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Environmental Toxicology

Making the Invisible Visible? Using Stable Isotope Analysis to Detect Indirect Toxicant Effects

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Abstract: Although stable isotope analysis (SIA) is widely used to address ecological research questions, its application in an ecotoxicological context has been limited. Recent studies have proposed an effect of chemical stressors on an organism's isotope signature, questioning the use of SIA in food webs impacted by toxicants. Against this background, the present study investigates 1) whether trophic enrichment factors (TEFs; i.e., the offset in stable isotope signatures of a consumer to its diet) are altered by the neonicotinoid thiacloprid, and 2) whether tracking toxicant effects on an organism's diet composition (i.e., indirect effect) with SIA fits direct observations of consumption. To address the former, the amphipod *Gammarus fossarum* (Koch) was exposed to three levels (0, 0.75, and 5 µg L^{-1}) of thiacloprid and fed with either black alder leaves or *Baetis rhodani* (Pictet) larvae over 6 weeks ($n = 35$). The thiacloprid-induced changes in TEFs that we found were statistically significant but small compared with other factors (e.g., resource quality, consumer, and physiological condition) and thus likely of minor importance. To address the latter issue, gammarids were exposed to two levels of thiacloprid (0 and 0.75 µg L^{-1}) and fed with either black alder leaves, live *B. rhodani* larvae, or both over 2 weeks ($n = 10$). Dietary proportions as suggested by SIA were indeed in agreement with those derived from direct observation of consumption. The present study consequently suggests that SIA is as a robust tool to detect indirect toxicant effects especially if TEFs are assessed in parallel. *Environ Toxicol Chem* 2023;42:1937–1945. © 2022 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Indirect effects; mixing models; stable isotope analysis; thiacloprid; trophic enrichment factor

INTRODUCTION

Chemical pollution is recognized as a major threat to the ecological integrity of aquatic and terrestrial ecosystems (Millennium Ecosystem Assessment, 2005), although only a fraction of species might be directly affected, due to differing sensitivities (Newman et al., 2000). However, co-existing species interact in various ways (e.g., via predation or competition), and direct toxic effects in one population can result in indirect effects in other (more tolerant) populations. Such effects may be immediate or propagated via intermediary populations (Preston, 2002) and can be of the same or even higher biological significance as direct chemical effects (Fleeger et al., 2003). However, indirect effects have received far less attention than direct effects (Köhler & Triebskorn, 2013). Moreover, most methods available to detect indirect effects are either not capable of tracking species interactions in complex communities (e.g., video tracking; Gómez et al., 1997; Preston et al., 1999) or do not allow for an unambiguous distinction between direct and indirect effects (e.g., monitoring abundances; Hatakeyama & Yasuno, 1987; Sarma et al., 1998).

Surprisingly, although stable isotope analysis (SIA) has been used for decades to tackle stress-related ecological questions (among other issues; Ehleringer et al., 1986; Martínez del Rio et al., 2009), it is still little used in ecotoxicology (cf. Jardine et al., 2006). By disentangling the food web structure (cf. Vander Zanden & Rasmussen, 2001; Vander Zanden et al., 1997), for instance, SIA has a promising potential to identify indirect implications of chemical stressors in complex communities. These opportunities arise from the phenomenon that

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the stable isotope signature of consumers reflects that of their food source(s), usually with just a little offset, the so-called trophic enrichment factor (TEF, Minagawa & Wada, 1984; Post, 2002). This is because biomolecules with chemical bonds involving lighter isotopes are usually metabolized faster, resulting in the enrichment of the heavier isotope in the consumer (i.e., discrimination; Peterson & Fry, 1987). However, this, in turn, means that any stressor affecting metabolism, as is the case for many chemical contaminants (Stenersen, 2004), could potentially affect stable isotope signatures, as has been indicated by recent studies (Ek et al., 2015; Staaden et al., 2010). Hence, the use of SIA for tracing indirect effects of chemical stressors (and other related uses such as trophic level estimation for bioaccumulation assessments, for example [Broman et al., 1992; Eulaers et al., 2014; Watanabe et al., 2008]) might be impeded.

The goal of the present study was 1) to assess the implications of a toxicant in the TEF for an omnivorous model consumer (i.e., the amphipod *Gammarus fossarum* Koch), and 2) to test whether SIA is capable of detecting an indirect toxicant effect in a multispecies experiment involving two alternative food sources. In a first experiment, *G. fossarum* was exposed to the neonicotinoid insecticide thiacloprid for 6 weeks at either 0, 0.75, or 5 $\mu g\ L^{-1}$, while being fed with either mayfly (*Baetis rhodani* Pictet) larvae or leaves of black alder (*Alnus glutinosa* (L.) Gaertn.). Two food sources were used because food quality might modulate the effects of toxicants, a factor important to consider (Hansen et al., 2008; Ieromina et al., 2014). In our second experiment, which was run for 14 days, *G. fossarum*, as well as its potential food sources (i.e., *B. rhodani* larvae, leaves, or both), were exposed to the lower thiacloprid concentrations (0 and 0.75 $\mu g\ L^{-1}$) to test whether the expected indirect effect was to be detected through SIA. The test item and concentrations were selected based on published data: At 0.75 $\mu g\ L^{-1}$, gammarids' increased consumption of animal prey is anticipated when they are offered *B. rhodani* larvae and leaves, which is likely related to a reduced predator avoidance behavior of the sensitive insect prey (i.e., an indirect effect; Englert et al., 2012). At 5 $\mu g\ L^{-1}$, direct toxic effects on *G. fossarum* are expected. Against this background, we anticipated direct toxicity to result in only minor implications in TEFs (cf. Ek et al., 2015) and expected stable isotope signatures to reflect the expected indirect effect of thiacloprid (i.e., a higher share of *B. rhodani* larvae in the diet of *Gammarus*; Englert et al., 2012).

MATERIALS AND METHODS

Model toxicant

The model toxicant thiacloprid was applied as a commercially available formulation (Calypso® 480 SC; 480 g thiacloprid L^{-1}; Bayer CropScience), which rendered the use of further solvents unnecessary. The formulation was serially diluted in amphipod medium (SAM-5S; Borgmann, 1996) to receive the respective nominal concentrations of 0.75 or 5 $\mu g\ L^{-1}$ (all concentrations refer to active ingredient). To verify exposure at these nominal concentrations, at the start of the experiments and during the medium exchanges, triplicate 10-mL samples were taken from the insecticide-free controls and the thiacloprid treatments. Samples were stored at −20 °C until analysis via an ultra-high-performance liquid chromatography system coupled to a mass spectrometer (EQuan MAX; Thermo Scientific) as described in detail by Englert et al. (2012; trap column: Hypersil Gold aQ, 20 × 2.1 mm, particle size 12 mm; analytical column: Hypersil Gold C18, 50 × 2.1 mm, particle size 1.9 mm; Thermo Scientific; for further information, see the Supporting Information, Table S1). The analyses revealed adequate thiacloprid dosing during the experiments because measured concentrations ranged between 86% and 123% of the nominal concentrations and were below the limit of detection (<0.05 $\mu g\ L^{-1}$) in controls (Supporting Information, Table S2).

Preparation of leaf discs

Leaf discs were prepared as described by Zubrod et al. (2010). Shortly before leaf fall, black alder leaves from trees near Landau, Germany (49°11′N, 8°05′E) were collected and stored at −20 °C until use. Discs of 2.0-cm diameter were cut from the leaves with a cork borer, excluding the main vein. Leaf discs were subjected to microbial colonization (i.e., conditioning) for 10 days in a nutrient medium (Dang et al., 2005) using leaf material previously exposed in a near-natural stream (Rodenbach, Germany, 49°33′N, 8°02′E) as inoculum. After conditioning, leaf discs were dried to a constant weight (for ~24 h at 60 °C) and weighed to the nearest 0.01 mg. Approximately 48 h prior to the start of each experiment, leaf discs were resoaked in test medium (SAM-5S; Borgmann, 1996) to prevent floating during the experiments. Several sets of leaf discs (see the following section, *Test organisms*) were then dried again (for ~24 h at 60 °C) for SIA.

Test organisms

Gammarus fossarum were kick-sampled in a near-natural stream upstream of any settlement, wastewater treatment plant effluent, or agricultural land use close to Landau, Germany (49°14′N, 8°03′E; cryptic lineage B; Feckler et al., 2014), 7 days prior to each experiment. Gammarids were immediately divided into size classes (Franke, 1977), and only adult males—identified by their position in precopula pairs—of approximately 6–8 mm body length being visually free of acanthocephalan parasites were used to reduce variability in feeding behavior during the experiments (Naylor et al., 1989; Pascoe et al., 1995). Throughout the acclimation phase in the laboratory, animals were kept in the aerated medium in a climate-controlled chamber at 16 ± 1 °C in total darkness, and they were fed ad libitum with preconditioned black alder leaves and gradually adapted to SAM-5S. To receive weights as well as stable isotope signatures at the start of the experiments, 10 gammarids were placed individually in cylindrical stainless-steel cages (Zubrod et al., 2010) to prevent coprophagy. The cages were situated in aerated SAM-5S for 24 h, and no food was provided, to purge the animals' guts. This step is required because undigested food in the gut can affect stable isotope signatures (Feuchtmayr &

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Grey, 2003). Afterward, gammarids were dried at 60 °C (for ~24 h) and weighed to the nearest 0.01 mg.

The mayfly (*B. rhodani*) larvae were obtained from a restored stretch of the Eußerbach stream near Landau, Germany (49°15′N, 7°57′E). Animals were collected 24 h prior to their use in the experiments. In the laboratory, mayfly larvae were size-separated (selecting only animals of 7–10 mm length). Afterward, animals were kept in aerated water from the sampling site at 16 ± 1 °C in total darkness, and algae-covered stones from the same site provided food. At the start and the medium exchanges of each experiment (i.e., when fresh food was also provided), several sets of three larvae each were subjected to gut purging and drying as just described for gammarids.

Experiment 1: Effects of thiacloprid on TEFs

For the first experiment, we used the test set-up described by Zubrod et al. (2010) in combination with a full-factorial design, exposing gammarids to either 0, 0.75, or 5 µg thiacloprid L^{-1}, while offering them three *B. rhodani* larvae or two leaf discs ($d = 2.0$ cm) as food (Figure 1A). Each of the six resulting treatments (three levels for thiacloprid exposure × two levels for the provided food) was replicated 35 times. In each replicate, one *G. fossarum* was placed together with the respective food in a 250-mL glass beaker containing 200 mL of SAM-5S with the respective thiacloprid concentration. Moreover, for each thiacloprid concentration (0, 0.75, and 5 µg L^{-1}), five replicates without animals were set up, accounting for abiotic and microbial leaf mass losses. Test vessels were placed under constant aeration in a climate-controlled chamber at 16 ± 1 °C in total darkness and were covered with plastic lids to prevent evaporation of the test medium and the loss of emerging mayflies. After 7 days, the test medium (including the respective thiacloprid concentration), and food (leaf discs or *B. rhodani*), were renewed to ensure adequate water and food quality. At the same time, leaf consumption and predation on *B. rhodani* were quantified. Mayfly larvae were considered consumed if less than 50% of their bodies remained. Consumed mayflies were replaced on a daily basis to ensure ad libitum feeding conditions. Gammarids were also checked for mortality daily, being considered dead if no response was observable after several gentle touches with the tip of a glass pipette. To quantify leaf consumption, remaining leaf discs and any leaf tissue shredded off were removed from the test vessels, dried at 60 °C (for ~24 h), and weighed to the nearest 0.01 mg. After every second week, up to 10 gammarids/treatment (depending on survival) were gut-purged as described previously in *Test organisms* and used for SIA (see the *SIA* section following). After 6 weeks, the experiment was terminated, and all remaining gammarids were sampled.

Experiment 2: Suitability of SIA to track indirect effects

For this experiment, we used the set-up of Englert et al. (2012) in combination with a full-factorial design, exposing three gammarids to either 0 or 0.75 µg L^{-1}, while offering them seven leaf discs, nine *B. rhodani* larvae, or both as food (Figure 1B). Each of the six resulting treatments (two levels for thiacloprid exposure × three levels for the provided food) was replicated 10 times. In each replicate, three *G. fossarum* were placed together with either seven resoaked and preweighed leaf discs and/or nine *B. rhodani* larvae in a 900-mL crystallizing dish containing 500 mL SAM-5S with the respective thiacloprid concentration. Moreover, for each thiacloprid concentration (0 and 0.75 µg L^{-1}), five replicates without animals were set up accounting for abiotic and microbial leaf mass losses. Test vessels were placed under constant aeration in a climate-controlled chamber at 16 ± 1 °C in total darkness and were covered with plastic wrap to prevent evaporation of the test medium and the loss of emerging mayflies. The numbers of consumed *B. rhodani*, as well as alive and dead gammarids,

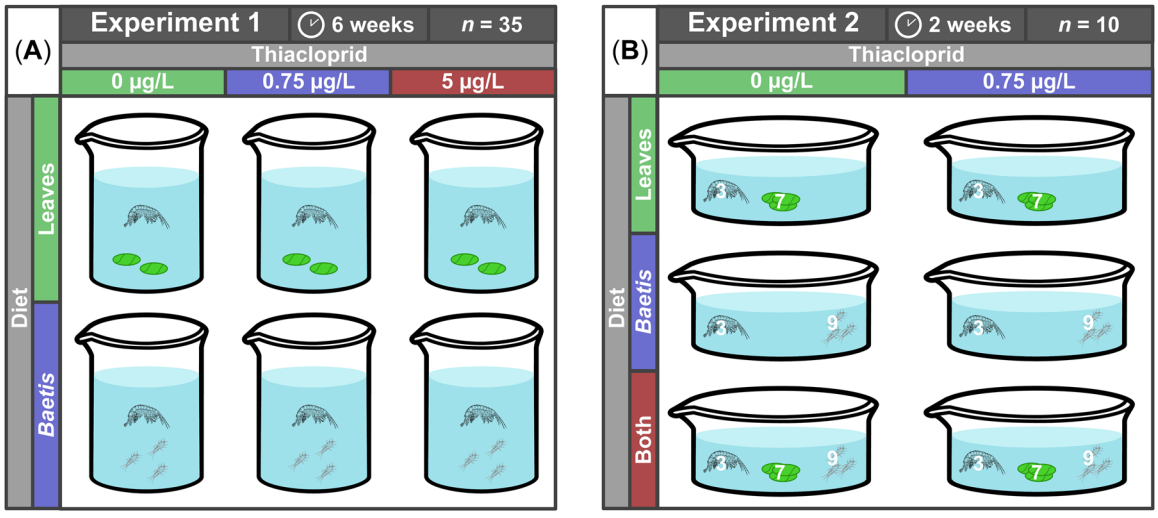

FIGURE 1: Experimental designs of experiment 1 (**A**) and experiment 2 (**B**). Experiment 1 features two levels of food sources and three levels of thiacloprid and was conducted over a duration of 6 weeks in 250-mL beakers ($n = 35$). Experiment 2 features three levels of food sources and two levels of thiacloprid and was conducted over a duration of 2 weeks in 900-mL crystallizing dishes ($n = 10$).

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were recorded daily. Replicates with dead or missing gammarids were removed from all further analyses because cannibalism might have occurred (MacNeil et al., 1997), which could interfere with the assessed endpoints. After 7 days, the test medium (including the respective thiacloprid concentration), as well as food, was exchanged to ensure adequate water and food quality. At the termination of the experiment (after 14 days), gammarids' guts were purged as described in *Test organisms* before animals were dried at 60 °C (for ~24 h) and weighed replicate-wise (i.e., in sets of three), as also described in *Test organisms*.

Stable isotope analyses

For SIA, leaf discs (sets of two and seven for the first and second experiments, respectively), *B. rhodani* larvae (sets of three for both experiments), and gammarids (single organisms and sets of three for the first and second experiments, respectively) were ground to a fine powder. Afterward, approximately 0.5 and 1.0 mg of animal and plant material was weighed (to the nearest 0.0001 mg) into tin cups (5 × 9 mm; IVA). The stable isotope signatures and elemental contents for nitrogen (N) and carbon (C) were determined using a Delta V Advantage isotope ratio mass spectrometer coupled to a Flash HT Elemental Analyzer (Thermo Scientific). An internal reference material (casein) was measured in duplicate every 10 samples, revealing an average precision (±1 SD) of 0.05‰ and 0.03‰ for N and C, respectively. The stable isotope signatures were expressed using the delta notation (δ; in per mil) relative to the respective international standards (atmospheric air for N and Vienna Pee Dee Belemnite for C).

Calculations and statistics

Leaf consumption rates (ξ, in mg d^{-1}) in both experiments were calculated as

$$\xi = \frac{m_{start} - m_{end}}{t} \times f \quad (1)$$

where m_{start} and m_{end} are the weights of provided leaf discs at the start and the end of the term t, respectively, and f is a factor correcting for mass losses due to abiotic and microbial leaf mass losses. For both experiments, effects on leaf consumption and consumed *B. rhodani* larvae between treatments were tested via two-factorial models with post hoc testing via Tukey-adjusted least-square contrasts using the R package "emmeans" (Ver 1.5.2-1; SAS Institute 2012) after visually checking normality and homoscedasticity. Trophic enrichment factors ($\Delta^{15}N$, $\Delta^{13}C$) were calculated as

$$\Delta^{15}N = \delta^{15}N_{Consumer} - \delta^{15}N_{Diet} \quad (2)$$

$$\Delta^{13}C = \delta^{13}C_{Consumer} - \delta^{13}C_{Diet} \quad (3)$$

and effects on TEFs were tested via a three-factorial (i.e., time, diet, and thiacloprid concentration) multivariate analysis of variance (MANOVA). Normality and homoscedasticity were tested prior to MANOVA via (multivariate) Shapiro–Wilk test and visually, respectively.

For experiment 2, the proportion of leaves and mayfly larvae in gammarids' diet was estimated with Bayesian mixing models for each treatment with a generalist prior to using the R package "MixSIAR" (chainLength = 100 000, burn = 50 000, thin = 50, chains = 3; Ver 3.1.12; Stock et al., 2018). To account for trophic enrichment, studies often refer to the literature instead of determining the TEFs experimentally. Therefore, we compared model outcomes using commonly used literature values (i.e., 0.5 ± 0.19‰ for $\delta^{13}C$ and 2.3 ± 0.24‰ for $\delta^{15}N$; McCutchan et al., 2003), as well as average TEFs using all replicates of both Experiments 1 and 2. To evaluate the consensus of SIA and direct consumption, the maximum a posteriori probability estimate (generated from mixing models) of the contribution of mayflies to the diet of *G. fossarum* was then compared with the proportional consumption of mayflies (f_{Baetis}, calculated from consumption and elemental content), which is estimated as

$$f_{Baetis} = \frac{1}{2}\left(\frac{n_{Baetis} \times \bar{m}_{Baetis} \times \%N_{Baetis}}{n_{Baetis} \times \bar{m}_{Baetis} \times \%N_{Baetis} + \kappa_{leaf} \times \%N_{leaf}}\right) + \frac{1}{2}\left(\frac{n_{Baetis} \times \bar{m}_{Baetis} \times \%C_{Baetis}}{n_{Baetis} \times \bar{m}_{Baetis} \times \%C_{Baetis} + \kappa_{leaf} \times \%C_{leaf}}\right) \quad (4)$$

where n_{Baetis} is the total number of consumed *B. rhodani* larvae, $\bar{m}_{Baetis}$ is the average dry mass per *B. rhodani* larva, $\%N_{Baetis}$ and $\%C_{Baetis}$ are the elemental contents of N and C of *B. rhodani* larvae, respectively, κ_{leaf} is the leaf consumption, and $\%N_{leaf}$ and $\%C_{leaf}$ are the elemental contents of N and C of leaf discs, respectively. Because outputs from mixing models deviate from reality for single-diet treatments (i.e., proportions of 0 and 1), raw dietary proportions from SIA were subsequently corrected via linear regression of single-diet treatments. This step was done to set single-diet treatments to their actual values of 0 or 1.

RESULTS AND DISCUSSION

Experiment 1: Effects of thiacloprid on trophic enrichment factors

Mortality and feeding. The survival rate of gammarids was 83%–97% higher in treatments with leaves as a food source than in treatments with *B. rhodani* (31%–64%) after 6 weeks. Nevertheless, no statistically significant effect of thiacloprid on mortality at the tested concentrations (up to 5 µg L^{-1}) was found, which is in agreement with earlier studies (Beketov & Liess, 2008). Consumption of *B. rhodani* larvae by *Gammarus* (Figure 2A) was reduced by 52% at 5 µg thiacloprid L^{-1} compared with the control over the entire study duration of 6 weeks ($p < 0.001$), whereas no statistically significant difference occurred between the low concentration (0.75 µg L^{-1}) and the control ($p = 0.124$). Leaf consumption (Figure 2B) was increased by 48% in the low concentration and decreased by 49% in the high concentration ($p < 0.001$, both compared with the

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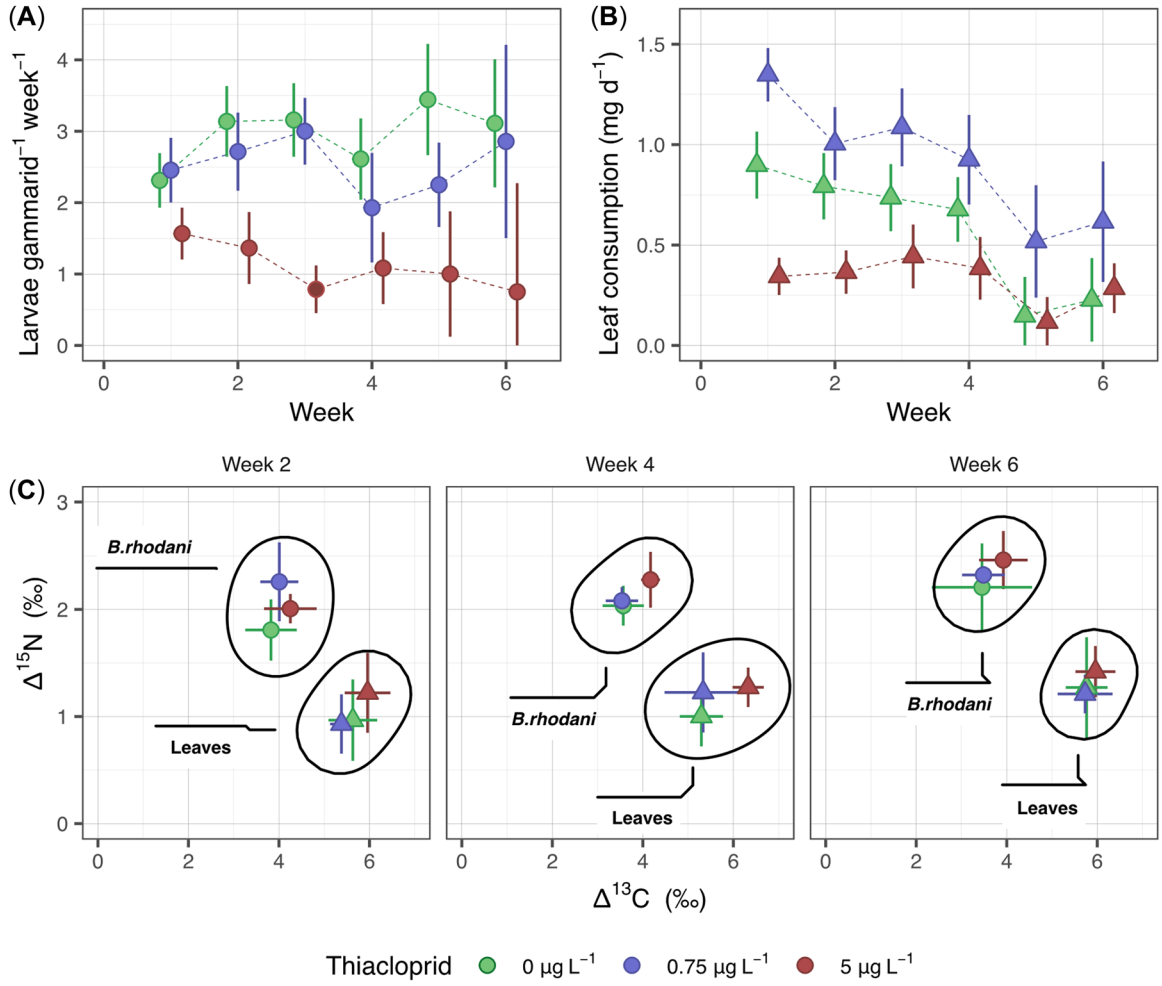

FIGURE 2: Experiment 1: Consumption of *Baetis rhodani* larvae (**A**), leaf consumption (**B**), and biplot showing trophic enrichment factors for ^{15}N and ^{13}C separately plotted for each week (**C**) for the control (green), low (blue), and high (red) thiacloprid concentrations. Data associated with *B. rhodani* and leaves are depicted as circles and triangles, respectively. Data are shifted symmetrically around the actual week of assessment (**A** and **B**) and highlighted by labeled circles (**C**) for readability. All values are shown as mean with 95% confidence interval.

control). Stress-induced metabolic costs (Calow & Sibly, 1990) are often balanced via compensatory feeding and the production of detoxification proteins (Maltby, 1999). Because the production of these proteins requires nitrogen, compensatory feeding was observed in leaf treatments (lower N content, Supporting Information, Figure S1) but not in *B. rhodani* treatments (higher N content, Supporting Information, Figure S1). The lower consumption rates for both diets at the high concentration are in accordance with other studies (Englert et al., 2012; Feckler et al., 2012) and are indicative of a direct sublethal effect of thiacloprid on *G. fossarum*.

Trophic enrichment factors. The TEFs were significantly modified by the 1) type of diet ($p < 0.001$), 2) study duration ($p = 0.008$), and 3) thiacloprid treatment ($p < 0.001$).

Type of diet. The Δ^{15}N values were between 2.2‰ and 2.5‰ for *B. rhodani* treatments and 1.2‰ and 1.4‰ for leaf treatments, which is in the range reported by other studies (Hellmann et al., 2015; Mancinelli, 2012; Remy et al., 2017). Remy et al. (2017) also reported higher Δ^{15}N with animal food compared with leaves, which contradicts the assumed general tendency of decreasing Δ^{15}N values with increasing food quality (Adams & Sterner, 2000; Hobson et al., 1993; Robbins et al., 2010). Differences between food sources were also observed for C: the TEFs were vastly different from reported values (−1.9‰ to 3.27‰; Hellmann et al., 2015; McCutchan et al., 2003; Remy et al., 2017). The Δ^{13}C values in the *B. rhodani* diet were closer (3.5‰–3.9‰) to this range than the Δ^{13}C values in the leaf diet (5.7‰–6‰). Both the degree of respiration versus assimilation, as well as the lipid content of a consumer (often derived from C:N ratios; McConnaughey & McRoy 1979), can influence the offset of a consumer's δ^{13}C to its diet. Because gammarids did not gain weight and their C:N ratios dropped during the study term ($p = 0.0096$; data not shown), it can be assumed that lipid reserves were respired, which discriminates against ^{13}C (i.e., disproportionally higher use of ^{12}C; DeNiro & Epstein, 1978; Post et al., 2007) and consequently results in higher values of δ^{13}C in the organism. In line with this, the higher Δ^{13}C for leaf treatments might indicate a higher respiration rate (Hessen et al., 2004) and thus a disproportionate release of $^{12}CO_2$ (DeNiro & Epstein, 1978) as a response to the lower nutritious value compared with

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B. rhodani larvae. Consequently, this results in higher TEFs than what would be expected from organisms that net-assimilate C. Both lipid respiration and the decrease in gammarids' weight could be induced by the inadvertently applied stress (handling and temperature) during handling.

Study duration. Because TEF values changed over the course of our study (and stable isotope signatures of resources did not; Supporting Information, Figure S2), it can be assumed that gammarids did not reach isotopic equilibrium with their resources. On the other hand, half-life periods are estimated to be approximately 20 and 14 days for N and C, respectively (Kaufman et al., 2008), which suggests close-to-constant stable isotope signatures toward the end of the experiment (Hellmann et al., 2015). Both increasing N turnover (e.g., in response to stress; Heugens et al., 2001) and depletion of own resources (Hobson et al., 1993) discriminate against ^{15}N and could explain the tendency of $\Delta^{15}N$ to increase over the test duration. Conversely, $\Delta^{13}C$ values were by tendency decreasing over the course of our study. An increase in lipid reserves, which are depleted in ^{13}C, would explain the observed decreasing $\Delta^{13}C$ values. However, C:N ratios indicate the opposite (i.e., a decrease in lipid content). Therefore, this effect is most likely explained by slowly approximating equilibrium with the ^{13}C-depleted resources (compared with gammarids).

Thiacloprid treatment. Shifts in TEFs with exposure to stressors are in accordance with the literature (Ek et al., 2015; Shaw-Allen et al., 2005; Staaden et al., 2010). Because TEFs were by tendency increasing with higher thiacloprid exposures, it can be assumed that (as discussed previously in the *Mortality and feeding* section) increasing detoxification protein production (Maltby, 1999) increases N turnover and consequently gammarids' $\delta^{15}N$ (cf. Heugens et al., 2001). Accordingly, higher $\Delta^{13}C$ values could also be explained by a lower proportion of the resource being assimilated and a higher degree of respiration (Ek et al., 2015). However, thiacloprid-induced effect sizes for both $\Delta^{15}N$ and $\Delta^{13}C$ were lower than between-resource differences (by ~25% and ~50%, respectively), suggesting a rather moderate impact of thiacloprid on TEFs. Furthermore, effect sizes are small compared with what is expected from changes in physiological characteristics that are not necessarily toxicant related (e.g.,

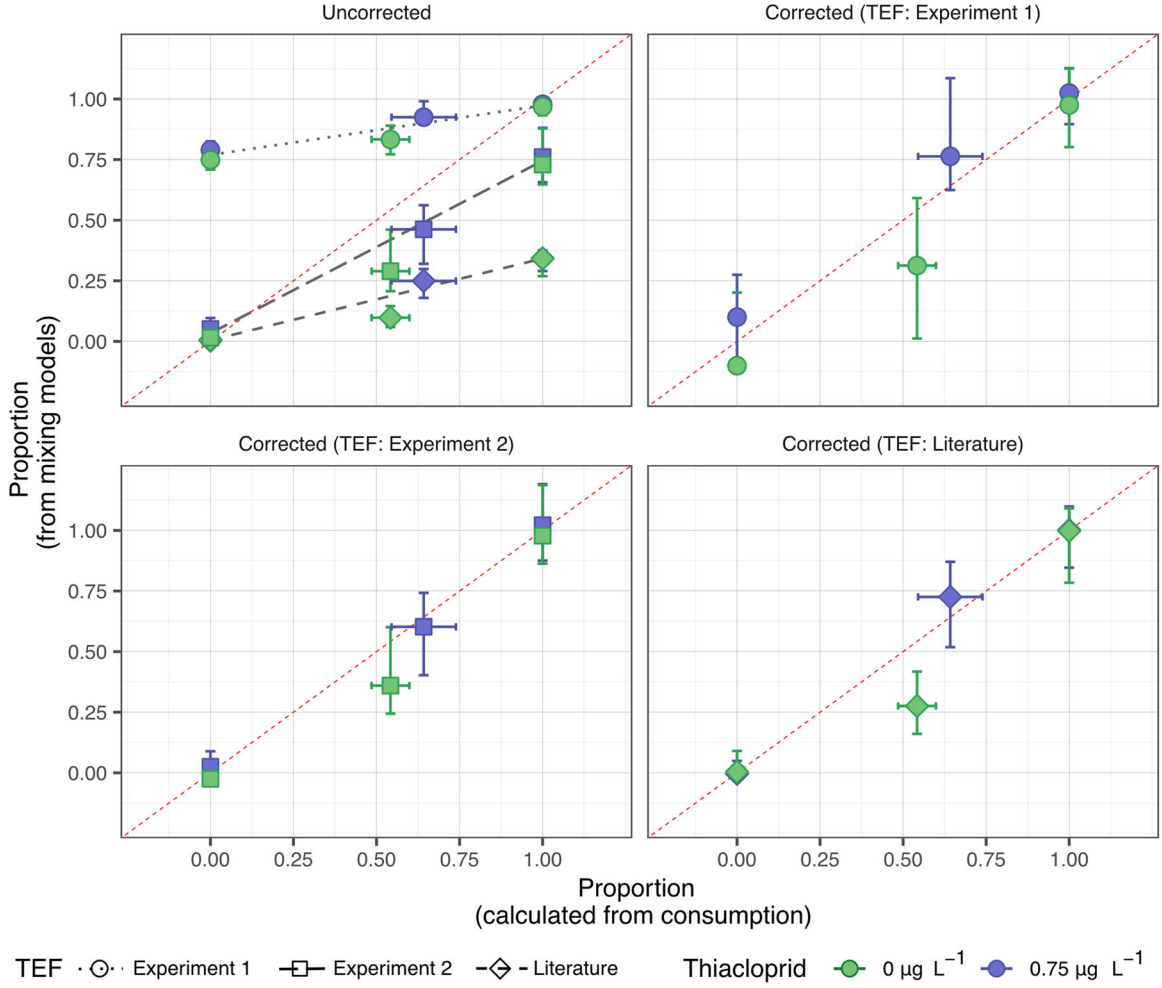

FIGURE 3: Proportion of *Baetis rhodani* larvae estimated from mixing models (maximum a posteriori estimate with 50% highest density interval) versus proportions estimated from consumption data (mean with 95% confidence interval if applicable). Data points are plotted for control (green) and low (blue) thiacloprid concentration as well as trophic enrichment factors (TEFs) from experiment 1 (circles, dots), experiment 2 (squares, large dashes), and McCutchan et al. (2003; diamonds, short dashes) separately. A dotted line indicating a 1:1 fit is plotted for orientation.

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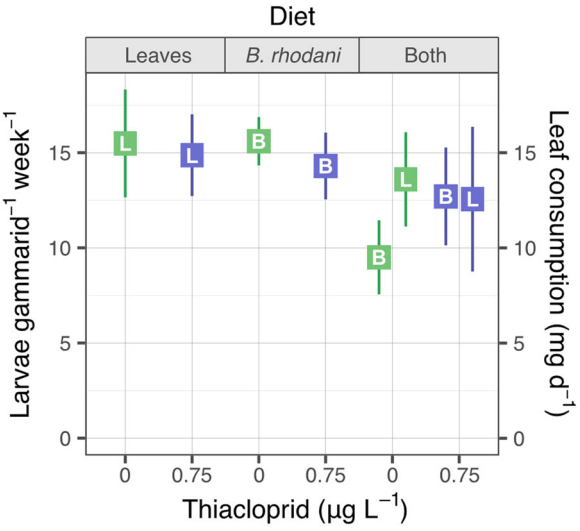

FIGURE 4: Experiment 2: Consumption of *Baetis rhodani* larvae and leaves by gammarids as mean with 95% confidence interval. Data are depicted for the control (green) and low (blue) thiacloprid concentration and labeled "L" and "B" for data of leaves and *B. rhodani* larvae, respectively.

N balance and growth rate; Del Rio and Wolf 2005). Nevertheless, effects on TEFs could be more pronounced for other stressors that directly impact metabolism. Because predicting a toxicant's effect on TEFs based on its mode of action will likely be intricate and associated with an unknown dimension of uncertainty, we recommend parallel assessment of toxicant impacts on TEFs for the use of SIA in ecotoxicological studies.

Experiment 2: Indirect effects assessed via stable isotope mixing models

In contrast to Experiment 1, gammarids were assimilating C and N (i.e., gaining weight; Supporting Information, Figure S3), which could be caused by seasonal differences. This resulted in gammarids' Δ^{15}N being 1.96‰–2.13‰ for the *B. rhodani* diet and 2.09‰–2.26‰ for the leaf diet, and Δ^{13}C values of 3.21‰–3.29‰ for the *B. rhodani* diet and 0.58‰–0.77‰ for the leaf diet. These values are closer to literature values (McCutchan et al., 2003) than what was observed in Experiment 1. Consequently, the estimated proportion of *B. rhodani* larvae in gammarids' diet was highest in mixing models with TEFs from Experiment 1 and lowest with TEFs from the literature (Figure 3A).

In mixed-diet controls, consumption of mayfly larvae was reduced by 39% (compared with single-diet treatments, $p < 0.001$; Figure 4), while being near-significantly increased by 24% in the thiacloprid treatment (compared with mixed-diet control, $p = 0.0568$). This indirect effect is in accordance with the results of Englert et al. (2012) and is likely explained by *B. rhodani* (96-h median lethal concentration [LC50]: 4.6 µg L^{-1}) being more sensitive to thiacloprid than *Gammarus* sp. (96-h LC50: 350 µg L^{-1}; Beketov & Liess, 2008), which leads to a reduced predation avoidance of the former and the observed increased predation in the latter. Irrespective of the TEFs used, all stable isotope mixing models detected a higher dietary proportion of larvae in the thiacloprid treatment (compared with control, Figure 3A). However, using the TEFs from Experiment 2 resulted in the highest accuracy (i.e., closeness to proportions estimated from consumption; Figure 3A), which indicates that the assessment of TEFs parallel to the experiment is vital for the validity of mixing model outputs. This accuracy can be further improved by correcting maximum a posteriori probability estimates by the intercept and slope from a linear regression of single-diet treatments (Figure 3B–D). Moreover, effect sizes were also most consistent with consumption data when using TEFs from Experiment 2. This is even the case even though applying TEFs from Experiment 2 results in the lowest Euclidian distance (as in a δ^{15}N vs. δ^{13}C biplot) of resources from one another (i.e., 1.38‰ distance compared with up to 1.18‰ SD of resources), which reduces the precision of mixing models (i.e., range of credible intervals; Figure 4A). This suggests that SIA is an applicable and robust tool to assess indirect effects.

The accuracy and precision could be constrained if TEFs are not determined experimentally and stable isotope signatures are sufficiently separated from one another (which is also the case in a nonecotoxicological context), respectively. Because the maximum number of unidirectional interspecific links (N_{max}) increases disproportionately with the number of species (n) in the food web (i.e., $N_{max} = n(n-1)/2$), assessment of TEFs for each consumer–diet relationship and each experimental treatment in more complex designs (e.g., multiple prey and consumers, feeding across multiple trophic levels, cannibalism) requires an experimental effort that (in many cases) cannot be accomplished. In addition, mixing models become more underdetermined with multiple sources (Phillips & Gregg, 2003), leading to lower precision, and sufficient separation of stable isotope signatures of a multitude of prey organisms is not always present, which could be a major limitation of the utility of SIA. An approach that will counteract all these limitations could be the labeling of resources with heavy stable isotopes. The consequence is twofold: 1) resources are sufficiently separated, which increases the precision of mixing models, and 2) differences and uncertainties regarding TEFs become vanishingly small compared with the between-resource differences, making them insignificant. With this technique, SIA could even resolve indirect effects in high-complexity food webs.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://10.1002.etc.5502.

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Conflict of Interest—The authors declare no conflict of interest.

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Data Availability Statement—Data are available on https://github.com/EricBollingerResearch. Data, associated metadata, and calculation tools are also available from the corresponding author, Mirco Bundschuh (bundschuh@uni-landau.de).

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