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Single and mixed effects of an herbicide and fungicide on green and brown fluvial food chains

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ABSTRACT

Single and mixed effects of an herbicide and fungicide on green and brown fluvial food chains.

Leaf litter and algae are the most important basal resources for invertebrate primary consumers in freshwater ecosystems. These basal source-consumer links can be affected by chemical pollutants, such as pesticides, that are widely used in agricultural activities. Pesticides are usually present in complex mixtures which potentially modifies the toxicological effect of the single compounds. In this study, we focused on the effects of two pesticides: terbuthylazine (1 µg/L), an herbicide that inhibits photosynthesis, and tebuconazole (13 µg/L), a fungicide inhibitor of ergosterol biosynthesis in fungi. Our aim was to evaluate the effects of these two pesticides on stream biofilms (photosynthetic activity, C:N ratio and chlorophyll-*a*) and leaf litter (mass loss, fungal biomass, C:N ratio) as well as diet-related effects on the consumption and growth rates of the snail *Physella acuta* (grazer feeding strategy) and the crustacean *Echinogammarus* sp. (shredder). We conducted a 14-day experiment using artificial channels where biofilm, leaf-litter and invertebrates were exposed to control conditions and to both pesticides, individually and in combination. The pesticides did not affect leaf litter measures or leaf litter processing by the shredder, either separately or in combination. However, both pesticides and their mixture reduced the chlorophyll-*a* concentration in the biofilm when grazers were present. Indirect, but likely also direct, effects of the fungicide on grazers reduced their respiration rate and increased their growth rate. These effects indicate that low concentrations of pesticides can have unexpected effects when considering top-down interactions, mainly on biofilms under the effects of grazing. Our pesticide concentrations were below those detected in some European surface waters, so it cannot be ruled out that more negative responses would exist if higher concentrations were maintained over time.

KEY WORDS: consumption rate, growth rate, river, invertebrate, leaf litter, biofilm, terbuthylazine, tebuconazole.

RESUMEN

Efectos por separado y de la mezcla de un herbicida y un fungicida en cadenas tróficas fluviales, herbívoras y detritívoras.

La hojarasca y las algas son los recursos basales más importantes para los invertebrados en los ecosistemas de agua dulce. Estas relaciones tróficas entre recurso y consumidor pueden verse afectadas por contaminantes, como los pesticidas, que se utilizan ampliamente en las actividades agrícolas. Los pesticidas suelen estar presentes en mezclas complejas que potencialmente modifican el efecto toxicológico de los compuestos individuales. En este estudio, nos centramos en los efectos de dos pesticidas: terbutilazina (1 µg/L), un herbicida que inhibe la fotosíntesis, y tebuconazol (13 µg/L), un fungicida

*inhibidor de la biosíntesis de ergosterol en hongos. Nuestro objetivo fue evaluar los efectos de estos dos pesticidas sobre el biofilm fluvial (actividad fotosintética, relación C:N y clorofila-*a*) y la hojarasca (pérdida de peso, biomasa fúngica, relación C:N), así como los efectos relacionados con la dieta en las tasas de consumo y de crecimiento del gasterópodo* Physella acuta *(raspador, como estrategia de alimentación) y el crustáceo* Echinogammarus *sp. (triturador). Realizamos un experimento de 14 días utilizando canales artificiales donde el biofilm, la hojarasca y los invertebrados se expusieron a condiciones control, y a ambos pesticidas, individualmente y en combinación. Los pesticidas, por separado o en combinación, no afectaron las características de la hojarasca ni el procesamiento de la misma por parte de los trituradores. Sin embargo, ambos pesticidas y la mezcla disminuyeron la concentración de clorofila-*a *en el biofilm en presencia del raspador. A su vez, los efectos indirectos, pero probablemente también directos, del fungicida sobre el gasterópodo, disminuyeron su tasa de respiración y aumentaron su tasa de crecimiento. Estos efectos indican que bajas concentraciones de pesticidas pueden tener efectos inesperados cuando se consideran las interacciones top-down, principalmente en el biofilm fluvial bajo los efectos del ramoneo. Nuestras concentraciones estaban por debajo de las detectadas en aguas superficiales en Europa, por lo que no se puede descartar que puedan existir más respuestas negativas si se mantienen concentraciones más altas en el tiempo.*

PALABRAS CLAVE: *tasa de consumo, tasa de crecimiento, río, invertebrado, ergosterol, descomposición de hojas, clorofila, biofilm, C:N ratio, terbutilazina, tebuconazol.*

INTRODUCTION

Leaf litter and algae are the most important basal resources for invertebrate primary consumers in freshwater ecosystems.This first level in the food chain is essential for nutrient and energy transfer to the entire food web. In streams, algae mainly grow on streambeds and jointly with other microorganisms (i.e. bacteria, fungi, and microinvertebrates) and mucilaginous compounds constitute the biofilm, a nutrient rich food for invertebrate grazers (Lamberti et al., 2007). The energy stored in coarse particulate organic matter (e.g. leaf litter) is released to aquatic ecosystems by breakdown processes involving microorganisms and aquatic invertebrates and contributes to the recycling of organic carbon and nutrients from the riparian vegetation (Graça & Canhoto, 2006). Leaf litter is colonised by microbial decomposers (aquatic hyphomycetes and bacteria) which produce extracellular enzymes that degrade plant constituents and transform leaf materials into highly nutritive and palatable material for invertebrate shredders (Kaushik & Hynes, 1971; Mas-Martí et al., 2015). The activity of shredders on leaf litter transforms leaves into fine particulates that are transported downstream and deposited at the river bottom being an important food source for collectors. The trophic role of grazers and shredders is essential for ecosystem functions such as recycling nutrients for primary producers and processing of dead organic matter.

These basal source-consumer links can be affected by chemical pollutants such as pesticides, that are widely used in agricultural activities. These pollutants reach surface waters from field runoff treated with these pesticides, spray drift, leaching, drainage flows and atmospheric deposition (Reichenberger et al., 2007). They are usually present in complex mixtures that interact with the environment, potentially modifying the toxicological effect of single compounds (Altenburger et al., 2015). The study of the combined effects of different pollutants is a more realistic assessment of their risk to organisms. Herbicides can affect algal growth in stream biofilms and fungicides may alter the leaf-associated fungal community, thus affecting both food quantity and quality for invertebrate consumers. Invertebrates can suffer from exposure to pesticides via the water phase (López-Doval et al., 2010; Solis et al., 2019). However, less is known about their effects through the diet, especially when the exposure concentration is low but continuous, which is the most realistic scenario under natural conditions.

In this study, we focused on the effects of two pesticides. Terbuthylazine, which belongs to the group of Chlorotriazine herbicides, is one of the most commonly used pesticides (Fingler et al., 2017). Its mechanism of action is the inhibition of photosynthesis (photosystem II) due to the alteration of chloroplast membrane proteins (Queirós et al., 2022). The fungicide tebuconazole (a group of triazoles), is an inhibitor of ergosterol

biosynthesis in fungi disrupting cell membrane synthesis and permeability. Both pesticides are routinely used in agriculture and are frequently detected in streams and rivers. Maximum concentrations (approximately 100 µg/L) were detected in European rivers (Wenneker et al., 2010) although the most common concentrations in rivers are lower, between 0.05 and 2.4 µg/L (Herrero- Hernandez et al., 2017; Hermosin et al., 2013). Terbuthylazine had effects on the growth rate of algae at 0.01 mg/L (Junghans et al., 2006) and on *Daphnia* mobilization at 5 mg/L (Marchini et al., 1988). Tebuconazole can inhibit leaf degradation through a reduction in fungal and bacterial biomass on leaves, changes in microbial community structure, and decreases in the microbial extracellular enzyme activities responsible for cellulose and hemicellulose degradation (Bundschuh et al., 2011, Zubrod et al., 2011). Effects on fungal biomass were observed at 0.06 mg/L and in the feeding behaviour of *Gammarus fossarum* at 0.09 mg/L (Zubrod et al., 2011). In general, effects were observed at higher concentrations than those usually found in rivers. To our knowledge, no studies have investigated the effects of these two pesticides acting together on different trophic resources and their consumers. Evaluating the effects of environmental concentrations and including trophic interactions between species may help to elucidate complex effects occurring in natural freshwater ecosystems.

Our aim was to evaluate the effects of these two pesticides on river biofilms (photosynthetic activity, biomass and C:N concentration) and leaf litter (mass loss, ergosterol and C:N concentrations) as well as diet-related effects on the consumption and growth rates of the snail *Physella acuta* (grazer) and the crustacean *Echinogammarus* sp. (shredder). We conducted a 14-day experiment using artificial channels where biofilm, leaf-litter and invertebrates were exposed to control conditions and to both pesticides, either individually and in combination, at environmentally realistic concentrations. Concentrations found in the field are usually well below the effective concentration for these organisms; thus, we expected that effects on basal resources would indirectly affect consumers. Specifically, we expected that i) the photosynthetic activity and algal biomass (chlorophyll-*a* concentration) would decrease in the biofilm due to the herbicide effect; these effects would be more evident due to grazing activity; ii) the fungal biomass (ergosterol concentration) and the quality of the leaf litter would decrease because of the effect of the fungicide on leaf-associated fungi; iii) despite each pesticide having a different target group, exposure to both pesticides would cause interaction effects; and finally, iv) the former effects would negatively affect the growth rate of both invertebrates.

MATERIALS AND METHODS

Pesticide concentration

Nominal pesticide concentrations (1 µg/L for terbuthylazine and 12 µg/L for tebuconazole) were based on bibliographic reports of concentrations found in rivers around Europe (ECOTOX-USEPA). These nominal concentrations were verified by water analysis during the experiment. Samples were randomly collected (three replicates) from the control and each treatment on the first day of the experiment and after 6 and 14 days. Water was collected before water renewal. Pesticides were analysed by means of high-performance liquid chromatography-mass spectrometry (HPLC-MS) at the Scientific and Technological Centers of the University of Barcelona.

Experimental design

The experiment was conducted in a set of 12 indoor experimental channels, each being 100 cm long, 10 cm wide, and 10 cm deep, for 14 days. The channels received a recirculated flow of dechlorinated tap water from individual aquaria, each holding 10 L of water. The water in the channels was changed every three days. The water temperature, oxygen concentration, conductivity, pH, and NH_3/NH_4^+ concentration were monitored every two days. The water velocity in the channels was maintained at 1 cm/s. Light was provided by LED lamps, and the photoperiod was set at 12 h light:12 h dark.

The bottom of each channel was covered with sandblasted glass substrata, which served as sub-

strata for algal attachment. An inoculum of biofilm from a natural, unpolluted stream (Arbúcies stream, 41.823133, 2.452826) was added to each channel three times, at weekly intervals, before the experiment started. Each week, at least five stones from the streambank were gently cleaned with a toothbrush, and the recovered biofilm was transported to the laboratory under cool conditions. The biofilm was mixed in a vortex mixer, and 1 mL of the suspension was added to each channel.

In autumn 2019, black poplar leaves freshly abscised were collected from the riparian area of the same stream and dried at room temperature until needed. Dried leaf sets (15-20 leaves) were inserted into mesh bags (0.5 mm mesh size) and transferred to the stream for microbial colonisation for 20 days. Afterwards, the mesh bags were transferred to the laboratory. Leaves from half of the mesh bags were promptly used to extract 16 mm diameter leaf discs using a cork borer, avoiding veins. The other half of the mesh bags was maintained in a container filled with 20 L of stream water and kept at 18 ºC under permanent aeration and total darkness for an additional 7 days before being used for the second week of the experiment, when we repeated the process to obtain leaf discs.

Seven days before the start of the experiment, individuals of *Echinogammarus* sp. and *P. acuta* were collected from two streams, Vallvidrera (41.437639, 2.052167) and Llémena (41.988773, 2.750359), respectively, in clean water upstream of any effluent. In the laboratory, the test organisms were acclimatised to laboratory conditions (18 ºC) and fed *ad libitum* with conditioned black poplar leaves or stones with natural biofilm, collected in the same streams. To stimulate feeding during the experiment, invertebrates were not fed for 24 hours before the experiment. Only gammarid males were used, and to avoid within-treatment variability, invertebrates of similar size were selected. An extra of 20 individuals were dried at 60 ºC until constant weight to determine initial dry mass (DM) (3.0 ± 0.7 mg for gammarids, 2.6 ± 1.4 mg for snails).

A total of 4 treatments were randomly distributed among the 12 channels: control treatment (C); fungicide treatment (F), where channels received tebuconazole; herbicide treatment (H), where channels received terbuthylazine; and finally, mixture treatment (FH), where channels received both pesticides (Fig. 1). The experimental setup included three channels for each treatment. In each channel, three glass substrata (a total of 0.3 m^2) were kept free of grazing, while five snails were randomly introduced in another three glass substrata (separated by a net to avoid crossing the snails). Three small mesh bags (9 x 4 cm), with 8 leaf discs each, were introduced to each channel for determination of leaf litter mass decomposition and related variables. Shredders were also introduced individually in teacup cages (5 cages in each channel) with 8 leaf discs for feeding. The leaf discs in bags and cages were replaced to new ones after 7 days to supply enough food to the shredder.

Biofilm analyses

The photosynthetic performance of the primary producers (photosynthetic yield, Yeff) was monitored daily in vivo using an amplitude-modulated fluorimeter (Diving PAM, Walz, Germany) as an approximation of the physiological status

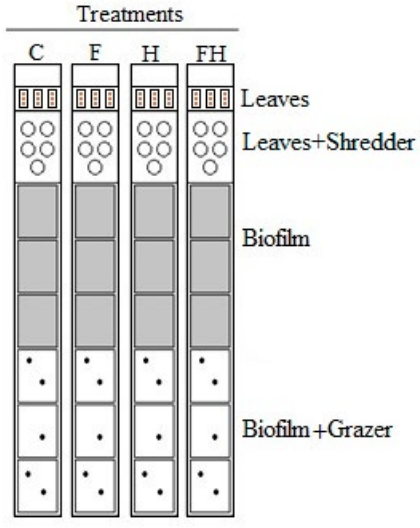

Fig. 1. Experimental design. Treatments: C (control), F (fungicide), H (herbicide), FH (mixture). Three channels per treatment completed the experimental design (12 channels). *Diseño experimental. Tratamientos: C (control), F (fungicida), H (herbicida), FH (mezcla). Configuran el diseño experimental tres canales por tratamiento (12 canales en total).*

of the algal community. The total chlorophyll-*a* (Chl-*a*) in the glass substrata was measured at the beginning of the experiment in each treatment. At the end of the experiment, it was measured in one glass substrata in each channel (3 replicates per treatment) and separately for glasses with and without snails. Total Chl-*a* was scraped off the glass substrata with a toothbrush and extracted in 90% acetone (10 mL) overnight at 4 °C in the dark. The extracts were filtered (Whatman GG/C) to reduce turbidity, measured spectrophotometrically (Perkin Elmer UV-VIS), and quantified following the method of Jeffrey and Humphrey (1975).

The biofilm from the third glass was extracted, dried, and ground into a fine powder to analyse the nitrogen (N) and carbon (C) concentrations using a Thermo Element Analyser 1108 (Thermo Scientific, Milan, Italy) at the Scientific and Technological Centers at the University of Barcelona. We expressed the results in terms of the C:N molar ratios.

Leaf litter analyses

Leaf mass loss due to microbial decomposition and physical processes was calculated with initial and final dry mass for each week and expressed in percentage. The initial dry weight of the leaf discs was estimated from 50 discs each week. Final leaf dry mass was measured using all the conditioned leaf discs (24) from each channel bag after 7 d and at the end of the experiment. Leaf discs were frozen, lyophilised and weighed to determine initial and final mass for each 7-day period. These leaf discs were later used for determination of ergosterol concentrations and C:N molar ratios (see below).

The ergosterol concentration in the leaf discs was determined as a proxy for fungal biomass (Gessner, 2020). At initial conditions, after 7 d and at the end of the experiment, five of the leaf discs used for mass determination for each bag were weighed and used for ergosterol extraction. We performed lipid extraction and saponification using 0.14 M KOH methanol (8 g/L) at 80 ˚C for 30 min in a shaking water bath. The extracted lipids were purified using solid-phase extraction cartridges (Waters Sep-Pak, Vac RC, 500 mg, tC18 cartridges, Waters Corp., Milford, MA, USA), and ergosterol was eluted using isopropanol. We used high-performance liquid chromatography (HPLC) to detect and quantify ergosterol by measuring the absorbance at 282 nm. We used a Jasco HPLC system (USA) equipped with a Gemini-NX 5 µm C18 250 × 4.6 mm column (Phenomenex, UK). The mobile phase was 100% methanol, and the flow rate was set to 1.2 mL/min. We expressed the results in µg of ergosterol per gram of dry mass leaf litter.

The other three leaf discs used for final mass determination for each bag and week were dried and ground into a fine powder for determination of the C:N molar ratios as described for the biofilm. The initial ratios were measured in three replicates with three leaf discs each.

Consumer analyses

The leaf discs offered to the shredder each week were weighed before and after their consumption to estimate the leaf DM change. At the end of the experiment, individuals were freeze-dried, weighed to the nearest 0.0001 g, ground and individually used for determination of C and N concentrations.

The consumption (C) was calculated as the loss of the biofilm's chlorophyll or leaf DM. Leaf values were corrected by the DM loss without shredder in the respective treatments. The relative consumption rate (RCR) was calculated as:

$$RCR = C/(DM_{cons} * day),$$

where DM_{cons} is the consumer dry mass (mg) at the end of the experiment and day is the number of days the test lasted. The relative growth rate (RGR) was estimated as follows:

$$RGR = (DM_f - DM_i)/(DM_{mean} * day),$$

where DM_f and DM_i are the final and initial dry mass (mg) of the consumer, respectively; DM_{mean} is the mean dry mass of the consumer between the start and the end of the test; and day is the number of days the test lasted (Mas-Martí et al., 2015). Survival was also recorded every other day throughout the experiment.

To measure consumer metabolism, via oxygen consumption, we used an optical oxygen microsensor adapted to a 20 mL glass vial (Fibox 4 PreSens, Regensburg, Germany) filled with oxygen-saturated water in which the grazer and shredder were individually introduced. The oxygen concentration was recorded every 10 seconds for 10 min. The results were expressed as µg O_2 L^{-1} mg DM^{-1} min^{-1}.

Grazer reproduction

At the end of the experiment egg clutches from snails were carefully collected from the channels and preserved with ethanol (70%) for analyses under the stereoscope. The number of egg masses, and the number of eggs per egg mass were counted. Embryos were differentiated in four developmental stages: morula, trocophore, veliger and juvenile.

Data treatment

Outliers were detected using the Interquartile Range (IQR) method and removed. To examine the effects of the herbicide terbuthylazine and the fungicide tebuconazole on the measured variables, two-way ANOVAs with interaction terms were conducted, allowing for the evaluation of both individual and combined treatment impacts. Due to the unbalanced nature of some data, Type III ANOVAs were utilized. Statistical significance was determined at a p-value of less than 0.05. To ensure the validity of our statistical analyses, diagnostic tests were performed: the Shapiro-Wilk test was used to assess the normality of residuals, and the Breusch-Pagan test was employed to evaluate the homogeneity of variances.

RESULTS

The average (±SD) concentrations of pesticides were 1.03 ± 0.53 and 13.2 ± 1.57 µg/L for terbuthylazina and tebuconazole, respectively. Fungicide and herbicide were only detected in the corresponding treatment, and no pesticides were detected in the control treatment.

There were no significant differences between treatments in terms of water temperature, pH, or dissolved oxygen concentration (n=12, p>0.05;

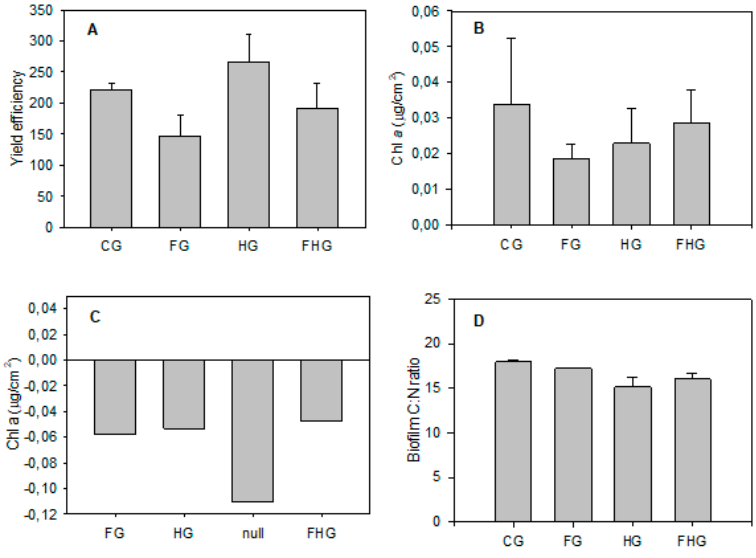

Fig. 2. Mean values and SEM for yield efficiency (photosynthetic performance of the primary producers) (A), chlorophyll-*a* concentration (B), and C:N ratio (D) in the biofilm with grazers. Figure C shows the reduction of chlorophyll-a in each treatment compared with that in the control treatment. The null bar shows the result of the simple addition of the effects from the two pesticides individually. C (control), F (fungicide), H (herbicide), FH (mixture). *Valores medios y SEM para la eficiencia fotosintética (rendimiento fotosintético de los productores primarios) (A), concentración de clorofila-*a *(B), y ratio C:N (D) en el biofilm con raspadores. La figura C muestra la reducción de la clorofila-a en cada tratamiento comparado con el tratamiento control. La barra nula muestra el resultado de la adición simple de los efectos de los dos pesticidas por separado. C (control), F (fungicida), H (herbicida), FH (mezcla).*

Supplementary material, Table S1, supplementary information, available at https://www.limnetica.net/en/limnetica). The ammonium concentration was always lower than 0.03 mg NH_4^+ /L and did not pose a risk for organisms according to OECD guidelines.

Effects of pesticides on biofilms and scrapers

The photosynthetic efficiency (photosynthetic yield, Yeff) decreased (p<0.0001) in the first 7 d of the experiment and remained stable thereafter (Fig. S1A, supplementary information, available at https://www.limnetica.net/en/limnetica). Only a slight decrease in herbicide treatments was observed but not statistically significant (Table 1, Fig. S1C). Grazing significantly reduced yield (p=0.011; Fig. S1B), but there were no significant effects of pesticides (Fig. 2A) (Table 1).

In the absence of grazing, the herbicide increased the biofilm biomass (p=0.022, Fig. S1E). Grazers significantly reduced the amount of Chl-*a* in the biofilm, with levels of 0.082 ± 0.050 µg/cm^2 without grazing and 0.033 ± 0.035 µg/cm^2 in the grazing treatments (p= 0.0028, Fig. S1D). Moreover, when grazers were present, a significant reduction in Chl-*a* was observed for both pesticides (Fig. 2B, Table 1). The effect of the fungicide on Chl-*a* was higher than that of the herbicide. However, the effect of the fungicide was more moderate in the mixture treatment. The reduction in Chl-*a* in the fungicide treatment compared to that in the control treatment was -0.06 µg/cm^2, while the reduction in the herbicide treatment was -0.05 µg/cm^2, and the reduction with both pesticides was -0.05 µg/cm^2 (Fig. 2C). The reduction in the mixture treatment (38%) was lower than the simple addition (54%) of the effects from the two pesticides separately (Fig. 2C).

The C:N molar ratio of the biofilm increased (initial mean value: 15.11 ± 1.10, final value 18.26 ± 2.05, p<0.0001) during the experiment, but no effects of pesticides were observed. The presence of snails reduced the C:N ratio of the biofilm compared to those of the treatments without snails (p=0.016, Fig. S1F). At the end of the experiment, the effects of the herbicide were relevant but marginally significant (p=0.06) when snails were present (Fig. 2D, Table 1).

There were no significant differences in the consumption rates of the snails among the treatments (Table 1). However, the RGR was higher in the fungicide treatment (~ 67% compared with the control, p=0.032, Fig. 3A). The oxygen consumption of snails exposed to fungicide was slightly lower (marginally significant, p=0.054; Fig. 3B). No significant differences were found in the C:N ratio of the snail (Fig. 3C). The total number of egg masses laid by snails in the channels at the end of the experiment was low, between 3 and 6 in average without differences between treatments (p>0.05; Table S2, supplementary information, available at https://www.limnetica.net/en/limnetica). The average number of eggs in each egg mass varied between treatments, without any pattern (p>0.05; Table S2). We found all embryo developmental stages in the egg masses, with veliger and juveniles being the most abundant. Only two snails (3 %) died during the experiment, and none died under control conditions. This percentage is well below the OECD recommendations.

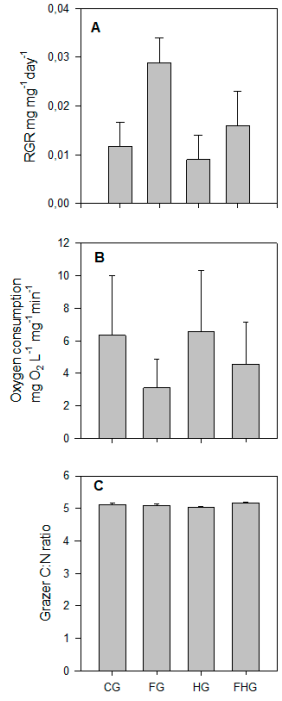

Fig. 3. Mean values and SEM for each treatment for the grazer growth rate (A), oxygen consumption (B), and C:N ratio (C). C (control), F (fungicide), H (herbicide), FH (mixture). *Valores medios y SEM para cada tratamiento de la tasa de crecimiento del raspador (A), consumo de oxígeno (B), y ratio C:N (C). C (control), F (fungicida), H (herbicida), FH (mezcla).*

Table 1. ANOVA test results for the different measured variables: for biofilms and grazers (A), and for leaves and shredders (B). Significant variables are in bold. C (control), F (fungicide), H (herbicide), FH (mixture). *Resultados del test ANOVA para las diferentes variables: para los biofilms y los raspadores (A), para las hojas y los trituradores (B). Las variables significativas se muestran en negrita. C (control), F (fungicida), H (herbicida), FH (mezcla).*

A) Biofilms and Grazers			
Response		F	p
Yield eff.			
	Time	38.83	**<0.0001**
	Grazing	7.08	**0.011**
	F	0.88	0.359
	H	0.01	0.922
	FH	0.24	0.627
	FG	0.05	0.830
	HG	0.01	0.910
	FHG	0.17	0.680
Chlorophyll- *a*			
	Grazing	10.68	**0.003**
	F	0.28	0.610
	H	4.40	**0.022**
	FH	1.90	0.210
	FG	10.33	**0.005**
	HG	8.79	**0.008**
	FHG	6.59	**0.018**
C:N ratio			
	Time	53.38	**<0.0001**
	Grazing	7.78	**0.016**
	F	2.68	0.140
	H	0.82	0.391
	FH	0.20	0.663
	FG	0.31	0.607
	HG	6.80	0.060
	FHG	1.04	0.365
Grazer RCR			
	FG	0.14	0.718
	HG	0.23	0.643
	FHG	0.002	0.962
Grazer RGR			
	FG	4.85	**0.032**
	HG	0.37	0.544
	FHG	0.005	0.945
Grazer oxygen consumption			
	FG	4.24	0.054
	HG	0.26	0.614
	FHG	0.99	0.331
Grazer C:N ratio			
	FG	0.12	0.646
	HG	2.95	0.096
	FHG	3.84	0.059

B) Leaves and Shredders			
Response		F	p
Dry Mass loss	F	2.376	**0.018**
	H	0.038	0.970
	FH	-1.372	0.171
Ergosterol	Initial-final	11.34	**0.001**
	Weeks	1.51	0.222
	F	0.002	0.958
	H	0.48	0.489
	FH	0.01	0.920
C:N ratio			
	Initial-final	1.28	0.261
	F	0.04	0.834
	H	0.43	0.517
	FH	0.07	0.785
Shredder RCR			
	F	1.78	0.197
	H	0.04	0.834
	FH	0.024	0.877
Shredder RGR			
	F	0.05	0.820
	H	0.15	0.704
	FH	0.04	0.849
Shredder oxygen consumption			
	F	0.48	0.494
	H	1.90	0.184
	FH	0.21	0.649
Shredder C:N ratio			
	F	6.30	**0.016**
	H	3.08	0.088
	FH	3.25	0.079

Effects on leaves and shredders

Leaf dry mass loss due to microbial decomposition was significantly lower in the fungicide treatment than in any of the other treatments (p=0.0182, Fig. 4A, Table 1).

There were no significant differences in the ergosterol concentration in the leaves collected during either week. Ergosterol increased during the experiment (p=0.0012, Table 1; e.g., control treatment initial: 4.85 ± 1.21, final: 6.16 ± 1.24 µg ergosterol/g leaf dry mass). The C:N molar ratio did not significantly differ among leaves during the experiment (Table 1). The C:N ratio and ergosterol concentration of the leaves did not significantly differ due to the presence of any of the pesticides (Fig. 4B, 4C, Table 1).

No significant effects were found in the consumption rates or RGR of shredders due to pesticides (Fig. 4D, Table 1). The fungicide increased the C:N ratio of the shredder body (p=0.036) (Fig. 4E), which was mainly related to an increase in Carbon. However, the differences from the control were minimal (only 0.1 higher in F). Shredder

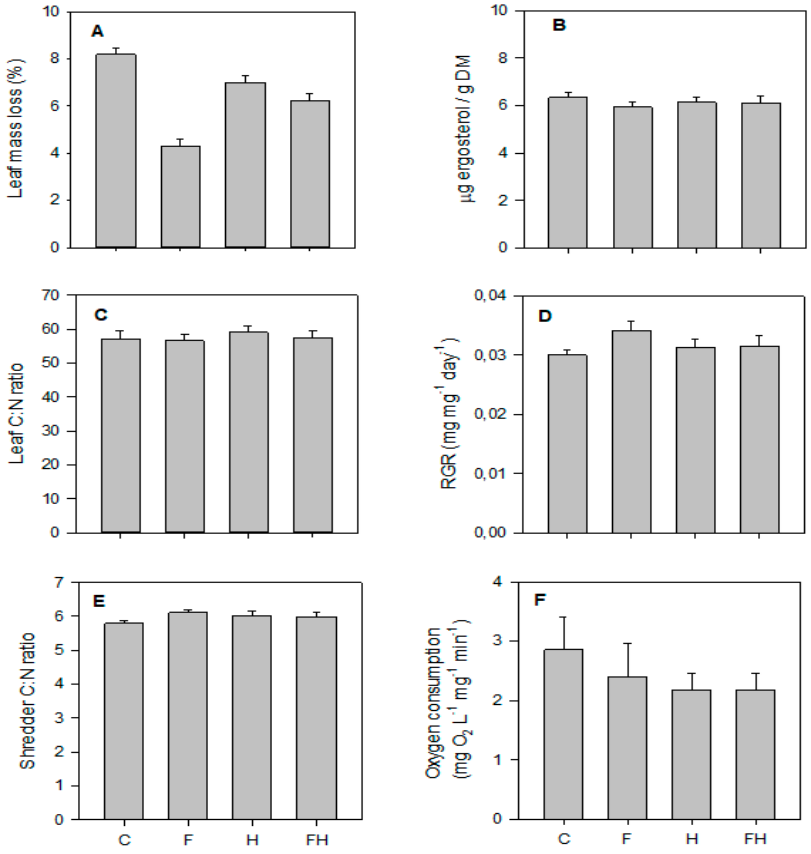

Fig. 4. Mean values and SEM for each treatment for the percentage of leaf mass loss (A), ergosterol concentration in leaves (B), leaf C:N ratio (C), shredder growth rate (D), shredder C:N ratio (E), and shredder oxygen consumption (F). C (control), F (fungicide), H (herbicide), FH (mixture). *Valores medios y SEM para cada tratamiento del porcentaje de pérdida de peso de hoja (A), contenido de ergosterol en las hojas (B) y ratio C:N en las hojas(C); tasa de crecimiento (D), ratio C:N (E), y consumo de oxígeno (F) del triturador. C (control), F (fungicida), H (herbicida), FH (mezcla).*

oxygen consumption decreased in the pesticide treatments but did not significantly differ among treatments (Fig. 4F, Table 1). No mortality was observed in shredder individuals during the experiment.

DISCUSSION

The present study showed that 1 µg/L of terbuthylazine did not have direct effects on biofilm. However, the presence of grazers caused a decrease in algal biomass when pesticides were present separately. Additionally, an antagonistic effect was observed with the mixture. Tebuconazole (13 µg/L) reduced leaf decomposition but did not cause changes in leaf-associated fungi. No effects on consumption rates were observed associated with the pesticides but the grazer growth rate was greater under the fungicide effect.

Pesticide effects on biofilm and leaves

The herbicide terbuthylazine without the presence of grazers increased the chlorophyll-*a* concentration but decreased the photosynthetic efficiency in the biofilm. Previous studies have reported similar results after long-term exposure of biofilms to low concentrations of diuron or other similar PSII inhibitor herbicides. Ricart et al. (2009) showed that 1 µg/L of diuron induced an increase in chlorophyll-*a* concentration. The mechanisms of homeostasis may explain these results. Photosynthetic pigments in phototrophic organisms can be environmentally regulated (e.g. by the presence

of herbicides). Phototroph organisms under stress have the ability to adjust their intracellular pigments (e.g. chlorophyll-*a*) to maintain efficient photosynthetic activity despite to a lowered level of electron transport due to inhibition by the herbicide (Tlili et al., 2011). Other studies related the increase in chlorophyll-*a* induced by pesticides to the formation of shade-type chloroplasts whose ultrastructure (broader grana and a higher staking degree of thylakoids) was less efficient for photosynthetic quantum conversion (Lopez-Doval et al., 2010). As expected, the fungicide Tebuconazole did not directly affect the biofilm.

When grazers were present, a significant decrease in chlorophyll-*a* was observed in biofilms with the pesticides, both individually and in combination. Compared with no grazing, grazing activity also reduced the biofilm photosynthetic efficiency. Grazing regulates biofilm biomass accumulation and removes senescent cells from the biofilm, facilitating toxicant exposure and increasing its effects (Steinman, 1996). Accordingly, several authors described similar results (i.e., a reduction in chlorophyll-*a* concentrations) when grazers and herbicides act together on biofilms (Muñoz et al., 2001; López-Doval et al., 2010). Contrary to our hypothesis, tebuconazole also significantly reduced the amount of chlorophyll-*a* in the biofilm with grazers, and the effects were more evident than those produced by the herbicide. This effect was probably indirect, resulting from the effect of fungicides on other components of the biofilm community, changing their relationships. Artigas et al. (2014) reported impaired photosynthetic activity and increased bacterial mortality in biofilms exposed to tebuconazole, suggesting that the effects of the fungicide on fungal community indirectly affect other biofilm communities. Similarly, other pesticides, such as the herbicide diuron, may cause a chain effect on biofilms, affecting both algal and bacterial activity (Ricart et al. 2009). Tebuconazole is a relatively lipophilic molecule (log Kow = 3.7) that rapidly adsorbs onto organic matter (Artigas et al., 2012). Biofilms possess a matrix of extracellular polymeric substances capable of adsorbing organic matter (Freeman & Lock, 1995), including organic pesticides such as tebuconazole, favouring the effects on biological communities within the biofilm.

When biofilm was exposed to both pesticides, the effects of the fungicide seemed to be moderated by the herbicide. The mixture reduced 16% the effects on chlorophyll-*a* concentration compared with the simple addition model, considering the separate effects of both pesticides, which suggests antagonistic effects (Coté et al., 2016) of the fungicide and herbicide mixture on the biofilm chlorophyll-*a* concentration. The non-specificity of the pesticide effects emphasizes the difficulty of predicting ecosystem impacts of pesticide mixtures (Flores et al., 2014).

Leaf decomposition (dry mass loss) decreased with the fungicide. Cornejo et al. (2020) also found a decrease of microbial decomposition in leaf litter pre-treated with the fungicide chlorothalonil. Leaf litter decomposition is sensitive to pollution and previous studies have demonstrated that metals (Bergmann & Graça, 2020), micro and nanomaterials (Trabulo et al., 2022; Seena et al., 2019) and other pollutants impair this freshwater ecosystem function. Surprisingly, no significant differences in the ergosterol concentration or C:N ratio of leaves were detected between treatments, although the ergosterol concentrations detected in this experiment were very low, which may have impaired the detection of any effects. Earlier studies also showed that in the presence of tebuconazole (238 µg/L), fungal biomass did not change (Dimitrov et al., 2014), while it significantly reduced fungal reproduction and changed community composition. However, other authors have shown that tebuconazole clearly inhibits fungal biomass in leaf litter (Artigas et al., 2012; Bundschuh et al., 2011; Zubrod et al., 2011). The fungicide concentration tested in those studies ranged between 33 and 65 µg/L, which were higher than the concentration used in the present study. Pimentão et al. (2020) also reported a reduction in fungal biomass at a concentration of 10 µg/L of tebuconazole but not with other fungicides, such as terbinafine. These authors highlighted that leaf litter decomposition and fungal biomass were less responsive to contaminants, especially fungicides, than was fungal reproduction. Salis et al. (2023) highlight that structural and functional responses to fungicides, as well as to other stressors, cannot be considered

in isolation. These complex relationships require a multifaceted approach to understand all the mechanisms involved.

Effects on consumers

The toxicant concentrations tested in this study did not cause lethal effects on consumers. The indirect effects of both pesticides on primary consumers were assessed by evaluating algal and leaf litter consumption by a grazer (*P. acuta*) and a shredder (*Echinogammarus* sp.), respectively. Contrary to our expectations and despite changes observed in the biofilm due to the herbicide, the grazers were not indirectly affected by the terbuthylazine tested concentration. However, the presence of tebuconazole in the water resulted in a higher growth rate (67%) of the snails and marginally significant lower metabolism (oxygen consumption). The observed changes in the biofilm (higher effects of the fungicide than the herbicide) and ultimately in the snail growth rate may be driven by a change in the proportion and relationships between biofilm biological components, as mentioned before. Pesticides can alter the composition of the community, leading to changes in the type of nutrients present in the biofilm, some of which are more nutritive for grazers. A complementary explanation may be related to the lower activity, expressed as oxygen consumption (marginally significant), of the grazers in the fungicide treatment where the algal biomass was reduced. With food limitations, a reduction in activity (lower metabolism) would allow more energy to be invested in growth (Auer et al., 2015). The basic response of an organism's metabolism to stress is carbohydrate mobilisation, which fuels the needed energy and provides a substrate for anabolic pathways (De Cohen et al., 2001). In our grazers, there was no carbohydrate loss due to pesticides, total C and N concentrations were similar to those under the control conditions, supporting the idea that consumers had enough carbohydrate reserves for growth demand in the fungicide treatment. High concentrations of tebuconazole (>0.40 mg/L) caused a reduction in the feeding rate of *Daphnia magna* (Sancho et al., 2009). The consumption rate of grazers in our study did not change due to pesticides; therefore, the growth rate changes observed in our study were not related to shifts in feeding rates.

No changes in fungal biomass or the C:N ratio were detected in the leaves, indicating that no changes in the nutritional value of the leaf material occurred in any of the treatments. Consequently, our expected hypothesis of indirect effects on shredders should not be supported. We did not observe changes in consumption rates, in accordance with Pimentão et al. (2020), who showed no significant effects on the feeding behaviour of *Chironomus riparius* and *Allogamus* sp. in the presence of tebuconazole-contaminated leaves. The consumption rates (0.05-0.25 mg leaf mg^{-1} animal day^{-1}) were within the reported range for invertebrate shredders (0.04 to 0.5 mg leaf mg^{-1} animal day^{-1}; Arsuffi & Suberkropp, 1989). In contrast, other studies (Zubrod et al., 2011, 2015) observed a reduction in leaf quality and a significant effect on the feeding rate of *Gammarus fossarum*. The higher concentrations used by Zubrod et al. (ranging from 65 to 500 µg/L) might explain these behavioural differences compared to those in the present study. There was no evidence of effects on the shredder growth rate. Other herbicides (i.e. glyphosate or propanil) at much higher concentrations than ours reportedly have negative effects on the growth of freshwater crustaceans (Annett et al., 2014; Villarroel et al., 2003).

The carbon concentration of shredders and consequently the C:N ratio, were slightly higher with fungicide addition. However, other authors (Villarroel et al., 2013; De Cohen et al., 2001) found that a reduction in carbohydrate content mitigated pesticide toxicity stress. Our results confirm that the low concentration of tebuconazole used in this study, which is more common in rivers, does not cause undesired effects on consumers (at least for *Echinogammarus* sp.).

The present study showed that the concentrations of terbuthylazine and tebuconazole (1 µg/L and 13 µg/L, respectively) did not affect leaf litter or leaf litter processing by *Echinogammarus* sp., either separately or in combination. These concentrations are below some of the highest concentrations detected in European surface waters, so it cannot be ruled out that negative responses may exist if higher concentrations are maintained over time, which is associated with the expansion

of agricultural activities in river basins. However, both pesticides and their mixture reduced the chlorophyll-*a* content in the biofilm when grazers were present. In turn, indirect but likely also direct effects of these pesticides on grazers led them to invest more energy in growth. These effects indicate that low concentrations of pesticides can have unexpected effects when considering top-down interactions, mainly on biofilms under the effects of grazing.

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AUTOR CONTRIBUTIONS

N.C.C.: Conceptualization, investigation, methodology, formal analysis, validation. Review and editing. M.S.: Conceptualization, investigation, methodology, data curation, validation. Review and editing. X.H.: Methodology, data curation. Review and editing. A.V.: Methodology. review and editing. M.M.: Methodology, investigation. Project administration. Review and editing. I. M.: Conceptualization, investigation, validation. Funding acquisition, project administration. Writing original draft, review and editing.

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