Response of the Mayfly (Cloeon dipterum) to Chronic Exposure to Thiamethoxam in Outdoor Mesocosms

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Abstract: Thiamethoxam is a widely used neonicotinoid insecticide that has been detected in surface water monitoring programs in North America and Europe. This has led to questions about its toxicity to nontarget insects, specifically those with an aquatic life stage. To address the uncertainty associated with possible impacts from environmental exposures, a chronic (35-d) outdoor mesocosm study with a formulated product containing thiamethoxam was conducted. The specific focus of the study was the response of mayflies (Ephemeroptera), which have been reported to be particularly sensitive in laboratory studies. A range of concentrations (nominally 0.1, 0.3, 1.0, 3.0, and 10.0 μg/L thiamethoxam), plus untreated controls were tested, and the abundance and emergence of mayflies (Cloeon dipterum) were assessed weekly for 35 d. Mean measured time-weighted average exposures were within 6% of nominal over the duration of the study, with the mean half-life of thiamethoxam in each treatment ranging from 7 to 13 d. Statistically significant reductions in both larval abundance and adult emergence were observed at 10.0, 3.0, and 1.0 μg/L following 1, 2, and 3 wk of exposure, respectively. Exposure to 0.1 and 0.3 μg/L thiamethoxam had no statistically significant effect on larval mayfly abundance or adult emergence at any point in the study. These findings support a 35-d no-observed-effect concentration (NOEC) of 0.3 μg thiamethoxam/L for mayflies (C. dipterum) under chronic conditions. Furthermore, because the 95th percentile of environmental concentrations has been reported to be 0.054 μg/L, these results indicate that populations of C. dipterum and similarly sensitive aquatic insects are unlikely to be significantly impacted by thiamethoxam exposure in natural systems represented by the conditions in our study. Environ Toxicol Chem 2018;37:1040–1050. © 2017 The Authors. Environmental Toxicology and Chemistry published by Wiley Periodicals, Inc. on behalf of SETAC.

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INTRODUCTION

Largely replacing organophosphate, carbamate, and organochlorine pesticides, neonicotinoid insecticides are used in agriculture worldwide for the broad-spectrum control of insect pests [1]. They are selective-action pesticides that act on insects’ nicotine acetylcholine receptors [2]. The first neonicotinoid insecticide, imidacloprid, reached agricultural markets in 1991, with 6 others coming into use in the following decade, including thiamethoxam (3-[(2-chloro-5-thiazolyl)methyl]tetrahydro-5-methyl-N-nitro-4H-1,3,5-oxadiazin-4-imine) in 1998 [2,3]. As of 2008, neonicotinoid insecticides were registered in more than 120 countries [4], with the most common uses including seed treatments and foliar and soil applications.

With the increased use of neonicotinoid insecticides, detections in environmental media samples have become more common. Morrissey et al. [5] conducted a global review, identifying 29 studies that reported on 63 neonicotinoid insecticide monitoring events. Eleven of these studies reported thiamethoxam detections in...
streams, rivers, wetlands, groundwater, ponds, and irrigation leachate between 2005 and 2013. Although one measurement as high as 225 \( \mu \text{g/L} \) was reported for a Texas (USA) playa wetland [6], 75% of grassland and 69% of crop playa water bodies contained no detectable residues. Recently, Finnegan et al. [7] summarized peer-reviewed, gray, and government literature for reported thiamethoxam concentrations in surface waters from North America and estimated the 90th, 95th, and 99th percentiles of those values to be 32, 54, and 400 \( \mu \text{g/L} \), respectively.

A recent review [7] of the acute toxicity of thiamethoxam to a range of aquatic organisms indicated that aquatic primary producers and fish were relatively insensitive to thiamethoxam (acute median lethal concentration [LC50] and median effect concentration [EC50] values exceeding 80 \( \mu \text{g/mL} \), as were annelids, molluscs, and rotifers. Generally, insects were the most sensitive group, but the crustaceans Asellus aquaticus and Ostracoda were also sensitive (acute EC50 < 1 \( \mu \text{g/mL} \)). Other studies reported in the peer-reviewed literature have assessed acute toxicity of thiamethoxam to crayfish (Procambarus clarkii) [8], 2 chironomids (Chironomus dilutus and tepperi) [9,10], a mosquito (Aedes aegypti) [11], a mayfly (Cloeon dipterum) [12], an amphipod (Gammarus kischineffensis) [13], and a snail and a mussel (Planorbera pilsbryi and Lampsilis fasciola) [14]. The LC50 values (24- or 48-h) exceeded 100 \( \mu \text{g/L} \) for all these organisms except C. dipterum, for which the 96-h EC50 (immobility) was 20 \( \mu \text{g/L} \) [12].

Although the focus on acute toxicity of thiamethoxam to aquatic invertebrates has been warranted owing to its rapid degradation through photolysis (Lu et al. [15] reported a half-life in water of 0.2–1.5 d), exposure modeling indicates the potential for protracted exposure to low concentrations in some scenarios. Morrissey et al. [5] suggest that although initial dissipation of neonicotinoid insecticides is rapid, there is evidence that longer term exposure may occur under certain circumstances, particularly conditions of low temperature and low pH. To date, only 3 studies with aquatic invertebrates have reported chronic-duration assessments for thiamethoxam. These consist of several 7- to 28-d tests with a chironomid (C. dilutes) [10], a mayfly (C. dipterum) [12], and a snail (P. pilsbryi) [14]. Of these, mayflies showed the greatest sensitivity to thiamethoxam exposure, with a reported 28-d EC50 of 0.68 \( \mu \text{g/L} \) for C. dipterum [12].

Considering the possibility of chronic-duration exposures, and the apparent sensitivity of mayflies [12], the potential for longer term effects of thiamethoxam on these insects under conditions more closely resembling edge-of-field surface waters, warrants further investigation, particularly at the population and community levels.

Although Robinson et al. [16] assessed impacts of thiamethoxam on larval wood frogs (Lithobates sylvaticus) in 300-L outdoor mesocosms, no publications have reported results of any micro- or mesocosm studies of thiamethoxam toxicity to potentially sensitive invertebrates [17]. To improve our understanding of toxicity of thiamethoxam to nontarget aquatic invertebrates, and to increase the information available for higher tier risk assessment and regulatory decision-making, we present the results of an outdoor aquatic mesocosm study that assessed the effect of continuous and chronic exposure to thiamethoxam on a population of the mayfly C. dipterum.

**MATERIALS AND METHODS**

**Mesocosm construction and preparation**

The present study was conducted following Good Laboratory Practice in a series of stainless steel enclosures (mesocosms) in a large artificial pond (comprising a concrete base and a circular wall of steel lined with glass-enamel) located at the MESOCOSM facility in Homberg/Ohm, Germany (see Supplemental Data, Figure S1, for photograph). The pond, with a diameter of approximately 9.39 m and a volume of approximately 76,000 L, was prepared in July 2014 by first filling with a clay layer (sourced on site from natural deposit) of approximately 10 cm to facilitate the sampling of robust sediment cores. A sediment layer approximately 10 cm deep was then added on top of the clay. The sediment (the upper 20-cm horizon) was collected from approximately 0.5 m below the water surface from a small lake on the test facility property with no known history of chemical contamination (see next paragraph for details regarding characterization). Washed, sieved sand (Lahn Sand sourced from a sand pit near the test facility) was also added to the sediment layer to lower the total organic carbon content of the sediment to 1 to 3%. Source water for the pond was then added from an on-site lake with no known history of chemical contamination. The pond was not used for any other study after preparation, and was allowed to equilibrate for approximately 1 yr.

Non-Good Laboratory Practice water and sediment characterization was conducted before the start of the study. The water was analyzed for metals (Mn, Pb, Cd, Cr, Cu, Ni, Hg, Zn), polychlorinated biphenyls (PCBs; 6 components), pesticides (27 components), and selected chemical parameters (Na, K, S, Cl). The sediment was analyzed for PCBs (6 components) and pesticides (27 components). All analyzed metals, PCBs, and plant protection products were below their respective limits of quantification (LOQs). The total organic carbon of the initial sediment was 1.6% of dry weight as determined by standard methods [18].

On 11 May 2015, 29 stainless steel enclosures were pressed through the sediment into the clay layer, such that the sediment and water in each enclosure was isolated. Each enclosure had a diameter of approximately 143 cm (surface area $\approx 1.60 \text{m}^2$), a height of approximately 150 cm, and a water depth of approximately 100 cm (volume $\approx 1600 \text{L}$). The study was conducted in 20 of these enclosures. The mesocosms contained a diverse assemblage of naturally growing macrophytes, algae, and invertebrates (zooplankton, macroinvertebrates), which originated from the sediment and water used to fill the mesocosm, and in the case of insects, from aerial colonization. These communities were assessed prior to study initiation to ensure uniformity and sufficient food resources for the mayflies (see Biological sampling section).
Prior to study initiation, a series of non-Good Laboratory Practice preliminary tests was conducted to characterize the dissipation of thiamethoxam within the test system using a randomly selected set of 5 non-test enclosures. It was observed that dissipation of the test compound in water occurred more rapidly with increasing pH values. Mesocosm pH values are mainly driven by photosynthetic activity of algae and macrophytes, and reached values >10 in summer, which has been observed elsewhere in similar systems [19,20]. To facilitate the maintenance of exposure concentrations close to nominal throughout the course of the 35-d study, a shade tent with open sides was installed above the pond on 3 June 2015, to reduce photosynthesis and avoid significant increases in pH. However, because of inclement weather, the tent was removed on 12 June, then reinstalled on 30 June, and finally removed again on 7 July 2015 for the same reasons.

Experimental design and thiamethoxam treatment

Mesocosms were treated with the formulated product thiamethoxam WG (formulation code A9584C; Syngenta; 25.0% thiamethoxam w/w, batch 731279), without the need for any organic solvent carrier. Test treatments comprised negative (water) controls (5 replicate enclosures) and 5 concentrations of test material (0.4, 1.2, 4.0, 12, and 40 μg A9584C/L) with 3 replicate enclosures/test concentration. These treatments corresponded to active ingredient concentrations of 0.1, 0.3, 1.0, 3.0, and 10 μg thiamethoxam/L. The lower concentrations of 0.1 and 0.3 μg/L are in the range of the maximum concentrations detected in surface waters, and the higher concentrations are in the range shown to have impacts on aquatic insects in chronic laboratory studies (e.g., Chironomus riparius 30-d emergence lowest-observed-effect concentration [LOEC] 20 μg/L [7]). No direct effects were anticipated on zooplankton or primary producers because of their lack of sensitivity to thiamethoxam [7]. The 6 treatments were assigned randomly to 20 of the enclosures in the pond, leaving 9 unused.

The application solutions were prepared at the test site just prior to dosing, by dilution of a stock solution of 100 mg thiamethoxam/L in tap water, prepared on each treatment day. Based on the results of 2 non-Good Laboratory Practice dissipation pretests, thiamethoxam was applied 9 times over the course of the study to maintain constant exposure levels close to nominal.

For the initial application on day 0 (9 June 2015), 120% of the nominal concentration was added to each mesocosm in an attempt to obtain time-weighted average concentrations until the next dosing event equivalent to nominal. Subsequently, thiamethoxam was added twice weekly (on Tuesday and Friday), based on concentrations measured on 2 sampling days before and extrapolated to the day of the application. Dissipation of thiamethoxam increases with pH; therefore, subsequent dosing levels also considered the actual pH values of the enclosures at the time of application. The dosing events are summarized in the Supplemental Data, Table S1. Between 0.3 and 193 mL of stock solution were applied to each enclosure during each dosing event.

Dosing was performed following the “toxicological approach” recommended by the Organisation for Economic Co-operation and Development (OECD) [21]. The thiamethoxam solutions (including the rinse water) were introduced directly into the water column by means of a specially adapted separating funnel whereby the glass tip was extended to 30 cm. A different funnel was assigned to each mesocosm. The solutions were distributed by moving the glass tip of the opened funnel in a circular pattern approximately 15 to 25 cm below the water surface while rinsing the application solutions. After application, the separating funnel was rinsed 3 times with nonchlorinated tap water, with all rinsates being released into the enclosure as described above. Tap water was applied to the control mesocosms in the same way as the thiamethoxam solutions.

Sampling and measurements

Water quality parameters. Temperature, dissolved oxygen (DO), pH, and conductivity were measured using Wissenschaftlich-Technische Werkstätten probes at approximately 0.5 m below the water surface at least once a week (as summarized in the Supplemental Data, Table S1). Measurements were taken at approximately the same time of the day in ascending order from the control to the greatest concentration of thiamethoxam, and probes were washed after use to prevent contamination. Ammonium, nitrate, phosphate, and water hardness were measured in sieved (mesh size, 60–70 μm) depth-integrated water samples taken on days −1 and 30. Separate depth-integrated samplers were used for each treatment group to avoid contamination (minimum 80-cm water column and ~ 40 mm inner diameter). Sampling was conducted with a depth-integrated sampling device (minimum 80-cm water column and ~ 40-mm inner diameter). Samples were taken from at least 3 locations/mesocosm, and combined to generate a single composite sample/mesocosm/sampling event. The nutrient parameters and hardness were determined photometrically using Wissenschaftlich-Technische Werkstätten cube tests (14739, 14556, P6/25 analogue ISO 6878, and 00961).

Measurement of thiamethoxam and its metabolite clothianidin in water samples. Water samples were taken just before each dosing event (except for the initial treatment on day 0), plus 3 h, 2, 3, and/or 4 d after each application (Supplemental Data, Table S1) to measure the concentration of thiamethoxam in the water column, to calculate the doses needed to maintain the target concentrations, and to check for the presence of clothianidin, an insecticidal metabolite of thiamethoxam. After the first dosing event, and subsequently once a week, the control mesocosms were sampled and analyzed to check for contamination. On the other sampling dates, only the treated mesocosms were sampled.

Sampling was conducted as described in Sampling and measurements section for water quality measures. At least 2 water subsamples (one for analysis and one spare) of 10 mL were taken from the composite sample and transferred to glass vessels (e.g., 40-mL EPA vial), to which 10 μL of acetic acid...
(100%, ACS grade, Merck) was added to reduce the pH to minimize degradation of thiamethoxam and clothianidin. The vessels were frozen and stored below –10 °C until analysis at the Fraunhofer IME (Schnallenberg, Germany). Clothianidin is a soil and plant metabolite that has comparable insecticidal activity to the parent compound thiamethoxam [22]. Therefore, water samples were also analyzed for this metabolite to ensure that any observed effects on mayflies could be attributed solely to thiamethoxam.

Both thiamethoxam and clothianidin were analyzed by liquid chromatography (LC) coupled to a triple quadrupole mass spectrometer (MS) using electrospray ionization (ESI); Waters, Acquity H-Class UPLC and Xevo TQ-S). The MS was operated in tandem mass spectrometry mode (MS/MS) and using internal standards delivered by Sigma-Aldrich (Thiamethoxam-D₃ and Clothianidin-D₃ with 99.1% purity each). Validation of the analytical method was performed according to the guidance document SANCO/3029/99 rev. 4 (11/07/00) [23]. The LOQ was 0.05 µg/L for both compounds in mesocosm water. Time-weighted average concentrations were calculated as described in OECD test guideline 211 for the Daphnia reproduction test [24].

Treatment means were generated from the 3 values (one/replicate enclosure) for each sampling point, and time-series plots were generated for the 5 nominal concentration levels (Figure 1). Attenuation rate constants and half-lives of thiamethoxam were estimated according to pseudo–first-order kinetics from the 5 enclosures used in the preliminary tests and the full study (as in Lu et al. [15]). The raw exposure data can be found in the Supplemental Data, Table S2.

**Biological sampling**

**Validation of the ecological integrity of the test system.**

Macroinvertebrates other than mayflies, zooplankton, phytoplankton, and periphyton were assessed twice each before the exposure period to characterize the enclosures. Assessments were conducted on days –11 and –1 for macroinvertebrates, days –13 and –4 for zooplankton, days –11 and –1 for phytoplankton, and days –12 and –4 for periphyton. Macrophytes were assessed once before exposure, on day –2. Periphyton and macrophyte coverage were also assessed once during the exposure period (on day 29) because of their relevance to mayflies as food source and habitat, respectively. Diversity indices (number of taxa, Shannon index, and evenness) were used to describe the zooplankton, macroinvertebrate, and periphyton communities, and were calculated based on pooled data from the 2 pre-exposure sampling events, as well as the day 29 event. Indigenous macrophytes were visually observed and mapped prior to treatment and on day 29. The following species were found: Ceratophyllum demersum, Potamogeton natans, and Zannichellia palustris. The alga Chara globularis was also identified and considered within the functional group of macrophytes.

**Mayflies**

Mayflies (C. dipterum) were sampled using several different approaches to characterize both early life stages as well as the successful emergence of the fully mature adult form. To determine the abundance of mayfly larvae in each experimental unit, 2 baskets (15 × 15 × 9 cm) containing stones and the shoots of the macrophyte C. demersus as suitable substrate were installed at depths of 20 and 60 cm beneath the water surface in each enclosure and remained in situ between samplings to allow recolonization. On each (weekly) sampling event, for each enclosure, both baskets were transferred to a single white plastic tray and flushed with pond water, and mayfly larvae were counted. Sweep netting (21 × 21-cm aperture, 25-cm length, 450-µm mesh size) was also conducted, with 3 net pulls/sampling event/enclosure, except for the last sampling, when 5 net pulls were completed because the number of larvae in the enclosures had generally decreased. For each sweep, the net was dropped to the sediment along the wall of the enclosure and then taken up to the water surface. Orientations of the pulls were the same in all enclosures. After each sweep, the net was rinsed through with water from the same enclosure into a white dish, and the total number of larvae from the combined sweeps was counted, before they were returned to the respective enclosure. Care was taken to ensure delicate handling of captured larval mayflies during enumeration. It is possible that handling of the larval mayflies resulted in some attrition; however, this factor would have been captured by observations within the control treatment. Overall, this was considered to have less impact than taking the sampled larvae out of the system without replacement. The counts from substrate baskets and sweep netting were added and expressed as the number of mayfly larvae/enclosure/sampling day.

Emergence of mayflies was measured with 2 emergence traps/enclosure, one situated over open water and one over macrophytes. The emergence traps were stainless steel constructions with a trapping device at the apex of the structure containing tap water with a small amount of surfactant to aid in

**FIGURE 1:** The measured thiamethoxam concentrations at each treatment level over the 35-d study. Each point is the mean of 3 enclosures (n = 3). No thiamethoxam was detected (limit of quantification [LOQ] of 0.05 µg/L) in the controls (n = 5). The metabolite clothianidin was not detected in any of the samples (all samples < LOQ of 0.05 µg/L).
Data analysis

Water quality measures were analyzed in SigmaStat 3.5 (Systat Software) for differences before and after addition of thiamethoxam. Nitrate, ammonium, phosphate, and hardness were analyzed by Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks \((p < 0.05)\) on days –1 and 30. The mean DO, pH, conductivity, and temperatures were determined at each sampling event for each treatment replicate before and after addition of thiamethoxam and analyzed by one-way ANOVA followed by a Dunnett’s test \((p < 0.05)\).

Abundance data for each taxonomic group of periphyton (cells/cm²), phytoplankton (µg chl a/L), macrophytes (area coverage), zooplankton (no./L), and macroinvertebrates (no./sample) were transformed using natural logarithm \((\ln)\) and analyzed by 2-sided Dunnett tests to assess differences between treated and control mesocosms on each sampling date.

Numbers of larval and emerged mayflies were used to calculate no-observed-effect concentration (NOEC) and LOEC for abundance data \([25]\). Minimum detectable differences were calculated as described in Brock et al. \([26]\), and expressed as a percentage of the control mean abundance. These data analyses were conducted using Ver 4.3.14 of Community Analysis \([27]\). The total number of emerged mayflies at the end of the 35-d study was analyzed for a NOEC and LOEC by means of one-sided Williams tests \((p = 0.05)\), along with the minimum detectable difference.

## RESULTS

### Water quality, periphyton, macrophytes, and zooplankton

For water quality parameters, there were no statistical differences among treatment levels before or after addition of thiamethoxam to test enclosures (Table 1). Dissolved oxygen was above guideline values \((e.g., >9.5 \text{ mg/L}[28])\) and nitrate \((e.g., <3.0 \text{ mg/L NO₃} [29])\), and ammonia \((e.g., <1.0 \text{ mg/L NH₃} [30])\) values were below levels of concern for aquatic life. The pH values ranged between 7.71 and 9.87 over the course of the study.

The outcomes of the statistical analysis for biological measures other than mayflies can be found in the Supplemental Data, Table S3. In all, 366 ANOVA comparisons were conducted.

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**TABLE 1:** Mean standard water quality measures taken from the mesocosms

| Treatment (µg/L) | Temperature (°C) | Dissolved oxygen (mg/L) | Conductivity (µS/cm) | pH | Ammonium (mg/L) | Nitrate (mg/L) | Hardness (g/L) | Phosphate (mg/L) | Calcium (mg/L) | Chlorophyll a (µg/mL) |
|------------------|------------------|------------------------|---------------------|----|----------------|---------------|---------------|-----------------|-----------------|---------------------|
| 0.1              | 14.9 (0.5)       | 11.20 (1.66)           | 11.48 (1.75)        | 9.12 (0.25)    | 9.15 (0.25)    | 9.32 (0.27)   | 9.29 (0.19)   | 9.57 (0.25)    | 9.15 (0.25)    | 224 (4)            |
| 0.3              | 14.8 (0.5)       | 11.20 (1.66)           | 11.48 (1.75)        | 9.12 (0.25)    | 9.15 (0.25)    | 9.32 (0.27)   | 9.29 (0.19)   | 9.57 (0.25)    | 9.15 (0.25)    | 224 (4)            |
| 1                | 14.8 (0.5)       | 11.20 (1.66)           | 11.48 (1.75)        | 9.12 (0.25)    | 9.15 (0.25)    | 9.32 (0.27)   | 9.29 (0.19)   | 9.57 (0.25)    | 9.15 (0.25)    | 224 (4)            |
| 3                | 15.0 (0.5)       | 11.20 (1.66)           | 11.48 (1.75)        | 9.12 (0.25)    | 9.15 (0.25)    | 9.32 (0.27)   | 9.29 (0.19)   | 9.57 (0.25)    | 9.15 (0.25)    | 224 (4)            |
| 10               | 14.9 (0.5)       | 11.20 (1.66)           | 11.48 (1.75)        | 9.12 (0.25)    | 9.15 (0.25)    | 9.32 (0.27)   | 9.29 (0.19)   | 9.57 (0.25)    | 9.15 (0.25)    | 224 (4)            |

The "pre-" values are the data prior to the introduction of thiamethoxam and the "post-" values are those after exposure. The \(n\)-value represents the number of times a measure was taken, with the standard deviation in brackets. There were no statistical differences at each measurement event from control mesocosms \((p > 0.05)\). The means were compared using Kruskal–Wallis one-way analysis of variance. **The moisture was calculated as described in Brock et al. \([26]\), and expressed as a percentage of the control mean abundance.**
There were no statistically significant differences in macrophyte cover between treated and control mesocosms before or during the study (days –2 and 29). Concentrations of chlorophyll a in phytoplankton groups (total, blue-green, green, Chromophytes, Cryptophytes) were measured prior to study initiation (days –11 and –1) and were not statistically different. For abundance of periphyton, 9 of 62 taxonomic groups had a treatment that was statistically different from control on day –12. No differences were found on day –4 (58 species), and 3 groups had a treatment that was different from controls on day 29 (out of 53 species). However, the total abundance of periphyton was not significantly different at any point (days –12, –4, or 29). Macroinvertebrate total abundance was not significantly different on days –11 or –1. The abundance of 2 (out of 51) and 4 (out of 54) macroinvertebrate taxonomic groups was statistically different in at least one treatment on days –11 and –1, respectively. The abundance of 4 (out of 33) and 1 (out of 35) zooplankton taxonomic groups was statistically different from control mesocosms in at least one treatment on days –13 and –4, respectively. Zooplankton total abundance was not significantly different on either sampling date. Overall, although several significant differences were detected in individual taxonomic groups of periphyton, macroinvertebrates, and zooplankton, there was no consistency among species, time points, or direction of change. Of the 366 contrasts performed, 94% were not significant, which is very close to the 5% expected just by chance (Supplemental Data, Table S3). Thus, the conditions in the mesocosms prior to dosing were deemed sufficiently similar for testing the effects of thiamethoxam on *C. dipterum*.

### Thiamethoxam exposure and fate

Dosing procedures were considered successful at maintaining concentrations of thiamethoxam within an acceptable range of target nominal values (Figure 1). Minimum and maximum concentrations of thiamethoxam in the analyzed samples ranged from 45 to 135% of nominal, respectively, with less than 11% of samples measured below 80% of target (Supplemental Data, Table S2). The time-weighted mean measured concentration for all enclosures was 101% of nominal, ranging from 93 to 108% for the 15 individual thiamethoxam-treated enclosures, and from 98 to 106% when averaged across a treatment (n = 3; Table 1). Calculated mean thiamethoxam half-lives ranged from 6.9 to 12.9 d (Table 2) during the full study. The calculated mean half-lives from the 5 test ponds used in preliminary tests were 2.72, 3.08, 3.58, and 29.21 (n=3) and 5.5 (n=1). No thiamethoxam was detected in the controls (<LOQ of 0.05 μg/L). The metabolite clothianidin was not detected in any of the samples (<LOQ of 0.05 μg/L).

### Cloeon dipterum responses

Because time-weighted mean measured values of thiamethoxam for each treatment were within 6% of target, NOECs and LOECs are reported as nominal concentrations. Responses of larval abundance over the course of the study are shown in Figure 2. At day 6, mean larval abundance in the 10.0 μg/L enclosures was reduced to less than 50% of control abundance, whereas no significant effects were found in the other treatments. On all subsequent sampling dates, no larvae were found in the 10.0 μg/L enclosures. By day 13, mean larval abundance decreased significantly in the 3.0 μg/L treatment to 35% of the control value, but no effect was found at lower test concentrations by this time point. At days 20 and 27, abundance was significantly reduced compared with controls at concentrations ≥1.0 μg/L (i.e., 71 and 64% for 1 μg/L, respectively, and 100% reduction at 3.0 and 10 μg/L for both dates), whereas abundance at 0.1 and 0.3 μg/L was still similar to controls (NOEC = 0.3 μg/L). Over the first 4 wk of the exposure period, the minimum detectable difference was always below 50%. Thus, according to the categorization of minimum detectable difference proposed by the European Food Safety Authority (EFSA) [31], it was possible to detect small effects (<50%). On day 34, average larval abundance had also decreased in the controls to values below 5 individuals/sampling, which resulted in an increase of the minimum detectable difference to 87%. The mean larval abundance at 3.0 μg thiamethoxam/L was 68% of the controls (i.e., the difference was less than the minimum detectable difference), and therefore the NOEC at this final

![Figure 2](image)

**Figure 2:** The geometric mean of numbers of mayfly (Cloeon dipterum) larvae from outdoor mesocosms treated with thiamethoxam at 0.1, 0.3, 1.0, 3.0, and 10.0 μg/L (n = 3), plus controls (n = 5) over the course of 35 d. The no-observed-effect concentration (NOEC) at each sampling point is provided at the top of the figure. The NOEC was determined by a one-sided Williams test (α = 0.05).

| Treatment (μg/L) | Mean measured time-weighted average (μg/L) | Mean measured time-weighted average (%) | Mean half-life (d) with standard deviation (n = 8) |
|-----------------|-------------------------------------------|----------------------------------------|-----------------------------------------------|
| Control         | Not detected                              | Not detected                           | Not detected                                  |
| 0.1             | 0.10                                      | 100                                    | 8.9 (4.2)                                     |
| 0.3             | 0.31                                      | 102                                    | 9.4 (3.5)                                     |
| 1               | 1.06                                      | 106                                    | 12.9 (4.0)                                    |
| 3               | 3.03                                      | 101                                    | 9.1 (3.3)                                     |
| 10              | 9.76                                      | 98                                     | 6.9 (3.1)                                     |
sampling point increased to 3.0 μg thiamethoxam/L. Visual observations at the end of the study indicated that multiple larval stages were present in the test systems. Calculated NOECs and LOECs for larval abundance and adult emergence at each sampling point are shown in Table 3 (raw data are in the Supplemental Data, Tables S4 and S5, respectively). The overall NOEC for abundance of larval *C. dipterum* was 0.3 μg/L. Emergence of *C. dipterum* was not affected over the 5 wk of exposure at 0.1 and 0.3 μg/L, whereas at 10.0, 3.0, and 1.0 μg/L, emergence dropped to zero in weeks 2, 3, and 4, respectively (Figure 3, and see the raw data in Supplemental Data, Table S5). On day 7, the minimum detectable difference was slightly above 50%, whereas later it was always <50%. On days 21 and 28, the minimum detectable difference was <30%. Thus, the emergence data also allowed detection of “small effects” [31]. As with larval abundance, the overall NOEC for emergence of *C. dipterum* was determined to be 0.3 μg thiamethoxam/L.

Emergence of *C. dipterum* was not affected over the 5 wk of exposure at 0.1 and 0.3 μg/L, whereas at 10.0, 3.0, and 1.0 μg/L, emergence dropped to zero in weeks 2, 3, and 4, respectively (Figure 3, and see the raw data in Supplemental Data, Table S5). On day 7, the minimum detectable difference was slightly above 50%, whereas later it was always <50%. On days 21 and 28, the minimum detectable difference was <30%. Thus, the emergence data also allowed detection of “small effects” [31]. As with larval abundance, the overall NOEC for emergence of *C. dipterum* was determined to be 0.3 μg/L. Calculated NOECs and LOECs for adult emergence at each sampling point are provided in Table 3 (raw data are in the Supplemental Data, Table S5).

The total number of emerged mayflies over the 35 d of the study can be seen in Figure 4. The 35-d NOEC and LOEC values for adult emergence were 0.3 and 1.0 μg/L, respectively. The minimum detectable difference was calculated to be 43%.

**DISCUSSION**

The present study characterized the response of the mayfly *C. dipterum* to chronic (35-d) exposure to thiamethoxam in an outdoor mesocosm. Pre- and post-application biological sampling across a range of taxa relevant to edge-of-field surface waters verified the ecological integrity of the test system and its suitability for investigating effects of thiamethoxam on mayflies. The test system was adequately colonized by *C. dipterum*, as representative of Ephemeroptera, at the start of the study. This is indicated by sampling on average just under 30 larvae/enclosure on the day before application, resulting in a minimum detectable difference of 45%, which allowed detection of small effects (according to EFSA [31]). Both larval abundance and adult emergence responded to the presence of thiamethoxam, with a clear and consistent concentration–response relationship, allowing determination of a NOEC population of 0.3 μg thiamethoxam/L.

### TABLE 3: Calculated no-observed-effect concentrations (NOECs) and lowest-observed-effect concentrations (LOECs) for *Cloeon dipterum* larvae and emergence over the course of a 35-d chronic thiamethoxam exposure mesocosm study

| Duration of exposure (d) | MDD % for larvae | Larvae NOEC (μg/L; % of control) | Larvae LOEC (μg/L; % of control) | Duration of exposure (d) | MDD % for emergence | Emergence NOEC (μg/L; % of control) | Emergence LOEC (μg/L; % of control) |
|-------------------------|-----------------|----------------------------------|----------------------------------|-------------------------|---------------------|------------------------------------|------------------------------------|
| 6                       | 45              | 3.0 (89)                         | 10.0 (42)                        | 7                       | 53                  | 10.0 (118)                         | >10.0 (118)                        |
| 13                      | 47              | 1.0 (104)                        | 3.0 (34)                         | 14                      | 42                  | 1.0 (97)                          | 3.0 (19)                           |
| 20                      | 37              | 0.3 (73)                         | 1.0 (29)                         | 21                      | 26                  | 0.3 (139)                         | 1.0 (15)                           |
| 27                      | 44              | 0.3 (137)                        | 1.0 (37)                         | 28                      | 27                  | 0.3 (77)                          | 1.0 (0)                            |
| 34                      | 87              | 3.0 (14)                         | 10.0 (0)                         | 35                      | 45                  | 0.3 (148)                         | 1.0 (0)                            |

*The percentage of minimum detectable difference (MDD %) for each reported NOEC and LOEC is also provided.*

**FIGURE 3:** The geometric mean of numbers of emerged adult mayfly (*Cloeon dipterum*) from outdoor mesocosms treated with thiamethoxam at 0.1, 0.3, 1.0, 3.0, and 10.0 μg/L (*n* = 3), plus controls (*n* = 5) over the course of 35 d. The no-observed-effect concentration (NOEC) at each sampling point is provided at the top of the figure. The NOEC was determined by a one-sided Williams test (*α* = 0.05).

**FIGURE 4:** The arithmetic mean number for total emerged adult mayfly (*Cloeon dipterum*) and from outdoor mesocosms over 35 d of exposure to thiamethoxam at 0.1, 0.3, 1.0, 3.0, and 10.0 μg/L (*n* = 3), plus controls (*n* = 5). The asterisks represent a statistically different treatment from control by one-sided Williams test (*α* = 0.05).
Concentrations of thiamethoxam in the treated enclosures were adjusted frequently throughout the course of the study (typically every 3–4 d), to maintain steady concentrations of thiamethoxam, with an overall mean of 101% of nominal. The regular additions of thiamethoxam made the calculation of a half-life more difficult, but it was still possible to estimate the stability of thiamethoxam in the test system: on average, the treatment half-lives ranged from approximately 7 to 13 d. In the preliminary tests with 5 enclosures, the mean responses were more consistent, with degradation time (DT)50 values of approximately 3 d, with the exception of one enclosure that had an average DT50 of 29 d (n = 3). Previous work on the fate of thiamethoxam in mesocosms has reported its half-life to be approximately 1 d, with photolysis being the primary mechanism of degradation; no hydrolysis was observed [15]. The thiamethoxam in this test system was more stable, likely because of a number of additional physical factors that reduced incident light. Lu et al. [15] used experimental systems that were approximately 30 cm in depth, and concluded that the majority of photolysis in their systems appeared to be occurring in the top 8 cm of water. By contrast, our test systems were 100 cm in depth, and incident light was further reduced by shading, both from an upper lip approximately 40 cm above the water surface, and from the addition of a shade tent. Although this is an interesting contribution to the literature, the DT50s reported in the present study should be interpreted with caution, as the calculation of this value was not a part of the study design, and the factors that can drive thiamethoxam degradation (e.g., light intensity) were not controlled or measured.

In a previous mesocosm study, Robinson et al. [16] reported measureable concentrations of the thiamethoxam metabolite clothianidin in 2 out of 3 of their exposure studies, although levels were <1% relative to measured concentration of thiamethoxam over >55 d of exposure with weekly additions to maintain thiamethoxam concentrations. This observation may have been because of contamination, as a simultaneous imidacloprid study had measurable thiamethoxam and clothianidin in the treatment vessels, as well as controls (Table 1) [16]. Although its presence has also been reported in insects, plants, soil, and some animals [22], clothianidin is regarded as only a minor metabolite of thiamethoxam in water and water/sediment systems, resulting from photolysis (≤3% formation). To ensure there was no unintentional clothianidin exposure and any effects could be solely attributed to thiamethoxam, water samples from our test system were analyzed for clothianidin throughout the study, but it was not detected in any of the 420 samples (LOQ = 0.05 μg/L). These observations are further supported by earlier work on this matter [32], as well as a mesocosm study by Lu et al. [15], which reported 2 main photoproducts from the photolysis of thiamethoxam, neither of which was clothianidin. Finally, in acute laboratory mixture toxicity studies with thiamethoxam and other neonicotinoid insecticides as well chronic studies of neonicotinoid insecticide toxicity, no detection of clothianidin was reported in test exposures [10,33].

Previous laboratory acute toxicity testing has shown C. dipterum to be relatively sensitive to thiamethoxam. Van den Brink et al. [12] observed that mayflies are among the most sensitive taxa to thiamethoxam and reported a 96-h EC50 (immobility) for C. dipterum of 20 μg/L. Finnegan et al. [7] reported similar results, with a 48-h EC50 (immobility) of 14 μg/L for a Cloeon sp. and 48-h EC50 (immobility) values ranging from 21 to 44 μg/L for C. dipterum. Finnegan et al. [7] also reported a 48-h LC50 of 53 μg/L for C. dipterum larvae. In our mesocosm study, after 7 d of exposure, the NOEC for emergence was ≥10.0 μg/L. At 6 d, the NOEC for larval abundance was 3.0 μg/L and the LOEC was 10.0 μg/L, which corresponded to a 68% decline from control. The short-term response (i.e., ≤7 d) of C. dipterum in our mesocosm study was therefore consistent with available acute toxicity data for this species. Finally, Finnegan et al. [7] calculated hazard concentrations (HC5s) based on insect acute laboratory toxicity data including C. dipterum. The HC5 based on 48-h EC50s for insects was estimated to be 0.6 μg/L, which would have been protective of these mayflies under the present test conditions with an acute exposure (i.e., ≤7 d). Overall, this provides some confidence in using laboratory data in setting water quality guidelines that will be protective of sensitive species.

Under the chronic exposure conditions of this outdoor study, the NOECPopulation for C. dipterum was determined to be 0.3 μg/L, based on larval abundance and adult emergence results from individual sampling days. When total emerged adults over the full course of the study are considered (Figure 4), the NOEC was also 0.3 μg/L, indicating that a chronic NOECPopulation of 0.3 μg/L is robust. By comparison, Van den Brink et al. [12] reported a 28-d EC50 of 0.68 μg/L for C. dipterum (winter generation) exposed to thiamethoxam in the laboratory. To our knowledge, there are no other field or mesocosm studies assessing chronic exposure of C. dipterum to thiamethoxam.

In laboratory tests with imidacloprid, Van den Brink et al. [12] observed a 5-fold greater sensitivity of their summer generation test organisms compared with their winter generation (EC50 = 0.13 and 0.68 μg/L, respectively) when exposed for 28 d. Although they did not perform a seasonal comparison with thiamethoxam, the winter generation laboratory EC50 value for thiamethoxam reported by Van den Brink et al. [12] lies well within the range of responses observed in our study with summer generation Cloeon. To some extent the absence of a significant difference in these winter/summer values may reflect the different complexity and realism of the test systems (laboratory compared with mesocosm), although our verification of test concentrations indicates that exposure was maintained throughout the study. In any case, if summer generation mayflies are more sensitive to neonicotinoids, as reported by Van den Brink et al. [12] for imidacloprid, the no-effect concentrations determined in our summer generation study should be protective of exposed winter populations. Moreover, as noted above, measured environmental concentrations of thiamethoxam are typically in the low ng/L range, with 95% of concentrations being <0.054 μg/L [7]. In Canadian Prairie wetlands, Main et al. [34] reported a 10% detection rate with measured median concentrations of 30 ng/L from water bodies surrounded by canola, wheat, oats, and other crops. Struger et al. [35] monitored 15 creeks from agricultural areas (mainly row crops such as corn and soybean, plus greenhouse activities) in
Ontario, Canada for a variety of neonicotinoids and reported a maximum concentration of 1.34 μg thiamethoxam/L, with creek means ranging from <0.00139 to 0.172 μg thiamethoxam/L (n = 440 across all creeks sampled). Even if these concentrations were sustained for significant periods, our mesocosm study provides evidence that C. dipterum are able to develop and successfully emerge at these levels of thiamethoxam exposure.

Other insect species, including midges (Chironomidae), have been examined for their response to thiamethoxam. Cavallaro et al. [10] reported the 14-d LC50 and the 40-d EC50 (emergence) for C. dilutus exposed to thiamethoxam as 23.60 and 4.13 μg/L, respectively, whereas Finnegan et al. [7] reported a 48-h LC50 of 260 μg/L for C. riparius, indicating that both acute and chronic responses of Chironomids are less sensitive than Cloeon. The greater sensitivity of mayflies relative to chironomids has also been observed with imidacloprid, with a 96-h EC50 of 0.65 μg/L for later instars of the mayfly Epeorus longimanus [36], and a 14-d LC50 of 1.65 μg/L for C. dilutus [10]. Roessink et al. [37] also found C. dipterum to be the most sensitive of 7 invertebrate species (2 macrocrustaceans and 5 insects) to imidacloprid in their evaluation of chronic toxicity, with a 28-d EC50 (immobility) and 28-d LC50 of 0.123 and 0.195 μg/L, respectively. Ephemeroptera (e.g., Baetidae) have also been observed to be more sensitive to insecticides (e.g. imidacloprid, deltamethrin) compared with other aquatic insect species in mesocosm studies [38,39]. As mayflies are known to be particularly susceptible to the effects of neonicotinoids [5], data from the present mesocosm study, notwithstanding its limitations, will be an important contribution to environmental risk assessments of thiamethoxam. Considering the available acute and chronic data from laboratory and mesocosm studies, it would seem likely that a chronic community NOEC for aquatic insects is not less than 0.3 μg/L for thiamethoxam.

Initially, the present study was planned to assess C. dipterum response to thiamethoxam over an 8-wk exposure period. However, the gradual reduction in larval mayfly abundance, in controls as well as treated enclosures, meant the eventual loss of appropriate statistical power. The minimum detectable differences were <50% over the first 4 wk of the study, meaning that small effects on abundance could be detected [31], and robust NOECs had been determined by days 27 and 28, for larval abundance and adult emergence, respectively. As a consequence, and given that larval abundance of mayflies was unlikely to rebound until late summer/autumn because of natural population cycling, the study was terminated on day 35. Water quality parameters in our test system were within acceptable ranges and were consistent across treatments and throughout the study, in respect to ambient conditions (e.g., temperature). Environmental conditions within the test system are therefore unlikely to explain the reduction in mayfly abundance in controls over the course of the study (June–July), which was not entirely unexpected. Several historical studies of the life history characteristics of a multivoltine Ephemeroptera report absence of larvae from samples taken in June, July, and August [40,41]. In their study of the effects of imidacloprid on insects in a stream mesocosm, Mohr et al. [42] observed a significant decline in numbers of Ephemeroptera, stocked in late May, starting 50 to 60 d later (i.e., from late July onward). Echols et al. [43], assessing the suitability of 2 mayfly species for whole effluent testing, also reported reduced availability of 2 bivoltine native North American mayfly species, in June and July.

Although significant declines in emergence and abundance of C. dipterum were observed because of thiamethoxam treatment, the ability of the species to recover should be considered when one is contextualizing the impacts of thiamethoxam on aquatic ecosystems, and on Baetidae specifically. Mayflies in this family are multivoltine, with the potential to produce 2 or more generations/year, and therefore recover relatively rapidly following significant impacts. For example, Caquet et al. [39] observed full recovery of baetids 84 d after the near total loss of all individuals following exposure to the pyrethroid insecticide deltamethrin. Similarly, Van den Brink et al. [44] reported full recovery for C. dipterum at 24 wk post exposure to the insecticide chlorpyrifos (Dursban) following the near complete removal of the species from their experimental systems. In both studies, recovery dynamics were driven in large part by the length of the life cycle, with those organisms having multiple breeding events recovering more rapidly. Given the observed half-lives for thiamethoxam in the present study and elsewhere (typically within several days), it is likely that concentrations causing an effect (1.0, 3.0, and 10.0 μg/L under chronic exposures, >20 d) would quickly decline to values below effects thresholds reported in the present study, allowing rapid and successful recolonization of bio- and multivoltine species like C. dipterum. Univoltine species, such as Caenoids, should they be sensitive at these concentrations under field conditions, might take longer to recover in the event of significant impacts to a population. Although other exposure scenarios (e.g., pulsed) may occur, we have assessed the response of a species with known sensitivity to thiamethoxam under a worst-case scenario. Based on these results, it is reasonable to predict that C. dipterum should be able to recolonize once concentrations of thiamethoxam drop to or below 0.3 μg/L, although the potential for surface waters to exceed this value is low (e.g., only 1.2% of the North American surface water samples had detections >0.3 μg/L [7]).

In conclusion, the response of a population of C. dipterum, a representative species of Ephemeroptera, to chronic thiamethoxam exposure was assessed in an outdoor mesocosm study. Continuous exposure to thiamethoxam at concentrations up to and including 0.3 μg/L for 35 d had no effects on larval abundance or adult emergence of C. dipterum. Although the present study focused on survival and overall abundance of the species, considered typical protection goals in a pesticide regulatory framework, it should be noted that our study did not assess mass or size at emergence. Considering the relative sensitivity of mayflies to neonicotinoids, as well as measured concentrations of thiamethoxam in North American surface waters [7,34,35], these data indicate that direct impacts of thiamethoxam on these and similarly sensitive aquatic invertebrates are unlikely to occur under the field conditions represented by the present study.
Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4028.

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Data availability—We provide core raw data in the Supplemental Data as tables. Other ancillary data are available on request (meaghean.finnegan@syngenta.com).

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