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Evaluating herbicidal risks of the fungicide tebuconazole: differential sensitivity of dicot and monocot macrophytes in freshwater mesocosms

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Abstract

Background The triazole fungicide tebuconazole (TBZ) has plant growth regulatory properties and is used as such in, e.g., oilseed rape crop production. To date, very little is known about the phytohormonal effects of TBZ on macrophytes. There are only data for the monocot macrophyte *Lemna* sp. available in the literature, which indicate a moderate risk for macrophytes. However, dicot macrophyte species are known to generally be more sensitive to growth regulating pesticides, due to the phytohormonal effects of these substances. Therefore, this study aimed to investigate if TBZ may impact monocot and dicot aquatic macrophytes differently and to quantify these effects. Two in situ experiments were conducted as part of a comprehensive study in freshwater mesocosms to evaluate direct and indirect effects of TBZ on five macrophyte species, particularly comparing the dicot species *Myriophyllum spicatum* with monocot duckweed species and the macrophyte standing stock of the mesocosms. For this, six naturally established freshwater mesocosms were dosed once with six different concentrations of TBZ (5–5000 µg/L), two served as controls.

Results The dicot *M. spicatum* was the most sensitive species with the lowest EC₅₀ (14 d) of 228 µg/L TBZ. In contrast, the monocot broad-leaved pondweed *Potamogeton natans* and two duckweed species reacted far less sensitive in this study with *Spirodela polyrhiza* being the most sensitive monocot species (EC₅₀ (14 d): 1487 µg/L TBZ). Shoots of *M. spicatum*, which were grown directly as standing stock in the mesocosms, developed strong growth deformities in the form of shorter stems and condensed leaf whorls during the course of the study in the higher TBZ treatments. This effect can be linked to the anti-gibberellic mode of action of TBZ.

Conclusion The results of this study highlight that short-term in situ experiments are a valuable tool that can efficiently be integrated into comprehensive mesocosm studies investigating aquatic communities, in order to gain reliable EC_x data for several non-standard macrophyte species with different growth forms under near-natural conditions. The results of this research underscore that for regulatory risk assessment of fungicides with a phytohormonal mode of action, dicot aquatic macrophytes need to be included in the first tier of the risk assessment.

Keywords Fungicide, Growth regulator, Risk assessment, In situ experiments, Aquatic plants, *Myriophyllum spicatum*

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Background

Aquatic macrophytes form the basis of many food webs in aquatic ecosystems [1]. Because they are important for sustaining the functionality and diversity of the aquatic community, macrophytes are considered as key organisms. Consequently, they are among the organisms used in the risk assessment of plant protection products and their active substances [2] and are considered key drivers for setting specific protection goals [3]. For pesticides with herbicidal mode of action and for plant growth regulators, further primary producers need to be tested in addition to the standard test with a green alga [2]. These include *Lemna* sp. (*L. gibba* or *minor*) and a non-green alga, which can be, e.g., the diatom *Navicula pelliculosa* [3]. If substances exhibit herbicidal mode of actions and *Lemna* sp. is not sensitive or if there is an expected uptake from the sediment by the roots of aquatic macrophytes, a toxicity test with a sediment-rooted aquatic macrophyte is required, either *Myriophyllum spicatum* [4] or *Glyceria maxima*. This approach acknowledges the need for considering a range of different growth forms of aquatic macrophytes in regulatory studies [2, 3, 5].

Fungicides are a group of pesticides that, in comparison to herbicides and insecticides, have received less attention in the past [6]. Although many fungicides act as biocides and do exert effects on a range of different taxa [6, 7], aquatic macrophytes are often not considered in experimental studies with fungicides. Tebuconazole (TBZ) is a triazole fungicide that, at the molecular level, inhibits the demethylation at the C14 position in fungal sterol biosynthesis by inhibiting the enzyme sterol 14 α -demethylase [8, 9]. In plants, sterol 14 α -demethylase is involved in the synthesis of phytosterol [10, 11]. As a result, TBZ exhibits plant growth regulatory properties [11] and is used as such in oilseed rape crop production [12]. Although growth regulation in monocots and dicots exhibits many similarities at the molecular level [13], it has been shown that dicot macrophytes such as the Eurasian watermilfoil *M. spicatum* respond significantly more sensitive to growth regulators than the standard monocot test species *Lemna* sp. [5, 14].

Up to now, quantitative effect concentration studies with TBZ on aquatic macrophytes are scarce. In the latest publicly available draft of the renewal assessment report (RAR) for TBZ according to the Commission Regulation (EU) N 1107/2009, two tests with the monocot duckweed *L. gibba* showed varying results with ErC_{50} (7 d) values of 237 μ g/L and 2280 μ g/L, respectively [15]. Unfortunately, no dicot macrophyte test is specified in the draft of the renewal report so far, which seems to be a significant gap, considering that TBZ is a phytohormonal acting fungicide.

In order to investigate if dicot macrophytes may be more sensitive to TBZ than monocot species, two in situ macrophyte experiments were run as part of a comprehensive fungicide study, investigating the effects of TBZ as active ingredient of the product Folicur® on fungal, plankton and macroinvertebrate communities in freshwater mesocosms, simulating stream lakes over a period of 8 months. Direct effects were studied at the individual level, using in situ tests with different macrophytes displaying particular growth forms, and at population level, evaluating the free-growing aquatic macrophyte standing stock in the mesocosms. Water chemistry was also monitored to obtain information on possible additional effects on the aquatic macrophyte community in the mesocosms.

Methods

Experimental design

Within the 8-month mesocosm study, two in situ experiments were run in the artificial pond and stream mesocosm facility, located at the field station of the German Environment Agency in Berlin, Germany [16]. For this, a regression design (EC_x) was used, for which six TBZ concentrations and two controls were randomly allocated to eight mesocosms. TBZ was dosed once as formulation Folicur® (Co. Bayer, Germany) in the systems, using the nominal concentrations 5, 50, 100, 500, 1000, and 5000 μ g/L TBZ. Dissipation of TBZ as well as physicochemical and nutrient parameters were monitored regularly in water. The two in situ experiments with *M. spicatum* started on the day of TBZ application (day 0) and differed in terms of exposure time (experiment 1: 14 days, experiment 2: 35 days; Table 1). Two duckweed species *Spirodela polyrhiza* and *Lemna trisulca* were exposed for 14 days and assigned to experiment 1 (Table 1). In addition to the in situ experiments, *Potamogeton natans* and *Nymphoides peltata*, which had been planted in the mesocosms three months prior to TBZ dosing as standing stock, were monitored weekly over the experimental time by means of photo-documentation. Moreover, a standard *M. spicatum* test was performed according to the OECD test guideline 239 [4]. Results of the test are publicly available on Zenodo [17].

Mesocosm set-up

The mesocosms were built of glass-fiber reinforced polyester material. Each mesocosm consisted of one pond (6.90 m $\times$ 3.25 m $\times$ 2.50 m; length $\times$ width $\times$ height) connected to a stream of 40 m length to simulate a stream-lake, which was operated in a circular mode (see SI Fig. 1). A stream-lake mesocosm set-up was chosen, in order to have optimal growth conditions for fungal communities (stream section) as well as for macrophyte- and

Table 1 Experimental set-up of in situ experiment 1 and 2

Species	Macrophyte preparation/monitoring	Endpoints	Duration (d)
<i>Myriophyllum spicatum</i> (dicot)	10 × 10 cm shoots in 10 plant pots with sand/natural sediment mixture fixed in stainless-steel racks	MSL, TSL, DW, RGR, NL	Experiment 1: 14 Experiment 2: 35
<i>Spirodela polyrhiza</i> (monocot)	3–5 colonies (1 replicate per mesocosm) in floating baskets	NL, RGR	Experiment 1: 14
<i>Lemna trisulca</i> (monocot)	40 leaves of similar size per replicate (6 replicates per mesocosm)	NL, RGR	Experiment 1: 14
<i>Nymphaeodes peltata</i> (dicot)	photo-documentation of leaf development of standing stock in mesocosms	NL	35
<i>Potamogeton natans</i> (monocot)	photo-documentation of leaf development of standing stock in mesocosms	NL	35

MSL: main shoot length; TSL: total shoot length; NL: number of leaves/whorls; DW: dry weight; RGR: relative growth rate

plankton communities (pond section). The pond area of each mesocosm was illuminated with six 4-channel LED lamps (Neptun® Roschwege, Germany) with a photo-synthetically active radiation of 150 $\mu\text{mol}/\text{m}^2/\text{s}$ (PAR, 400–700 nm, two white channels), 200 $\mu\text{W}/\text{cm}^2$ ultraviolet (350–425 nm, UV channel), and 30 $\mu\text{W}/\text{cm}^2$ far red (650–800 nm, FR channel) at the water surface. The pond illumination included a twilight phase of 1 h at dawn and dusk, respectively, and was adjusted monthly to the respective daylight length.

Four months prior to experimental start, the pond mesocosms were filled with sand and a 5 cm layer of a natural sediment as nutrient supply for plants. The natural sediment was then covered with a 5 cm sand layer to prevent nutrient leaching into the water. The natural sediment originated from Lake Duckwitz (Mecklenburg-Western Pomerania, Germany) and was tested for standard pollutants according to LAGA [18]. Each pond was equipped with a stepped shore, resulting in three flat zones with different water depths (shore zone 1: 35 cm, shore zone 2: 85 cm, and zone 3: 135 cm water depth above sediment; see SI Figs. 1 and 2). The mesocosms were filled with a mixture of processed ground water and deionized water from the field station's own water work, to a conductivity of about 520 $\mu\text{S}/\text{cm}$ and a total water volume of 30.5 m^3 . Flow was generated by means of a screw pump in the stream section and was run at a flow velocity of 0.1 m/s in a circular flow. The pond section had an inflow and outflow in zone 3 from the stream section and relatively stagnant flow conditions in shore zones 1 and 2 (see sketch and photos of mesocosm set-up SI Fig. 1 and SI Fig. 2).

For the biological set-up of the systems, the macrophyte *N. peltata* was placed in shore zone 1, *M. spicatum* in shore zone 2, and *P. natans* in zone 3, each at ten pre-marked positions (40 cm distance between each position). At each position, one root stem of *N. peltata*, five 15 cm shoots of *M. spicatum*, and three 10–20 cm shoots of *P. natans* were planted. The water of each mesocosm

was inoculated with periphyton and plankton communities from two different mesotrophic lakes (Lake Britzer Garten and Teufelssee, Berlin, Germany) 14, 12, and 8 weeks prior to TBZ application. In addition, the mesocosms were stocked with macroinvertebrates 10 and 4 weeks prior to the experimental start from different reference streams (Lieberoser Mühlenfließ, Plane, Barolder Mühlenfließ, and Mühlenteich Wiesenburg in Brandenburg, Germany), by means of attraction traps made of polyethylene bags (5 mm mesh size) stuffed with 100 g organic triticale straw and by dip nets. Mesocosms were kept in a mesotrophic nutrient state by nutrient addition when necessary. Target concentrations were 0.01 mg/L PO_4 as K_2HPO_4 , 0.5 mg/L $\text{NO}_3\text{-N}$ as $\text{Ca}(\text{NO}_3)_2$, 2 mg/L Si as Na_2SiO_3 . Over the period of the in situ experiments, the water of the mesocosms was fertilized only once on day 16 after TBZ application with 1.03 g K_2HPO_4 , 128.56 g $\text{Ca}(\text{NO}_3)_2$ and 196 mL Na_2SiO_3 to achieve target concentrations. Consequently, only experiment 2 was affected by this nutrient dosing, as nutrients were applied directly after finalizing experiment 1.

Set-up of in situ experiments

Prior to the start of the in situ experiments, plant pots (9 cm × 9 cm × 9.5 cm; length × width × height) made of polypropylene (Co. Göttinger, Germany) were pre-watered for 6 weeks in deionized water to promote leaching of possible soluble additives. Over the 6 weeks, the water was renewed three times. Pots were then prepared by placing two layers of pond fleece over the perforated bottom of the pots and filling with one layer of sand (65 g), one layer of a sand and natural sediment mixture (3:1; 220 g), and another layer of sand (65 g). The same sediment as for the establishment of the mesocosm systems was used. Shoot tips of *M. spicatum* for experiment 1 and 2 were taken randomly from *M. spicatum* standing stock of the eight mesocosms from zone 2 prior to TBZ dosing. *Lemna trisulca* was collected from untreated ponds at the Sinderhoeve Experimental Station

in Wageningen (Netherlands), while colonies of *S. polyrhiza* were collected from an untreated outdoor pond at the field station of the German Environment Agency. For each species, plants and shoot tips were chosen with similar quality (fresh weight, number of whorls/leaves, vitality) and prepared, based on their unique morphology (Table 1).

For *M. spicatum*, side shoots were removed if present. Fresh weight, and number of leaves/whorls and total shoot length above sediment were documented at planting. The *M. spicatum* shoots were planted with the apical tip upwards in the allocated pots with two whorls in the sediment. The pots were placed in stainless-steel racks which were transferred into the mesocosms one day prior to the start of the experiments and positioned on the bottom of shore 2 (SI Fig. 1 and 2). The following endpoints were measured for each plant at the start and the end of the experiments after 14 (experiment 1) and 35 days (experiment 2): fresh and dry weight (FW and DW), main shoot length (MSL), total shoot length (TSL), and number of leaves/whorls (NL). Relative growth rates (RGR) were calculated for each endpoint (Table 1). Dry weight measurements were made after drying at 60°C for at least 24 h. In order to determine DW of *M. spicatum* at the start of the experiment, 40 randomly chosen plant shoots were dried and weighed and the mean taken as the start DW for experiment 1 and 2.

For *L. trisulca*, 40 conjoined leaves of similar size were chosen for each of the 6 replicates per mesocosm. The leaves were placed in stainless steel mesh baskets which hung in floating devices held in space by bamboo sticks in shore zone 1 (see SI Fig. 2). For *S. polyrhiza*, 3–5 colonies of similar sized leaves were placed in one stainless steel mesh basket per mesocosm. The mesh baskets were fixed in the mesocosms in the same way and position as for *L. trisulca*. At the start of experiment 1, 40 randomly selected leaves of both duckweed species were taken to record the initial DW. After 14 days of TBZ exposure, quantitative image analysis was used for counting the NL of both duckweed species and DW was determined. *Nymphoides peltata* and *P. natans* grew as standing stock in the mesocosms. The number of floating leaves was recorded by means of photo-documentation throughout the experiments up to day 35. The photos were used to evaluate leaf development by counting the number of leaves of each species.

Analysis of tebuconazole and physicochemical water parameters

In order to monitor uniform mixing of the active substance in the water body immediately after Folicur® application, water samples were analyzed for TBZ after 1 and 4 h in the ponds and corresponding stream areas.

Thereafter, water samples were taken from the bend of the stream section and the TBZ concentration was determined after 1, 3, 7, 14, 21, 28 and 35 days. Water samples for TBZ analysis, with TBZ-D6 as internal standard, were concentrated and purified using SPE (Strata XL, Phenomenex) and then 1 µl of the extract was analyzed using a 7890B GC system (Agilent) coupled to a 7010B triple-quadrupole mass spectrometer with HES ion source (Agilent). To ensure the accuracy of the method, fortified control samples were analyzed concurrently within each sample set. The sample components were separated using an OPTIMA 35 MS column (30 m×0.25 mm, 0.25 µm) at constant flow (1.5 mL/min helium). Electron impact ionization in MRM mode was used for detection and quantification (TBZ: m/z 250 → 125 and m/z 252 → 127; TBZ-D6: m/z 258 → 127). The limit of detection (LOD) was 0.15 µg/L and the limit of quantification (LOQ) was 0.5 µg/L TBZ for water.

The physicochemical water parameters oxygen, pH, electrical conductivity, and water temperature were measured hourly with online measurement devices (WTW, Germany). For each online parameter, a daily mean value was calculated for further evaluation. For nutrient and major ion analysis, water samples were taken every 2 weeks. Water samples were filtered (0.45 µm glass fiber filters, Macherey–Nagel, Germany) before further analysis. Concentrations of o-phosphate, ammonia, nitrite, nitrate, and silicate were quantified photometrically using continuous flow analysis (San++, Skalar, Netherlands) according to standard methods [19–22].

Statistical analysis

Time-weighted average (TWA) concentration was calculated employing the Double First-Order in Parallel (DFOP) equation, determined as most suitable TBZ degradation kinetic model:

$$c_w(t) = C_0 \left(g * e^{-k_1 t} + (1 - g) * e^{-k_2 t} \right),$$

where c_w is the concentration in water, C_0 the starting concentration [g/L], t is the time [days], g the fraction of C_0 applied to compartment one, k_1 and k_2 the respective rate constants in compartment one and two. Data were modeled with R (version 3.6.1) using the gmkim package [23]:

$$c_{twa} = \frac{C_0}{t} \left(\frac{g}{k_1} \left(1 - e^{-k_1 t} \right) + \frac{1 - g}{k_2} \left(1 - e^{-k_2 t} \right) \right).$$

For the different endpoints of each experiment, the relative growth rates (RGR) were calculated over the duration of each experiment (14 and 35 days). The RGR based on plant length (TSL RGR and MSL RGR) and on

number of leaves (NL RGR) for each replicate plant was calculated as follows:

$$\text{RGR} = [\ln(X_t) - \ln(X_0)]/t,$$

where X_t is the measurement of the endpoint at the end of the experiment (time t), X_0 is the measurement of the endpoint at the start of experiment (time 0) and t is the duration of the experiment (days).

EC_x values were calculated with the open source package openRtox1.1 tool based on Excel and R [24].

Results

TBZ concentrations in mesocosms 4 h after Folicur® application showed that a homogeneous distribution of the active substance in the systems was achieved, almost reaching the nominal concentrations (deviations of $\leq 16\%$ for all treatments; Table 2). During the course of the experiment, the TBZ concentrations decreased slowly, resulting in slightly different exposure concentrations for the macrophytes in experiment 1 and 2. As guidance for standard laboratory tests, the end concentration of the test substance in the medium should not decrease below 80% of the start concentration. This was the case for all treatments in this study except for the 5 $\mu\text{g/L}$ TBZ treatment for experiment 2 on day 35 (Table 2).

Start conditions concerning physicochemical water parameters and nutrient concentrations on day 0 were very similar in all mesocosms (Figs. 1 and 2). Mean water temperature varied slightly in experiment 1 compared to experiment 2 (18.7°C and 16.9°C, respectively; Fig. 1) due to longer experimental duration with decreasing temperature in autumn (September till October). We calculated EC_{10} and EC_{50} values for the tested macrophyte species using the start concentrations, as well as TWAs for comparison (Table 3). In general, EC_x values based on TWA were lower than values based on measured TBZ start concentrations, due to slight dissipation of the active ingredient over the experimental time (Tables 2 and 3).

Table 2 Measured concentrations of tebuconazole ($\mu\text{g/L}$ active ingredient of the product Folicur®) in treated mesocosm water on day 0, day 14, and day 35 as well as the time-weighted average (TWA) concentration for the different test durations

Nom	Day 0	Day 14	TWA 1–14	Day 35	TWA 1–35
5	4.2	3.6	3.5	2.8	3.2
50	46.3	41.3	40.0	38.1	38.5
100	93.2	79.5	77.1	75.2	76.6
500	418.2	409.6	368.7	353.1	367.8
1000	856.4	736.8	736.4	719.3	731.8
5000	4038.7	4009.0	3837.4	3834.6	3814.9

Growth rates of control plants differed for *M. spicatum* in experiment 1 and 2 while the RGRs were very similar for both duckweed species (Table 4). Overall, the coefficient of variation of control plants was very low and always below 15 %. Concentration dependent effects of TBZ were found on macrophytes used for the in situ experiments as well as on the macrophyte standing stock community in the mesocosms, and EC_x values for numerous endpoints and species could be calculated (Table 3 and Fig. 3). Of all tested macrophytes in the mesocosm study, the dicot *M. spicatum* was the most sensitive species with lowest EC_{50} for the endpoint MSL RGR after 14 d with 228 $\mu\text{g/L}$ TBZ based on TWA concentrations. A longer exposure time (35 d) did not increase the toxicity of TBZ and EC_{50} values ranged from 263 to 778 $\mu\text{g/L}$ TBZ for *M. spicatum* and were 368 $\mu\text{g/L}$ TBZ for the dicot *N. peltata* (endpoint NL based on TWA; Table 3). In contrast, the monocot species *P. natans*, *S. polyrhiza* and *L. trisulca* reacted far less sensitive than the tested dicot species. Of these monocot species, *S. polyrhiza* was the most sensitive with an EC_{50} of 1487 $\mu\text{g/L}$ TBZ (NL RGR, 14 d based on TWA).

In the highest treated mesocosm (5000 $\mu\text{g/L}$ TBZ), all dicot macrophytes died during the time of the in situ experiments (35 d) and macrophyte standing stock in the mesocosms did not recover at the end of the 8 months investigation time. These strong effects on dicot macrophytes and other organisms in the 5000 $\mu\text{g/L}$ TBZ treatment were also reflected in the water parameters O_2 , pH and conductivity (Fig. 1), as well as in the nitrite and ammonia concentrations directly after TBZ application (Fig. 2). Considering the log transformation of pH values, concentration dependent effects were also identified for this water parameter directly after TBZ application (Fig. 1C). In the 500 $\mu\text{g/L}$ and 1000 $\mu\text{g/L}$ TBZ treatment, dicot macrophytes, which were grown directly as standing stock in the mesocosms, developed strong growth deformities during the course of the 8-month mesocosm study (Fig. 3). *Nymphoides peltata* produced crinkled, yellow leaves that died off a few weeks after TBZ application, while *M. spicatum* displayed a noticeable morphological alteration in the form of shorter stems and condensed leaf whorls (day 134 of TBZ exposure, Fig. 3).

Discussion

Negative effects of TBZ on dicot species were identified in the standing stock of the mesocosms as well as in the in situ experiments (Table 3, Fig. 3), which underlines that this paraphyletic group appears to be sensitive to the fungicide TBZ, given its mode of action as phytohormone. Of the dicot species in the mesocosms, *M. spicatum* was most sensitive, followed by the dicot species *N. peltata*.

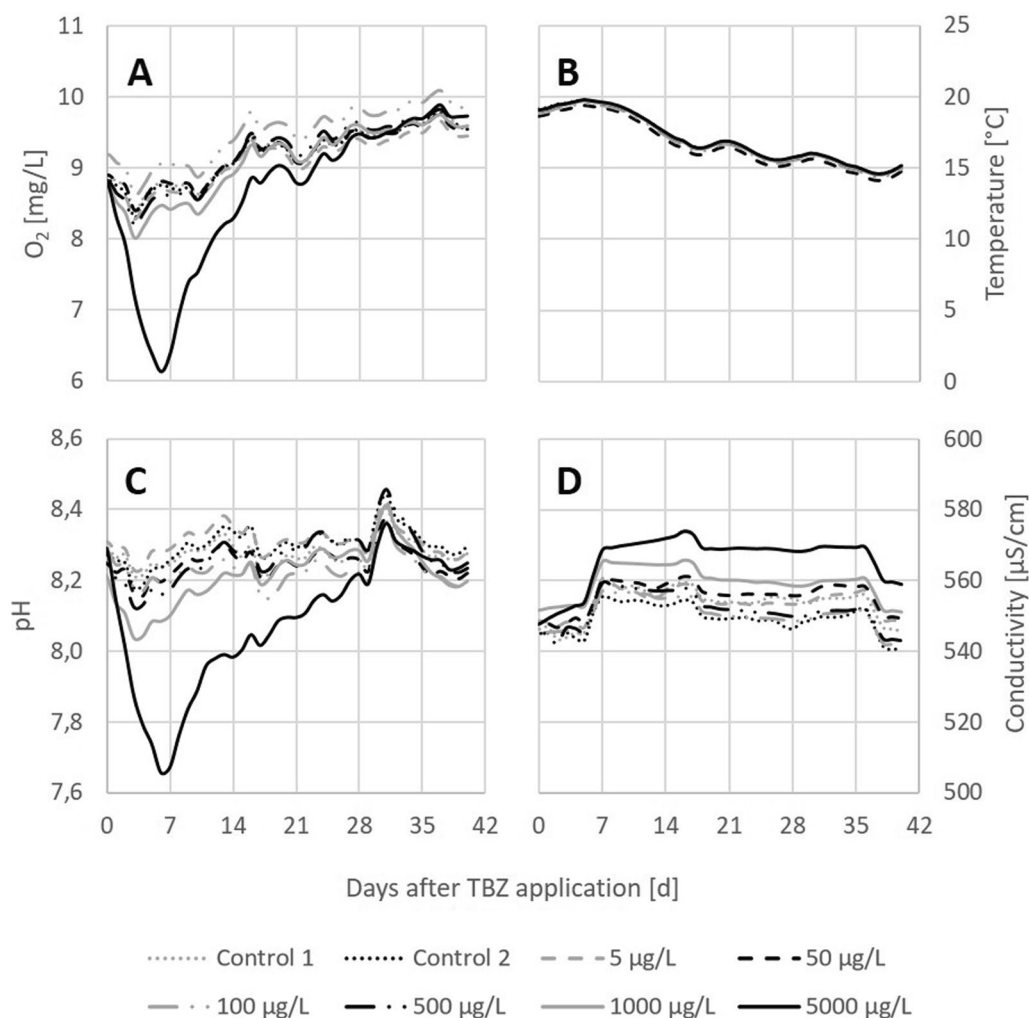


Fig. 1 Physicochemical parameters in mesocosm water (**A** oxygen, **B** temperature, **C** pH, and **D** conductivity) over the experimental time of in situ experiments

For *M. spicatum*, the EC_{50} ranges of 320–412 $\mu\text{g/L}$ TBZ, found in the in situ experiments, were in line with the EC_{50} ranges of 216–515 $\mu\text{g/L}$ TBZ for *M. spicatum* in the standard single-species water–sediment test according to OECD guideline 239 [17] (Table 3), which was carried out subsequent to the mesocosm study. The findings of this in situ study, showing that dicot species react sensitive to TBZ, are supported by Moreyra et al. [25]. The authors found that the roots of the dicot wetland plant *Bidens laevis* were very sensitive, with effects on the specific growth rate already starting at 0.1 and 10 $\mu\text{g/L}$ TBZ [25].

The fact, that a longer exposure time did not lead to a stronger effect on *M. spicatum* in our study (14 days compared to 35 days), may be explained by lower growth rates due to lower mean temperature and light conditions under progressing autumn conditions [26] (Table 4,

Fig. 1B). Potential recovery effects of *M. spicatum* after 35 days can be excluded, since TBZ concentrations decreased only slightly (Table 2) and effects on the standing stock of *M. spicatum* were still profound on day 134 of the comprehensive mesocosm study.

In comparison to the dicot species, the tested *Lemnaceae* displayed a much lower sensitivity and *P. natans* was not sensitive at all. Our results are in line with findings of Boros et al. [27]. Applying the OECD test 221, the authors found an EC_{50} (yield) of 1552 $\mu\text{g/L}$ TBZ for the duckweed species *L. minor*, which is in the same range as for *S. polyrhiza* (EC_{50} : 1487 $\mu\text{g/L}$ TBZ) for the endpoint NL-RGR in our study.

TBZ is known to have multiple modes of action affecting plants [9], but unfortunately little to nothing is known about specific modes of action on macrophytes. It remains unclear which mode of action of TBZ acted on

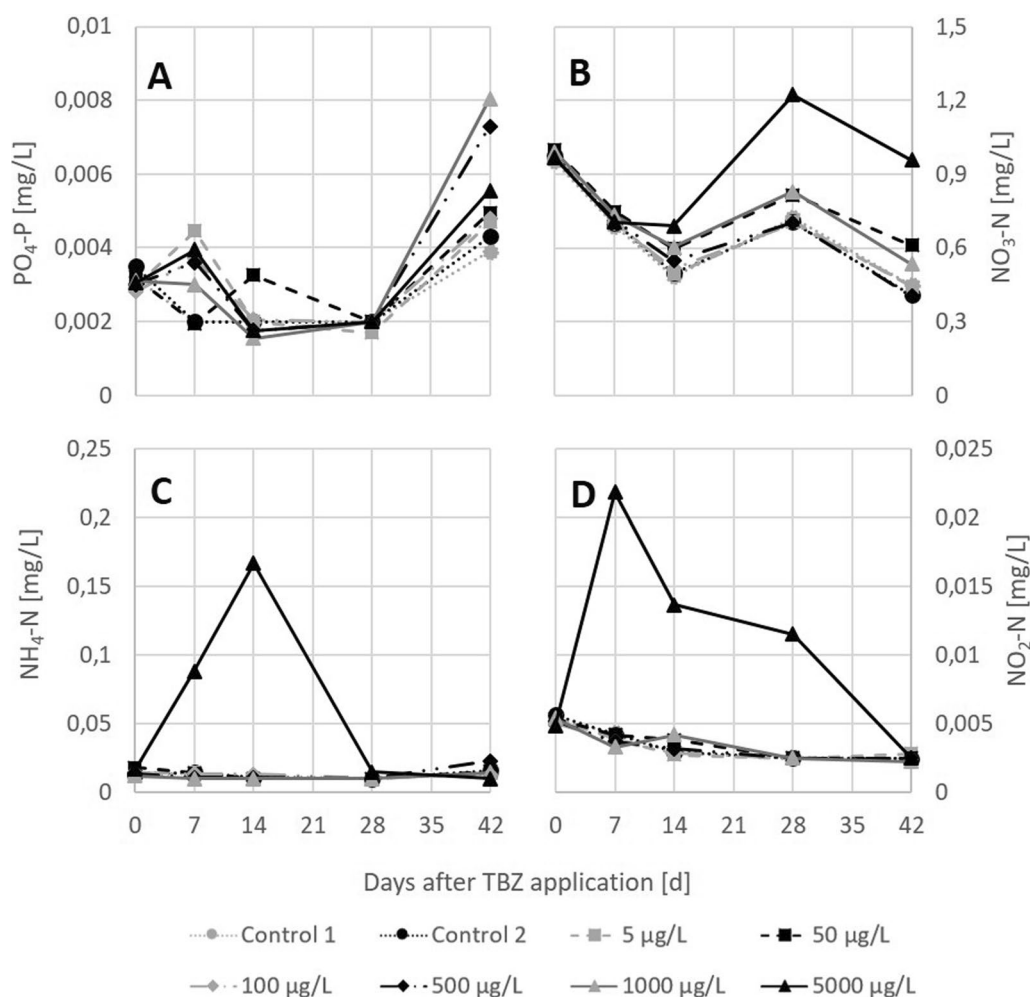


Fig. 2 Nutrient content in mesocosm water of phosphate (PO₄-P, **A**), nitrate (NO₃-N, **B**), ammonia (NH₄, **C**), nitrite (NO₂-N, **D**)

the metabolism of monocot species, while the effects on *Myriophyllum* can be linked to TBZ acting as phytohormone. The anti-gibberellic mode of action of TBZ leads to an inhibition of stem elongation and stimulates the formation of leaves and accumulation of biomass in dicot species, and is also known as “dwarfing effect” [11, 28]. In the present study, the length of *M. spicatum* shoots was found to be a more sensitive endpoint compared to biomass. Macrophyte length was also the most sensitive endpoint in the OECD test 239 [17], and can be explained by this dwarfing effect. Strong changes on *M. spicatum* plants, which manifested as short stems with dark green whorls and very short distances between nodes, were also observed in *M. spicatum* plant morphology of the mesocosm standing stock community after long-term exposure to TBZ in the 500 and 1000 µg/L treatment (Fig. 3). For tomato plants exposed to TBZ foliage treatment, Rogach et al. [11] confirm this observation. The authors found that treatment with TBZ did not change the

number of leaves and dry weight of whole tomato plants, but that chlorophyll content in the leaves increased and stem elongation was inhibited. While the use of TBZ optimized crop capacity of the treated tomato plants, it remains unknown if the observed growth deformities have an impact on performance and fitness of *M. spicatum*. The mesocosm study started in late summer and was run over the winter period without the possibility of monitoring flowering and fruit-setting of *M. spicatum* and the other dicot macrophyte species.

The situation in the field could be mimicked very well in terms of abiotic parameters such as pH and conductivity, as well as annual light regime and nutrient supply. The present study is therefore comparable to realistic outdoor conditions, where decreasing temperature and light conditions and competition for nutrients influence growth factors. The timing of the experimental start was chosen, consistent with the typical application periods for TBZ in the field. The

Table 3 EC₁₀ and EC₅₀ values calculated with measured start and time-weighted average (italic letters) tebuconazole concentrations (µg/L active ingredient of the product Folicur®) for different macrophyte species plus confidence interval (CI) for the experiment 1 (14 d) and 2 (35 d)

	Test species	Test duration	Endpoint	EC ₁₀ (CI)	EC ₅₀ (CI)
Dicot macrophytes	<i>Myriophyllum spicatum</i>	14 d	TSL RGR	71 (29–114) 61 (24–98)	333 (248–418) 289 (214–364)
		14 d	TSL	31 (–2 to 64) 26 (–2 to 54)	412 (248–576) 356 (223–500)
		14 d	MSL RGR	73 (31–116) 62 (26–99)	320 (240–401) 228 (207–349)
		14 d	MSL	35 (1.3–70) 27 (–2 to 55)	399 (251–547) 351 (213–490)
		14 d	NL	124 (63–186) 100 (47–152)	711 (574–847) 628 (504–752)
		35 d	TSL RGR	89 (55–123) 76 (46–105)	512 (436–588) 445 (279–512)
		35 d	TSL	50 (27–73) 42 (22–61)	340 (270–411) 294 (232–356)
		35 d	MSL RGR	75 (47–102) 63 (39–87)	402 (342–463) 349 (295–402)
		35 d	MSL	50 (11–71) 39 (21–57)	296 (239–354) 263 (208–318)
		35 d	NL RGR	200 (125–275) 176 (107–236)	778 (653–801) 676 (567–784)
	<i>Nymphoides peltata</i>	35 d	NL	29 (–36 to 94) 24 (–30 to 77)	424 (6–842) 368 (4–732)
	<i>Myriophyllum spicatum</i> (OECD test 239)	14 d	TSL RGR	44 (24–80)	515 (235–1088)
		14 d	TSL	23 (14–39)	216 (119–389)
		14 d	MSL RGR	37 (17–81)	372 (142–943)
		14 d	MSL	27 (12–60)	239 (93–600)
Monocot macrophytes	<i>Spirodela polyrhiza</i>	14 d	NL RGR	410 (–371 to 1191) 345 (–314–1003)	1660 (–140 to 3459) 1487 (–206 to 3180)
	<i>Lemna trisulca</i>	14 d	NL RGR	140 (–56 to 336) 121 (–51 to 293)	15,892 (689–31,095) 13,961 (1771–26,150)
	<i>Potamogeton natans</i>	35 d	NL	> 5000	> 5000
	<i>Lemna gibba</i> (ECHA 2013)	7 d	RGR	–	237
		14 d	RGR	–	144

MSL: main shoot length; TSL: total shoot length; NL: number of leaves/whorls; RGR: relative growth rate

Table 4 Mean growth rate (RGR), standard deviation (SD), and coefficient of variation (CV; %) of control plants in experiment 1 and 2

Species	<i>M. spicatum</i> 1 TSL	<i>M. spicatum</i> 2 TSL	<i>L. trisulca</i> 1 NL	<i>S. polyrhiza</i> 1 NL
Mean RGR	0.103	0.057	0.06	0.06
SD	0.015	0.003	0.007	0.009
CV	14.5	5.7	10.5	14.8

Test plants: *Myriophyllum spicatum*, *Lemna trisulca*, and *Spirodela polyrhiza*; TSL: total shoot length; NL: number of leaves/whorls

mesocosm study also showed several secondary effects on pH, oxygen and conductivity, as well as on nutrient concentrations in the water, due to the collapse of the

aquatic macrophytes at the highest TBZ concentrations. These types of effects can only be observed in higher-tier, realistic mesocosm studies [29, 30].

The toxic effects of TBZ on dicot macrophytes have largely been overlooked up to now. The current pesticide regulation [31] and data requirements [2] recommend a second test for a dicot species if a fungicide has an herbicidal mode of action. Given the fact that TBZ is also used as a growth regulator, a first-tier test with *M. spicatum* should be one of the data requirements. A draft of the renewal assessment report for TBZ is currently under annotation by the EU member states and has been made publicly available [15]. So far, a test with *Myriophyllum spicatum* is still missing. Results of an additional *L. gibba* test were added, but these resulted



Fig. 3 Growth deformations of *Myriophyllum spicatum* and *Nymphaeoides peltata* shoots during tebuconazole (TBZ) exposure in mesocosms. Top: *N. peltata* leaves in control and 1000 µg/L TBZ mesocosm on day 14; bottom: *M. spicatum* shoots in control and 1000 µg/L TBZ mesocosm on day 134

in 10 times higher EC_{50} (7 d) values of 2230 $\mu\text{g/L}$, compared to a *L. gibba* study published in former peer review documents and scientific opinions [10, 12]. The former study though, with an ErC_{50} (7 d) value of 237 $\mu\text{g/L}$, was ranked invalid due to analytical reasons [15]. The results of the present study and findings of Boros et al. [27] confirm this expert judgement, but also emphasize the need to implement data for dicot macrophyte species in the TBZ renewal assessment report.

Conclusions

The results of this study represent just one aspect of a comprehensive mesocosm experiment and emphasize the importance of mesocosm research, in which multiple factors, acting on biota as well as abiotic parameters, can be investigated on a scale larger than that of single-species laboratory experiments. The integration of short-term in situ experiments proved to be a valuable approach for generating reliable EC_x data for various non-standard macrophyte species with diverse growth forms under conditions that closely resemble natural environments. The findings of this study indicate that dicot macrophytes are more sensitive than monocots with respect to the phytohormonal effects of TBZ, and highlight the need to include effect studies on dicot macrophytes in the risk assessment of fungicides with phytohormonal mode of action.

Abbreviations

DW	Dry weight
EC_x	Effects concentration at which X% of organisms were affected
FW	Fresh weight
<i>L. trisulca</i>	<i>Lemna trisulca</i>
MSL	Main shoot length
<i>M. spicatum</i>	<i>Myriophyllum spicatum</i>
NL	Number of leaves/whorls
<i>N. peltata</i>	<i>Nymphoides peltata</i>
<i>P. natans</i>	<i>Potamogeton natans</i>
RGR	Relative growth rate
SI	Supplemental information
<i>S. polyrhiza</i>	<i>Spirodela polyrhiza</i>
TBZ	Tebuconazole
TSL	Total shoot length

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12302-025-01174-x>.

Additional file1 (PDF 637 KB)

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Author contributions

SM, MA, GA, US and VC were involved in the design, methodology and data processing of the experiment and wrote the manuscript. CP, SB and GA contributed to modifications of the manuscript. SB measured, analyzed and validated TBZ concentrations. CP was responsible for the evaluation of the OECD 239 test in addition to this experiment. All authors have reviewed and agreed to the published version of the manuscript.

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Availability of data and materials

No datasets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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