet, J.-L.; Texier, C.; Biron, D. G.; Blot, N.; El Alaoui, H.; Belzunces, L. P.; Delbac, F. Exposure to sublethal doses of fipronil and thiacloprid highly increases mortality of honeybees previously infected by Nosema ceranae. PLoS One 2011, 6 (6), e21550.

(6) Quarles, W. Pesticides and honey bee colony collapse disorder. IPM Practitioner 2008, 30 (9), 1–10.

(7) Decourtey, A.; Lefort, S.; Devillers, J.; Gauthier, M.; Aupinel, P.; Tisseur, M. Sublethal effects of fipronil on the ability of honeybees (Apis mellifera L.) to orientate in a complex maze. Julius-Kühn-Archiv. 2010, 423, 75.

(8) Mayer, D.; Lunden, J. Field and laboratory tests of the effects of fipronil on adult female bees of Apis mellifera, Megachile rotundata and Nomia melanderi. J. of Apicult. Res. 1999, 38 (3–4), 191–197.

(9) Li, X.; Bao, C.; Yang, D.; Zheng, M.; Li, X.; Tao, S. Toxicities of fipronil enantiomers to the honeybee Apis mellifera L. and enantiomeric compositions of fipronil in honey plant flowers. Environ. Toxicol. Chem. 2010, 29 (1), 127–132.

(10) Decourtey, A.; Devillers, J.; Genequeue, E.; Menach, K.; L.; Badzinski, H.; Cizeau, S.; Pham-Delegue, M. H. Comparative Sublethal Toxicity of Nine Pesticides on Olfactory Learning Performances of the Honeybee Apis mellifera. Arch. Environ. Contam. Toxicol. 2005, 48 (2), 242–250.

(11) Southwick, E. E.; Southwick, L. Estimating the economic value of honey bees (Hymenoptera: Apideae) as agricultural pollinators in the United States. J. Econ. Entomol. 1992, 85 (3), 621–633.

(12) 1155th Public Announcement. Ministry of Agriculture of China, 2009.

(13) Fipronil: European ban on bee-killing insecticides goes on. Pesticide Action Network; http://www.env-health.org/news/members-news/article/pan-europe-fipronil-european-ban (accessed on December 13, 2015).

(14) Budd, R.; Ensminger, M.; Wang, D.; Goh, K. S. Monitoring Fipronil and Degradates in California Surface Waters, 2008–2013. J. Environ. Qual. 2015, 44, 1233.

(15) Ensminger, M. P.; Budd, R.; Kelley, K. C.; Goh, K. S. Pesticide occurrence and aquatic benchmark exceedances in urban surface waters and sediments in three urban areas of California, USA, 2008–2011. Environ. Monit. Assess. 2013, 185 (5), 5697–5710.

(16) Van der Sluijs, J.; Amaral-Rogers, V.; Belzunces, L.; van Lexmond, M. B.; Bonmatin, J.; Chagnon, M.; Downs, C.; Furfaro, L.; Gibbons, D.; Giorio, C. Conclusions of the Worldwide Integrated Assessment on the risks of neonicotinoids and fipronil to biodiversity and ecosystem functioning. Environ. Sci. Pollut. R. 2015, 22 (1), 148–154.

(17) Chandler, G. T.; Cary, T. L.; Bejarano, A. C.; Pender, J.; Ferry, J. L. Population consequences of fipronil and degradates to copepods at field concentrations: an integration of life cycle testing with Leslie matrix population modeling. Environ. Sci. Technol. 2004, 38 (23), 6407–6414.

(18) Everts, J. W.; Mbaye, D.; Barry, O.; Mullie, W. Environmental Side-Effects of Land and Watercourse Control; LOCUSTOX Project: GCP/SEN/041/NET; FAO: Dakar, Senegal, 1998, 1998.

(19) Peveling, R.; Domba, S. A. Toxicity and pathogenicity of Metarhizium anisopliae var. acridum (Deuteromycotina, Hyphomycetes) and fipronil to the fringe-toed lizard Acanthodactylus dumerili (Squamata: Lacertidae). Environ. Toxicol. Chem. 2003, 22 (7), 1437–1447.

(20) Hayasaka, D.; Korenaga, T.; Suzuki, K.; Saito, F.; Sánchez-Bayo, F.; Goka, K. Cumulative ecological impacts of two successive annual treatments of imidacloprid and fipronil on aquatic communities of paddy mesocosms. Ecotoxicol. Environ. Saf. 2012, 80, 355–362.

(21) Gan, J.; Bondarenko, S.; Oki, L.; Haver, D.; Li, J. Occurrence of fipronil and its biologically active derivatives in urban residential runoff. Environ. Sci. Technol. 2012, 46 (3), 1489–1495.

(22) Heidler, J.; Halden, R. U. Fate of organohalogens in US wastewater treatment plants and estimated chemical releases to soils nationwide from biosolids recycling. J. Environ. Monit. 2009, 11 (12), 2207–2215.

(23) Weston, D. P.; Lydy, M. J. Toxicity of the insecticide fipronil and its degradates to benthic macroinvertebrates of urban streams. Environ. Sci. Technol. 2014, 48 (2), 1290–1297.

(24) Sengupta, A.; Lyons, J. M.; Smith, D. J.; Drewes, J. E.; Snyder, S. A.; Heil, A.; Maruya, K. A. The occurrence and fate of chemicals of emerging concern in coastal urban rivers receiving discharge of treated municipal wastewater effluent. Environ. Toxicol. Chem. 2014, 33 (2), 350–358.

(25) Heidler, J.; Halden, R. U. Mass balance assessment of triclosan removal during conventional sewage treatment. Chemosphere 2007, 66 (2), 362–369.
(26) Heidler, J.; Sapkota, A.; Halden, R. U. Partitioning, persistence, and accumulation in digested sludge of the topical antiseptic triclocarban during wastewater treatment. Environ. Sci. Technol. 2006, 40 (11), 3634−3639.
(27) Meakins, N. C.; Bubb, J. M.; Lester, J. N. The behaviour of the s-triazine herbicides, atrazine and simazine, during primary and secondary biological waste water treatment. Chemosphere 1994, 28 (9), 1611−1622.
(28) Sinclair, E.; Kannan, K. Mass loading and fate of perfluoroalkyl surfactants in wastewater treatment plants. Environ. Sci. Technol. 2006, 40 (5), 1408−1414.
(29) Mayer, P. W.; DeOreo, W. B.; Opitz, E. M.; Kiefer, J. C.; Davis, W. Y.; Dziegielewski, B.; Nelson, J. O. Residential End Uses of Water. AWWA Research Foundation and American Water Works Association; Denver, CO; http://www.waterrf.org/publicreportlibrary/rfr90781_1999_241a.pdf (accessed on December 13, 2015).
(30) Tomlin, C. The Pesticide Manual: A World Compendium 11 ed.; British Crop Protection Council and the Royal Society of Chemistry: 1997.
(31) U.S. Environmental Protection Agency. Estimation Programs Interface Suite for Microsoft® Windows, v 4.11. United States Environmental Protection Agency: Washington, DC, 2014.
(32) Chamberlain, E. F.; Wang, C.; Shi, H.; Adams, C. D.; Ma, Y. Oxidative Removal and Kinetics of Fipronil in Various Oxidation Systems for Drinking Water Treatment. J. Agric. Food Chem. 2010, 58 (11), 6895−6899.
(33) Brennan, A. A.; Harwood, A. D.; You, J.; Landrum, P. F.; Lydy, M. J. Degradation of fipronil in anaerobic sediments and the effect on porewater concentrations. Chemosphere 2009, 77 (1), 22−8.
(34) United States Geological Survey. NAWQA Data Warehouse; http://waterqualitydata.us/portal/.
(35) Aajoud, A.; Raveton, M.; Aouadi, H.; Tissut, M.; Ravanel, P. Uptake and xylem transport of fipronil in sunflower. J. Agric. Food Chem. 2006, 54 (14), 5055−5060.
(36) Chauzat, M.-P.; Faucon, J.-P.; Martel, A.-C.; Lachaize, J.; Cougoule, N.; Aubert, M. A survey of pesticide residues in pollen loads collected by honey bees in France. J. Econ. Entomol. 2006, 99 (2), 253−262.
(37) Kadar, A.; Faucon, J.-P. Determination of traces of fipronil and its metabolites in pollen by liquid chromatography with electrospray ionization-tandem mass spectrometry. J. Agric. Food Chem. 2006, 54 (26), 9741−9746.
(38) Bonmatin, J. M.; Marchand, P. A.; Cotte, J. F.; Aajoud, A.; Casablanca, H.; Goutailler, G.; Courtiade, M.; Colin, M.; Belzunces, L. Systemic insecticides (imidacloprid and fipronil) reach pollen. A worrisome situation for bees. In International Conference on Bees: Agriculture and Biodiversity, Mamer, Luxembourg, November 16−17, 2007.
(39) Pisa, L.; Amaral-Rogers, V.; Belzunces, L.; Bonmatin, J.-M.; Downs, C.; Goulson, D.; Kreutzweiser, D. P.; Krupke, C.; Liess, M.; McField, M.; et al. Effects of neonicotinoids and fipronil on non-target invertebrates. Environ. Sci. Pollut R. 2014, 22 (1), 68−102.
(40) Bonmatin, J.-M.; Giorio, C.; Girolami, V.; Goulson, D.; Kreutzweiser, D.; Krupe, C.; Liess, M.; Long, E.; Marzaro, M.; Mitchell, E.; et al. Environmental fate and exposure: neonicotinoids and fipronil. Environ. Sci. Pollut. R. 2015, 22 (1), 35−67.
(41) Peveling, R.; McWilliam, A.; Nagel, P.; Rasolomananana, H.; Rakotomianina, L.; Ravoninjatoovo, A.; Dewhurst, C.; Gibson, G.; Rafanonezana, S.; Tingle, C.; Raholijaona. Impact of locust control on harvester termites and endemic vertebrate predators in Madagascar. J. Appl. Ecol. 2003, 40 (4), 729−741.
(42) Lin, K.; Haver, D.; Oki, L.; Gan, J. Persistence and sorption of fipronil degradates in urban stream sediments. Environ. Toxicol. Chem. 2009, 28 (7), 1462−1468.