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Effects of the fungicide azoxystrobin in two habitats representative of mediterranean coastal wetlands: A mesocosm experiment

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ABSTRACT

This paper investigates the effects of the fungicide azoxystrobin, a compound widely used in rice farming, on aquatic communities representative of two habitats characteristic of Mediterranean wetland ecosystems: water springs and eutrophic lake waters. The long-term effects of azoxystrobin were evaluated on several structural (phytoplankton, zooplankton, macroinvertebrate populations and communities) and functional (microbial decomposition, macrophyte and periphyton growth) parameters making use of freshwater mesocosms. Azoxystrobin was applied in two pulses of 2, 20, 200 μg/L separated by 14 d using the commercial product ORTIVA (23 % azoxystrobin w/w). The results show that these two habitats responded differently to the fungicide application due to their distinct physico-chemical, functional, and structural characteristics. Although overall sensitivity was found to be similar between the two (lowest NOEC $<$ 2 μg/L), the taxa and processes that were affected differed substantially. In general, the most sensitive species to the fungicide were found in the water spring mesocosms, with some species of phytoplankton (*Nitzschia* sp.) or macrocrustaceans (*Echinogammarus* sp. and *Dugastella valentina*) being significantly affected at 2 μg/L. In the eutrophic lake mesocosms, effects were found on phytoplankton taxa (*Desmodesmus sp.* and *Coelastrum sp.*), on numerous zooplankton taxa, on chironomids and on the beetle *Colymbetes fuscus,* although at higher concentrations. The hemipteran *Micronecta scholtzi* was affected in both treatments. In addition, functional parameters such as organic matter decomposition or macrophyte growth were also affected at relatively low concentrations (NOEC 2 μg/L). Structural Equation Modelling was used to shed light on the indirect effects caused by azoxystrobin on the ecosystem. These results show that azoxystrobin is likely to pose structural and functional effects on Mediterranean wetland ecosystems at environmentally relevant concentrations. Moreover, it highlights the need to consider habitat-specific features when conducting ecotoxicological research at the population and community levels.

1. Introduction

Mediterranean coastal wetlands host a large diversity of habitats, which are determined by their ample hydrological, physico-chemical, and biological gradients (Gascón et al., 2009; Pérez-Ruzafa et al., 2011, 2019). Such high degree of environmental heterogeneity suggests that responses to disturbances may vary significantly across (micro-) habitats within the same study area (Ostrowski et al., 2021). One of the most studied Mediterranean coastal wetlands is the Albufera Natural Park (ANP), which is located in Eastern Spain (Martínez-Megías and Rico 2022). The ANP includes a polymictic and oligohaline lake dominated by phytoplankton and subject to a high eutrophication degree, as well as a complex system of water springs (locally named *Ullals*) surrounding the lake. Such water springs are dominated by macrophytes, have lower conductivity, and host a high number of endemic species (Soria et al., 2021). In this area, rice cultivation is a common agricultural practice, with more than 60 % of the surface of the ANP dedicated to this crop. This extensive cultivation renders both the lake and the surrounding water spring habitats susceptible to pesticide contamination during the rice growing period.

Fungicides are commonly used in rice cultivation due to the humid environment associated with this crop, which results in high fungal

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growth rates (Dean et al., 2005; Zheng et al., 2013; Bevitori and Ghini, 2014). The most used fungicides under Mediterranean conditions belong to the strobilurin and the azole groups, which have demonstrated to be highly effective against spore germination and early host penetration (Balba, 2007). Numerous studies have been carried out in the ANP aimed at quantifying the concentrations of pesticides in both water and sediment, with fungicides being the most ubiquitous and hazardous compounds (Calvo et al., 2021; Rodrigo et al., 2022). This circumstance has been attributed to their application mode, consisting of a double aerial application in early August when the rice fields are flooded (Rodrigo et al., 2022). Additionally, a recent modelling study shows that the strobilurin fungicide azoxystrobin is one of the most hazardous substances for aquatic organisms in the ANP (Martínez-Megías et al., 2023). This is mainly due to its intrinsic properties such as a the relatively slow dissipation from the water phase (DT_{50} water =14–18 days) and its broad-spectrum toxicity (Jones and Lake, 2000; European Food Safety Authority, 2009; Zafar Iqbal et al., 2012; Gustafsson et al., 2010), which involves inhibiting the transfer of electrons from cytochrome-B to cytochrome-C in eukaryotic organisms, thus impairing or totally inhibiting cellular respiration (Bartlett et al., 2002).

The fungicide azoxystrobin has proven to be toxic to a wide range of non-target aquatic organisms (Rodrigues et al., 2013). According to current literature, zooplankton seems to constitute the most sensitive group to azoxystrobin, with the cladoceran *Daphnia magna* showing physiological effects at environmentally relevant concentrations (Warming et al., 2009). Nonetheless, copepods have been found to be the most sensitive taxa in different experiments, showing chronic effects at concentrations around 3 μg/L (e.g., a LOEC of 10 μg/L was shown by Lauridsen et al. (2003); a LOEC of 3 μg/L by Gustafsson et al. (2010); and a NOEC of 1 μg/L by Van Wijngaarden et al. (2014). In addition, effects on phytoplankton communities have also been detected, with desmids and diatoms appearing to be the most sensitive taxa (e.g. an EC50 of 98 μg/L for *Skeletonema costatum* was reported by European Food Safety Authority, (2009); a NOEC of 0.33 μg/L for *Cosmarium moniliferum* by van Wijngaarden et al., 2014). Although the concentrations of azoxystrobin found in the Albufera lake are not particularly high (about 2 μg/L on average, with peaks of 5 μg/L according to the annual ANP management reports) they raise concerns as they can be considered pseudo-persistent during late summer and exceed some chronic risk thresholds, e.g., the HC5 (Hazardous Concentration for the 5% of species) of 1.87 μg/L derived by van Wijngaarden et al. (2014). Recent attempts to model rice field discharge from irrigation ditches reveal concentration peaks approaching 80 μg/L in the most conservative scenarios (Martínez-Megías et al., 2023). Given azoxystrobiǹs environmental concentrations and current literature, it is likely that azoxystrobin is causing detrimental effects on the aquatic communities within the ANP, but also in other Mediterranean wetlands of international relevance (e.g. see Mhadhbi et al., 2019; Čelić et al., 2021).

Most ecotoxicological tests designed to assess the impact of azoxystrobin on aquatic communities have been conducted in northern and central European regions. Hence, most of the ecotoxicological information available for azoxystrobin originates from this area. Given that variability in community response to pesticide disturbance is linked to multiple local/landscape variables (Schäfer et al., 2007), the risk associated with azoxystrobin in Mediterranean wetlands may differ from that estimated for other aquatic ecosystems. Thus, it is crucial to determine the sensitivity of communities in coastal Mediterranean environments and to assess the associated risks of this substance.

The aim of this study was to assess the effects of the fungicide azoxystrobin on aquatic communities' representative of two Mediterranean wetland habitats found in the ANP and to compare their sensitivity with that of other communities representative of other regions. A mesocosm experiment was designed with a range of azoxystrobin concentrations (including environmentally relevant concentrations) and two types of model ecosystems representative of the water spring and the eutrophic lake habitats typically found in Mediterranean coastal wetlands. Based on this experiment, we assessed short and long-term direct and indirect effects on structural and functional parameters of these ecosystems, and derived safe environmental concentrations for each of these two habitats. The hypothesis was that given the differences in the structure of aquatic communities representing both habitats and their trophic status, different direct and indirect effects may arise, and different environmental thresholds for this fungicide may be derived. The results of this study highlight the complex nature of ecological systems, shaping different chemical responses at different levels of biological organization, and contributing to refine the risk assessment of azoxystrobin for Mediterranean coastal wetland ecosystems.

2. Materials and methods

2.1. Experimental design

A mesocosm experiment was performed between May and August of 2022 at the Albufera Biological Station, which is located in the Technical Management Office of the ANP in El Palmar (Valencia, Spain). The experiment consisted of 24 outdoor mesocosms (diameter: 140 cm; height: 95 cm, water depth: 80 cm) initially filled with 5 cm of sediment and 1230 L of dechlorinated tap water. During the mesocosm preparation phase, 10 shoots of *Myriohyllum spicatum* and 10 of *Ceratophyllum demersum* were planted in each mesocosm. Macroinvertebrates, zooplankton, and phytoplankton were inoculated from different water bodies in the ANP, according to the different habitats represented in the experiment (Supplementary Information, Text S1). Water was circulated once a week among the mesocosms belonging to the same habitat using a pump with a maximum flow rate of 11,000 L per hour. This pump was connected to a 40 mm hose and operated for 10 min in every tank, covering all possible mesocosm-combinations within the same treatment, to ensure that the communities were homogeneously distributed. The acclimatization period lasted for 4 weeks in total. During the acclimatization phase and during the experimental phase water losses caused by evaporation were compensated weekly with dechlorinated tap water to guarantee a relatively constant water level.

To replicate the differences in salinity and nutrient content between the water spring and the eutrophic lake habitats, the mesocosms simulating the eutrophic lake were subject to a single sodium chloride application and a bi-weekly nutrient input (Supplementary Information, Text S2). Nutrient additions started 3 weeks prior to the pesticide application, and were maintained during the course of the experiment.

In the study, the treatments involving water spring and eutrophic lake each had control mesocosms and mesocosms exposed to three concentrations of azoxystrobin (2 μg/L, 20 μg/L, 200 μg/L), with both control and the contaminated mesocosms having three replicates each. Azoxystrobin was applied twice with a time interval between applications of 14 days, simulating water exposure peaks after application of the fungicide in the rice fields. The 2 μg/L concentration emulated the concentrations that are systematically measured in the lake or in other protected areas, while the 20 μg/L simulates peak concentrations found in drainage channels nearby rice fields. The experiment was run for 56 days after the first fungicide application.

The formulated product ORTIVA (22.8 % azoxystrobin w/w; Spanish registration number 22,000), developed by Syngenta (Basel, Switzerland), was used in this study. The formulated product includes C16–18 ethoxylated alcohols (10–20 %), methanol (0.1–1 %), and 1,2-benzisothiazol-3(2H)-one (0.025–0.05 %). Out of these components only 1,2-benzisothiazol-3(2H)-one is considered to pose aquatic toxicity, with a predicted no effect concentration of 4 μg/L. In this experiment, the highest exposure concentration of this compound was 0.1 μg/L, which is an order of magnitude lower than the predicted no effect concentration. Therefore, the highest toxicological impact of this formulated product on aquatic ecosystems may be attributed to the principal active ingredient (i.e., azoxystrobin).

2.2. Azoxystrobin application, sampling, and analysis

Before each fungicide application, a stock solution was prepared in distilled water by diluting 4 mL of the formulated product in 996 mL of water resulting in a concentration of approximately 1 mg/mL. From this stock solution, 2.46 mL, 24.6 mL, and 246 mL were diluted with 1 L distilled water to obtain a final concentration of 2460, 24,600 and 246,000 μg/L. These dose solutions were applied to the mesocosms, resulting in treatment concentrations of 2, 20, and 200 μg /L (mesocosm volume: 1230 L). The control systems received 1 L of the same water without pesticide. The glass bottles were carefully emptied onto the surface of the tank. Subsequently the water was stirred using a wooden stick to ensure a good distribution of the substance over the water column.

Azoxystrobin sampling was performed on days 0, 7, 14 (before and after the second pulse), 21 and 56 relative to the first fungicide application. The sampling method consisted of taking 5 depth-integrated water samples of approximately 1.5 L with a PVC tube, mixing them in a bucket, homogenizing the sample and taking a 250 mL sub-sample in a 1 L borosilicate bottle. Excess water in the bucket was then returned to the corresponding mesocosm. Each azoxystrobin concentration treatment had its associated PVC sampler to avoid cross-contamination. The samples were stored at −20 °C until further analysis.

The quantification of azoxystrobin was done using aliquid chromatograph coupled to a triple quadrupole mass spectrometer. The equipment and analytical conditions of the analysis are provided in the Supplementary Information (Text S3, Table S1).

The dissipation coefficients (k) for azoxystrobin in the different treatments were determined through linear regression analysis of ln-transformed concentrations over the sampling times, using Microsoft Excel version 2010, assuming first-order kinetics. The half-life (DT_{50}) were calculated by dividing Ln(2) by the dissipation coefficient (k).

2.3. Water quality parameters

Dissolved oxygen, pH, electric conductivity, and temperature were measured in each mesocosm using a HANNA HI0194 automatic probe at 30 cm depth. These parameters were measured in the morning on days −7, 7, 21, and 56 relative to the first fungicide application. Concentrations of ammonia, nitrate, nitrite, total nitrogen, orthophosphate, and total phosphorus were also evaluated to observe possible changes in the metabolism of the microbial community. The sampling process was conducted by collecting two samples (50 mL each) in high-density polyethylene (HDPE) bottles from the same integrated sample from which the azoxystrobin sample was obtained. One sample was filtered back in the lab, while the other remained unfiltered. Following collection, samples were frozen at a temperature of −20 °C. More details regarding the nutrient analysis are provided in the Supplementary Information (Text S4).

2.4. Phytoplankton, zooplankton and macroinvertebrates sampling and identification

Phytoplankton, zooplankton and macroinvertebrates were sampled on days −7, 7, 21, 35 and 56 relative to the first fungicide application. The methods used for sampling and identification are provided in the Supplementary Information (Text S5-S7).

2.5. Organic matter decomposition, macrophyte growth and biofilm colonization

Organic matter decomposition, macrophyte growth (*Myriophyllum spicatum* and *Ceratophylum demersum*) and biofilm colonization were assessed biweekly. More details on these analyses can be found in the Supplementary Information (Text S8-S10).

2.6. Data analyses

The Principal Response Curve (PRC) method, introduced by Van den Brink and Braak (1999), was used here to evaluate the impact of the test substance on the phytoplankton, zooplankton, and macroinvertebrate communities of each treatment. PRCs were computed to compare the control groups of each treatment and to examine differences in community composition over time. The PRC method helps to visualize the variation between the treated communities and the controls at the different sampling times (C_{dt}), so that the larger the C_{dt} the larger the structural variation of the treated communities as compared to the control. It also allows the calculation of the affinity of each taxon with the PRC (b_k), so that the species with the highest b_k values show a population decline, and the species with negative b_k values a population increase related to the chemical concentration (for further details see Van den Brink and Braak, 1999). For each sampling date and community (i.e. phytoplankton, zooplankton and macroinvertebrates), the results of the abundances of the different taxa were split by their respective treatments. In this way, two daily data sets were obtained for each community, each consisting of three replicates of the different concentration regimes. Consequently, the two final datasets comprised 12 daily observations across 5 sampling days, resulting in a total of 60 observations per treatment. To assess the overall significance of azoxystrobin on the variation in community composition, 499 Monte Carlo permutations were performed in each dataset. Furthermore, a Redundancy Analysis (RDA) was conducted to determine the influence of azoxystrobin on each community at each sampling date. A community NOEC was calculated for each sampling date and treatment using the sample scores of the first axis of the Principal Component Analysis (PCA) of the corresponding dataset and using the Williams test (Williams 1972) with such sample scores. These multivariate analyses were carried out using the CANOCO software, version 5 (Ter Braak and Smilauer, 2002), and the Williams test was conducted with the Community Analysis computer program, version 4.3 (Hommen et al., 1994). Prior to the analysis, the organism abundance data was ln (Ax+1) transformed, following the method described in Van den Brink et al. (2000). For phytoplankton, the A value was 33.3, while for zooplankton it was 10 and for macroinvertebrates it was 2. All statistical tests were performed using a significance level (α) of 0.05.

Effects of the test substance on the individual populations were deemed consistent and treatment-related when the four criteria shown in the Supplementary Information (Text S11) were met.

Although the PRC method provides valuable information regarding the toxicological effects, it falls short in providing insights into the underlying mechanisms that drive those impacts. To address this limitation and to gain a deeper understanding of the direct and indirect effects caused by azoxystrobin in the different treatments a Structural Equation Modelling (SEM) approach was used. SEM is a statistical technique used to examine the relationships between different variables and to test their causal connections, allowing he investigation of both direct and indirect effects among variables (Shipley, 2016). The process involves specifying an initial model based on existing theoretical knowledge and then assessing whether the model's implied covariance structures align well with the actual covariance structures observed in the dataset.

The SEM was carried out with the R *lavaan* package, incorporating a matrix comprising both structural and functional data. The different taxa were divided into trophic groups, distinguishing between phytoplankton, microzooplankton, macrozooplankton, herbivorous macroinvertebrates, detritivores, and predators, and the total abundance for each of them was calculated. Subsequently, data were log-transformed to fulfil SEM linearity and normality assumptions. The model was built using data from days 7 and 21 of the experiment, under the premise that the most significant effects were observed on this period. The model was built assuming an effect of azoxystrobin and the treatment water spring or eutrophic lake on all trophic group abundances and functional parameters. We also assumed a potential covariation between all trophic groups and functional parameters that could indicate the propagation of toxic effects along the food-web, as is commonly seen in ecotoxicological studies (Rumschlag et al., 2020; 2022). Thus, a first saturated model with perfect fit and 0 degrees of freedom was constructed. From this first saturated model, non-significant direct relationships were eliminated to ensure statistical robustness, resulting in a final unsaturated model. In evaluating the model, we utilized several fitting indices for a comprehensive assessment: the Chi-square test for overall model fit, the RMSEA (root mean square error of approximation) for model approximation error, and the CFI (comparative fit index) for comparative fit against a baseline model. The SRMR (standardized root mean squared residual) provided a standardized measure of the residual differences, while the GFI (goodness of fit index) and AGFI (adjusted goodness of fit index) evaluated the proportion of variance explained by the model (Kline, 2023). These indices collectively offered a complete understanding of the model's performance, highlighting model's strengths and weaknesses.

3. Results

3.1. Azoxystrobin concentrations in water

The measured azoxystrobin concentration after the first azoxystrobin application was 98 % of the nominal concentration in the eutrophic lake treatment and 91 % in the water spring treatment, indicating an accurate dosing of the test compound. The second application resulted in an increase in azoxystrobin concentrations above the nominal concentration due to compound build-up in the test systems (Fig. 1). The dissipation of azoxystrobin was observed to be higher in the eutrophic lake treatment ($k = 0.066 \pm 0.009$, $DT_{50} = 11$ days) compared to the water spring treatment ($k = 0.028 \pm 0.006$, $DT_{50} = 25$ days) across all tested concentration regimes (Fig. 1). This implied that, comparatively, the Time Weighted Average Concentrations (TWACs) obtained for the water spring treatment were higher than those in the eutrophic lake treatment (Table 1).

3.2. Water quality parameters

The eutrophic lake treatment showed a higher concentration of nutrients, particularly N, and higher conductivity than the water spring treatment. During the experiment, a significant rise in water temperature and conductivity in both treatments was observed, together with a decrease of oxygen concentrations (Table 2). Notably, on the last day of the experiment, the treatments converged in terms of nutrient concentrations. This convergence aligns with the shift in phytoplankton community dynamics shown in Figure S1, particularly the increased abundance of *Pediastrum* sp. within the eutrophic lake treatment. This shift in phytoplankton composition, likely due to the consistent nutrient supply, could have led to changes in nutrient cycling within the ecosystem. No consistent significant azoxystrobin-related effects were found on nutrient concentrations, electrical conductivity, and dissolved oxygen. Therefore, based on the results of this study it can be concluded that azoxystrobin NOECs for the measured water quality parameters are all higher than 200 μg/L (Table S2).

3.3. Phytoplankton effects

In total, 45 different phytoplakton taxa were identified in the mesocosms over the experimental period. Despite the differences in physico-chemical parameters and introduced inocula, taxonomic composition was found to be somewhat similar between treatments (Figure S1). The difference lies in the abundances at which the various taxa appear, with abundances being considerably higher within the eutrophic lake treatment. *Desmodesmus* sp. was highly prevalent in both treatments, alongside with the Cryptophyceae *Rhodomonas sp.* in the water spring treatment and *Cryptomonas* sp. in the eutrophic lake treatment. In the latter, colonial Chlorophytes such as *Pediastrum* sp. were also abundant, while in the water spring treatment smaller species such as *Oocystis* sp. predominated, especially as the experiment progressed.

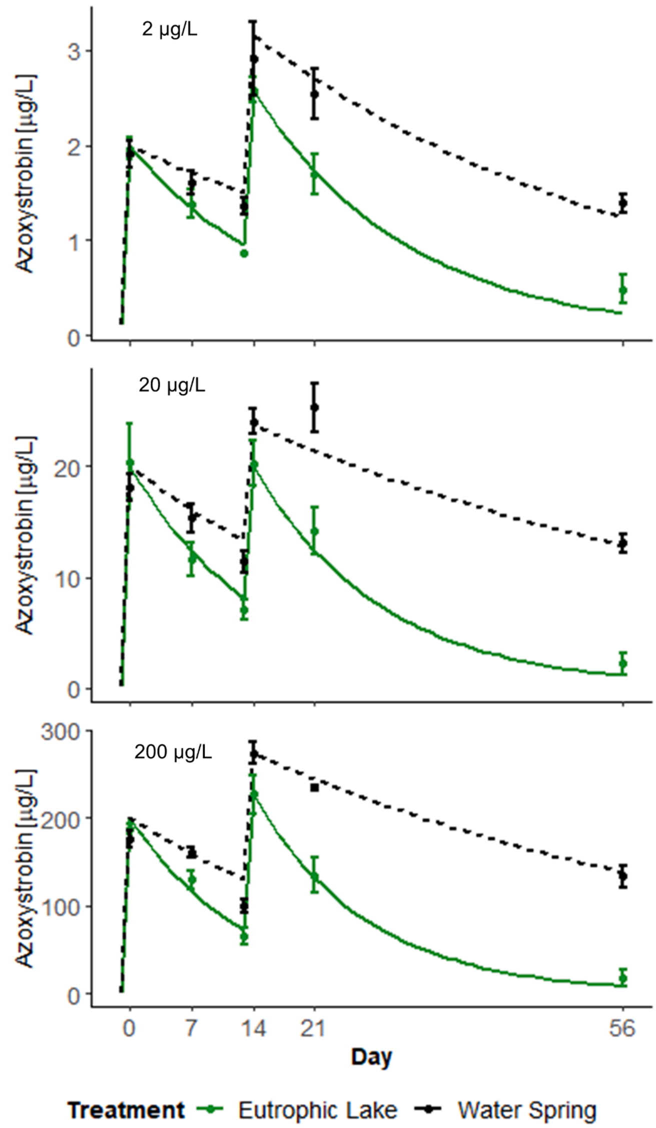

Fig. 1. Concentration dynamics of azoxystrobin in the different treatments. Dots indicate the mean measured concentrations with their standard deviation and the line the modelled concentration based on first-order kinetics.

The effects of azoxystrobin on the phytoplankton community corresponding to the eutrophic lake treatment were only significant on day 21 for the 200 μg/L concentration (Table 3). The species with the highest b_k scores were *Desmodesmus* spp., *Cryptomonas* sp.*, Coelastrum sp,* which indicate a decline in abundance, while *Rhodomonas sp.*, showed a negative one, indicating an abundance increase (Fig. 2A). Among these, only *Desmodesmus* sp. and *Coelastrum* sp. showed a signficant population decline with increasing azoxystrobin concentrations, with NOECs of 20 and 2 μg/L, respectively (Table 3).

Regarding the water spring treatment, significant effects on the phytoplankton community were found on day 35 for the 20 μg/L and 200 μg/L concentrations, and on day 56 for the 200 μg/L concentration (Table 3). A slight recovery was found in the 2 μg/L and the 20 μg/L concentrations on the last day of the experiment (Fig. 2B). The PRC graph shows that the species with the highest b_k scores were the diatom *Nitzschia* spp. and the chlorophyte *Desmodesmus sp.*, while *Cryptomonas sp.* was found to show the lowest b_k score (Fig. 2B); although the Monte Carlo permutation test indicated that the fist axis did not display a

Table 1
Calculated Time Weighted Average Concentrations (TWACs) for an experimental period of 7, 14, 21 and 56 days (D) after the first azoxystrobin application. Higher concentrations were observed within the water spring treatment due to slower dissipation.

Concentration	Eutrophic lake treatment				Water spring treatment			
	D7	D14	D21	D56	D7	D14	D21	D56
2 μg/L	1.7	1.4	1.7	1.3	1.8	1.7	2.0	2.0
20 μg/L	16.0	13.0	14.4	10.5	16.7	15.3	18.4	18.9
200 μg/L	158	131	148	103	168	153	187	185

Table 2
Physico-chemical parameters measured in the control mesocosms of the eutrophic lake and the water spring treatments throughout the experiment. The main differences between treatments are caused by the biweekly nutrient input to the eutrophic lake habitat and the simulated salinity difference. T: temperature.

	Eutrophic lake treatment				**Water spring treatment**			
Parameter	**D-7**	**D7**	**D21**	**D56**	**D-7**	**D7**	**D21**	**D56**
Temperature (°C)	21.4 ± 0.26	24.5 ± 0.17	25.1 ± 0.55	29.3 ± 0.25	21.4 ± 0.27	24.4 ± 0.21	25.53 ± 0.1	29.05 ± 0.05
Dissolved oxygen [mg/L]	6.94 ± 1.43	12.5 ± 2.13	8.59 ± 0.33	4.45 ± 3.31	8.67 ± 0.97	6.63 ± 1.53	7.39 ± 0.06	7.59 ± 0.49
Dissolved oxygen (%sat)	78.7 ± 15.99	150 ± 26.6	105 ± 4.36	58.9 ± 43.3	98.2 ± 11.3	79.53 ± 18.38	91.0 ± 0.00	99.7 ± 6.05
Electric conductivity (μS/cm)	1102 ± 41.2	1164 ± 8.39	1542 ± 25.8	1807 ± 15.7	606 ± 26.1	674 ± 27.8	925 ± 36.0	1123 ± 23.5
pH	8.77 ± 0.60	9.78 ± 0.05	9.66 ± 0.10	8.39 ± 1.01	9.24 ± 0.27	8.50 ± 0.45	8.90 ± 0.11	8.39 ± 0.04
$NH4^{+}$ [mg/L]	1.24 ± 0.91	0.28 ± 0.01	0.43 ± 0.27	0.10 ± 0.11	0.02 ± 0.04	0.00 ± 0.00	0.01 ± 0.01	0.19 ± 0.04
$NO3^{-}$ [mg/L]	2.52 ± 0.42	2.83 ± 0.54	8.26 ± 4.44	1.23 ± 0.47	0.42 ± 0.02	0.26 ± 0.02	1.30 ± 0.21	1.19 ± 0.13
Total N [mg/L]	14.1 ± 0.93	17.2 ± 2.07	23.9 ± 3.78	46.7 ± 3.96	2.15 ± 0.08	3.11 ± 0.43	9.87 ± 4.17	32.2 ± 8.95
Ortophosphate [mg/L]	0.14 ± 0.02	0.27 ± 0.12	0.06 ± 0.05	0.07 ± 0.08	0.07 ± 0.11	0.00 ± 0.00	0.01 ± 0.01	0.04 ± 0.01
Total P [mg/L]	0.17 ± 0.02	0.23 ± 0.11	0.16 ± 0.04	0.21 ± 0.076	0.06 ± 0.02	0.13 ± 0.07	0.07 ± 0.03	0.15 ± 0.06

Table 3
Calculated NOECs for the phytoplankton, zooplankton, and macroinvertebrate communities and populations. Only the taxa that met the criteria to characterize consistent azoxystrobin-related effects in at least one sampling date are displayed, while the rest are presented in the Supplementary Information. NOECs that were considered consistent and treatment-related are indicated in bold. The super-index refers to the exclusion criterion: [a]there were insufficient abundance of organisms; [b] effects did not occur in a dose-response manner. ↑: abundance increase; ↓: abundance decrease. D: day relative to the first azoxystrobin application. Empty cells refer to sampling dates in which the taxon was not present, so a NOEC could not be calculated. NOECs are expressed in μg/L.

	Eutrophic lake habitat					**Water spring habitat**				
	D-7	**D7**	**D21**	**D35**	**D56**	**D-7**	**D7**	**D21**	**D35**	**D56**
Phytoplankton										
Community	>200	>200	**20**	>200	>200	>200	>200	>200	**2**	**20**
Populations										
Desmodesmus spp.	>200	>200	20↓[b]	>200	**20↓**	>200	>200	>200	20↓[b]	20↓[b]
Coelastrum sp.	>200		>200	**2↓**	2↓[b]	>200	>200	>200	2↓[a]	>200
Nitzschia spp	>200	>200	>200	20↓[b]	200↑[b]	>200	>200	>200	**2↓**	**2↓**
Zooplankton										
Community	>200	**20**	>200	>200	>200	>200	**<2**	>200	**<2**	**2**
Populations										
Daphnia sp.	>200	<2↓[b]	>200	2↓[b]	>200	>200	20↓[b]	**<2↓**	>200	**<2↓**
Ceriodaphnia sp.	>200	20↓[b]	**<2↓**	**<2↓**	>200	>200	**<2↓**	20↓[b]	>200	>200
Scapholeberis sp.	>200	>200	>200	>200	20↑[a]	>200	20↓[b]	**<2↓**	>200	>200
Chydorus sp.	>200	>200	>200	**20↓**	>200	>200	<2↓[b]	>200	>200	2↑[b]
Calanoida	>200			>200	>200	>200	20↓[b]	**<2↓**	>200	**<2↓**
Nauplii	>200	>200	>200	20↓[b]	20↓[b]	>200	**<2↓**	200↑	20↑[b]	>200
Lecane lunaris	>200	>200	>200	>200	**2↓**	>200	>200	>200	2↑[a]	>200
Trichocerca sp.	>200	>200	>200	>200		>200		**<2↓**	>200	20↑
Hydra	>200	>200	>200			>200	>200	**<2↓**	**<2↓**	
Euchlanis sp.		>200	>200	>200	>200			>200		**<2↓**
Brachionus angularis				**2↓**	>200					2↑[a]
Macroinvertebrates										
Community	20↑	>200	**20**	**20**	**20**	>200	**20**	**<2**	**<2**	**2**
Populations										
Caenidae	>200	>200	2↓[b]	20↓[b]	20↓[b]	>200	>200	>200	**20↓**	>200
Cloeon sp.	>200	>200		>200	>200		>200	**2↓**	>200	>200
Chironomini	2↓[b]	>200	>200	>200	**20↓**	>200	>200	>200	>200	20↑[b]
Colymbetes fuscus (adult)	>200	>200	>200	**2↓**	2↓[a]	>200	>200	>200	20↑[a]	>200
Colymbetes fuscus (larvae)	>200	>200	>200	>200	**<2↓**	>200	>200	<2↓[a]	>200	
Echinogammarus sp.	>200	>200	>200	>200		>200	20↓[b]	20↓[b]	**20↓**	**<2↓**
Dugastella valentina	>200	20↓[b]	>200		>200	>200	20↓[b]	<2↓[b]	**<2↓**	2↓[b]
Micronecta scholtzi	20[b]	>200	**20↓**	20↓[b]	>200	>200	>200	**<2↓**	>200	2↓[b]
Orthocladineae	>200	>200	>200	2↓[a]	**20↓**	>200	>200	20↓[a]	2↑[a]	>200

significant amount of variance. Only *Nitzschia sp*. exhibited significant fungicide-related effects at concentrations exceeding 2 μg/L on days 35 and 56 (Table 3).

3.4. Zooplankton effects

We identified a total of 34 zooplankton taxa within our experimetnal set-up. In general, there was a higher overall abundance of cladocerans

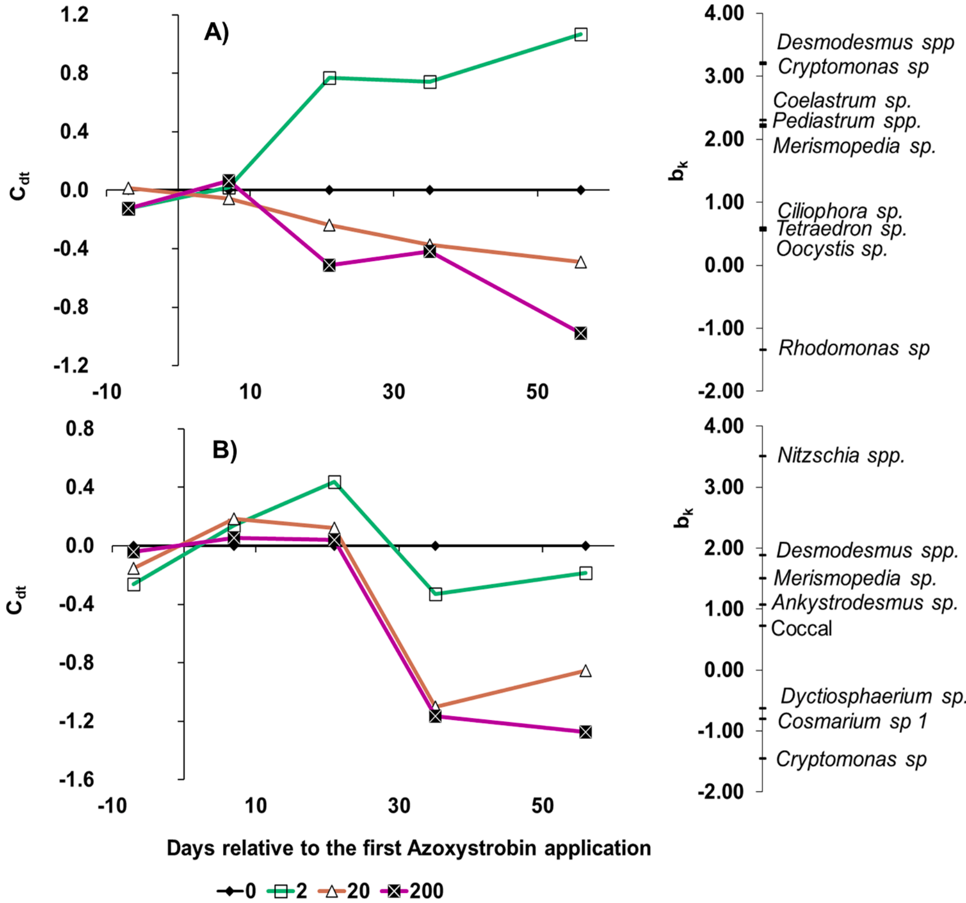

Fig. 2. Principal Response Curves showing the effects of azoxystrobin on the phytoplankton community of the eutrophic lake treatment (A) and the water spring treatment (B). The species weight (b_k) can be interpreted as the affinity of each taxon with the principal response curves (C_{dt}). Taxa with a species weight (b_k) between −0.5 and 0.5 are not displayed. In the case of eutrophic lake treatment, 24 % of all variance could be attributed to the sampling date (displayed on the horizontal axis) and 37 % to the fungicide application, out of which 56 % is displayed on the vertical axis. The Monte Carlo permutation test indicated that the diagram displays a significant amount of the variance explained by the fungicide application ($p = 0.024$). In the water spring treatment, 42 % of all variance could be attributed to sampling date (shown on the horizontal axis), and 21 % to the fungicide application, out of which 27 % is shown in the vertical axis. The Monte Carlo permutation test indicated that the diagram did not display a significant amount of variance ($p = 0.146$).

in the eutrophic lake treatment, as shown in Figure S2. Differences between the communities were explained by the greater abundance of *Ceriodaphnia sp*. and *Chydorus sp*. and a greater diversity of rotifera (dominated by *Lecane lunaris*) in the eutrophic lake treatment as compared to the water spring one. Conversely, *Synchaeta* sp. demonstrated a preference for the treatment that replicated water spring conditions.

The zooplankton community of the eutrophic lake treatment showed significant effects caused by the fungicide on day 7, with a NOEC of 20 μg/L (Table 3). After computing the PRC, the cladoceran *Ceriodaphnia*, copepod's nauplii, and two species of the genus *Brachionus* obtained the highest scores, while smaller rotifers such as *Lecane* or *Lepadella* obtained the most negative b_k values (Fig. 3A). Consistent treatment-related effects were found for the cladocerans *Ceriodaphnia* (NOEC <2 ug/L on days D21 and D35), and *Chydorus* (NOEC 20 ug/L on day D35), which showed significant abundance declines. Within microzooplankton, the affected taxa were *Lecane lunaris* (NOEC <2 ug/L on day D56), and *Brachionus angularis* (NOEC <2 ug/L on days D35).

Significant impacts of azoxystrobin on the zooplankton community representative of the water spring habitat were observed on days 35 and 56, with calculated NOECs of 2 μg/L and <2 μg/L, respectively (Table 3). The cladoceran *Chydorus sp*., the cyclopoid copepods, and the rotifer *Synchaeta sp*. were the taxa exhibiting the highest b_k scores. Conversely, the rotifers *Hexathra* and *Lecane lunaris* displayed negative b_k scores, indicating an abundance increase in the mesocosms with high fungicide concentrations (Fig. 3B). Multiple taxa, comprising cladocerans, copepods and rotifers, exhibited NOECs below 2 μg/L (Table 3). Consistent azoxystrobin-related abundance declines were found on *Daphnia* (NOEC <2 μg/L on days D21 and D56), *Ceriodaphnia* (NOEC <2 μg/L on day D7) and *Scapholeberis* (NOEC <2 μg/L on day D21). Regarding copepods, effects were found on Calanoida (NOECs <2 μg/L on days D21 and D56) and nauplii (NOEC <2 μg/L on day D7). Among microzooplankton, the affected taxa were *Trichocerca* (NOEC <2 μg/L on days D21 and D35), *Hydra* (NOEC <2 μg/L on days D21 and D35) and *Euchlanis* (NOEC <2 ug/L on day D56).

3.5. Macroinvertebrate effects

Twenty-nine macroinvertebrate taxa were identified. Different community structure was observed between the two treatments, with the water spring community showing a higher abundance of crustaceans and a greater diversity of gastropod species, including *Theodoxus* sp. and *Melanopsis sp*. On the other hand, the mesocosms representing the eutrophic lake habitat contained higher abundances of *Chironomidae* and *Micronecta scholtzi* (Figure S3).

The results of the PRC assessing the effects of azoxystrobin on the macroinvertebrate communities are shown in Fig. 4. In the case of the eutrophic lake treatment, the RDA yielded statistically significant effects

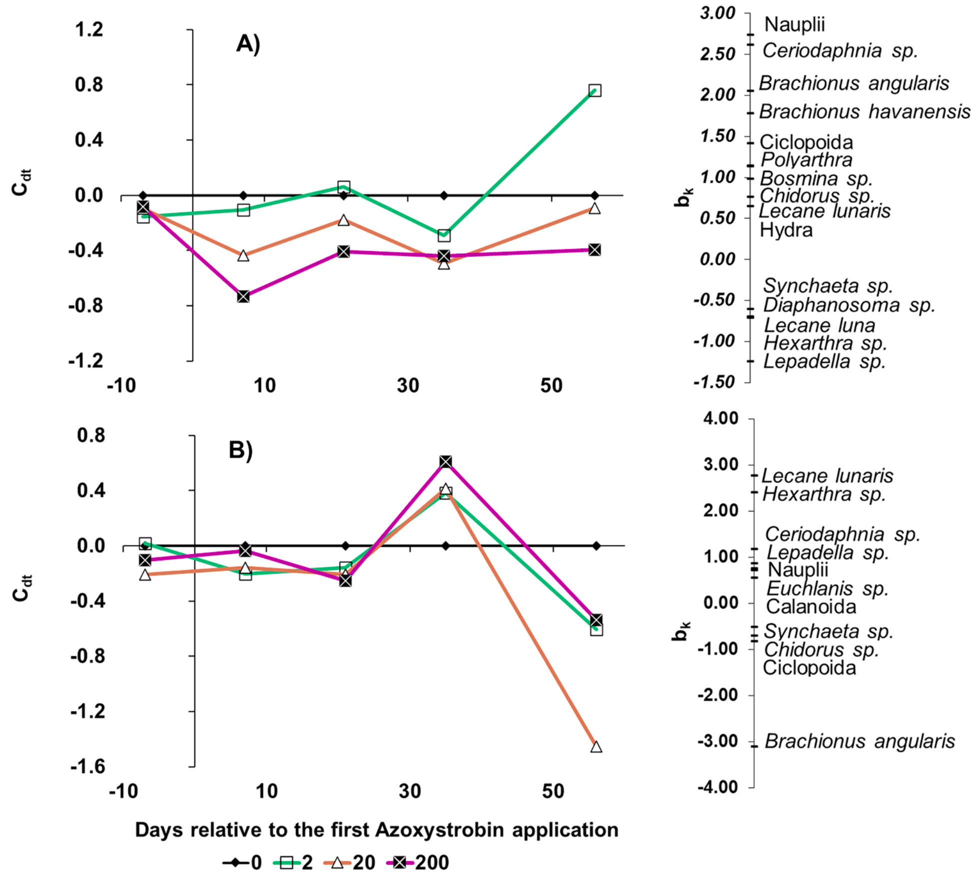

Fig. 3. Principal Response Curves showing the effects of azoxystrobin on the zooplankton community of the eutrophic lake treatment (A) and the water spring treatment (B). The species weight (b_k) can be interpreted as the affinity of each taxon with the principal response curves (C_{dt}). Taxa with a species weight (b_k) between −0.5 and 0.5 are not displayed. In the case of the eutrophic lake treatment (A), 21 % of all variance could be attributed to the sampling date (displayed on the horizontal axis) and 28 % to the fungicide application, out of which 27 % is displayed on the vertical axis. The Monte Carlo permutation test indicated that the diagram displays a significant amount of the variance explained by the fungicide application ($p = 0.002$). In the water spring treatment (B), 23 % of all variance can be attributed to sampling date (shown on the horizontal axis), and 32 % to the fungicide application, 28 % of which is shown on the vertical axis. The Monte Carlo permutation test indicated that the diagram displays a significant amount of the variance explained by azoxystrobin ($p = 0.010$).

of azoxystrobin on days 21, 35 and 56, with a community NOEC of 20 μg/L (Table 3). The hemipteran *M. scholtzi*, the *Caenidae* family (Ephemeroptera), and the diptera Orthocladinae and Chironomini exhibited the highest b_k scores, whereas the *Hydroglyphus geminus* (beetle) was associated to higher concentrations (Fig. 4A). Consistent azoxystrobin-related effects were only found for *Chiromini* (NOEC: 20 μg/L; D56), Orthocladinae (NOEC: 20 μg/L; D56), *Colymbetes fuscus* (Adult: NOEC: 2 μg/L; D35; Larvae: NOEC: <2 μg/L; D56) and *Micronecta* (NOEC: 20 μg/L; D35), which showed a population decline (Table 3). Fig. 5 illustrates the changes in abundance of *Chiromini* and *M. scholtzi* over the course of the experiment.

Azoxystrobin had a significant impact on the community structure of the water spring treatment, with community NOECs of 20 μg/L on day 7, <2 μg/L on day 21 and 35, and 2 μg/L on day 56 (Table 3). The macrocrustacean taxa (i.e., shrimps and gammarids) showed the highest b_k scores, indicating abundance declines due to the increasing azoxystrobin concentration, while at higher concentrations more chironomids appeared (Fig. 4B). Significant abundance declines were found for Caenidae (NOEC 20 μg/L on day 35), *Cloeon* sp. (NOEC 2 μg/L on day 21), *Echinogammarus* sp. (NOEC 20 μg/L on day 35, NOEC <2 μg/L on day 56), *Dugastella valentina* (NOEC <2 μg/L on day 35), and *Micronecta scholtzi* (NOEC <2 μg/L on day 21). Fig. 5 displays the variations in the populations of the macrocrustaceans *Echinogammarus sp* and *Dugastella valentina* throughout the experiment.

3.6. Organic matter decomposition

The mean organic matter decomposition rate in the eutrophic lake treatment (mean±SD: 0.11 ± 0.01 g/day) was similar to that of the water spring treatment (mean±SD: 0.11 ± 0.01 g/day; Fig. 6) throughout the experiment. The organic matter decomposition rate was significantly reduced by azoxystrobin in both treatments. In the eutrophic lake treatment, significant effects were determined from the first application of azoxystrobin to day 42, with the most pronounced effects being observed between day 14 and 28, with calculated NOECs droping to 2 μg/L (Table 4). Between days 14 and 28, the 20 μg/L concentration resulted in a mean inhibition of 16 %, while the 200 μg/L concentration showed a mean inhibition of up to 31 % (Fig. 6). In the case of the water spring habitat, such effects were significant from day 0 to day 42, but only for the highest test concentration (Table 4). In these mesocosms the inhibition varied between 13 % and 22 % over the experimental period (Fig. 6).

3.7. Macrophyte growth

The mean *M. spicatum* and *C. demersum* growth rate in the eutrophic lake treatment throughout the experiment were 0.11 ± 0.09 cm/day and 0.24 ± 0.19 cm/day, respectively, and in the water spring treatment they were 0.11 ± 0.10 cm/day and 0.20 ± 0.20 cm/day (mean±SD; Fig. 6). No significant impacts of azoxystrobin on *M. spicatum* were observed in any of the treatments. Regarding *C. demersum*, the largest

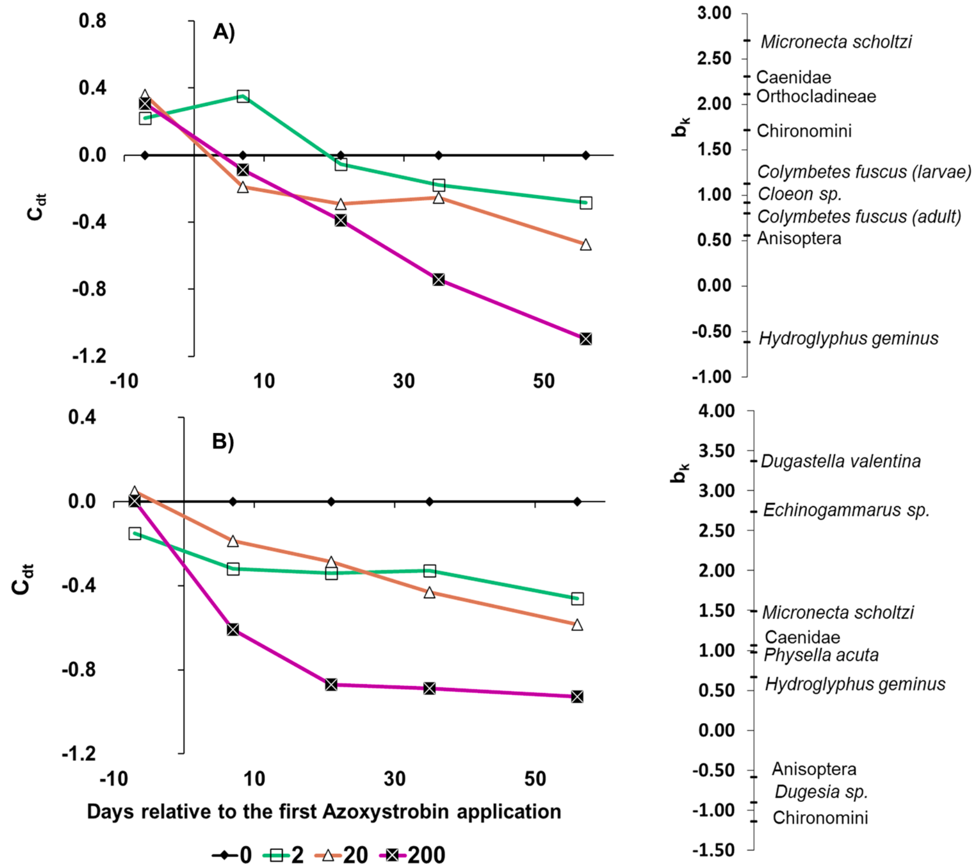

Fig. 4. Principal Response Curves resulting from the analysis of the macroinvertebrates dataset, indicating the effects of azoxystrobin in the eutrophic lake treatment (A) and the water spring treatment (B). The species weight (b_k) can be interpreted as the affinity of each taxon with the principal response curves (c_{dt}). Taxa with a species weight between −0.5 and 0.5 are not shown. In the case of the eutrophic lake treatment (A), 27 % of all variance could be attributed to the sampling date (displayed on the horizontal axis), and 28 % to the fungicide application, 35 % of which is displayed on the vertical axis. A Monte Carlo permutation test indicated that the diagram displays a significant amount of the variance explained the fungicide application ($p = 0.002$). Similarly, in the water spring treatment (B), 28 % of all variance can be attributed to sampling date (shown on the horizontal axis), and 38 % of all variance can be attributed to the fungicide application, 43 % of which is shown on the vertical axis. A Monte Carlo permutation test indicated that the diagram displays a significant amount of the variance explained by the treatment ($p = 0.002$).

effects were found after the first application of azoxystrobin (D0-D14), with a NOEC of 2 μg/L (Table 4). Effects were dose-dependent, but the direction of the effect was different between the treatments. Azoxystrobin had a negative effect on the plant growth in the eutrophic lake treatment, but a positive one in the water spring treatment. At concentrations of 20 μg/L and 200 μg/L, *C. demersum* growth was inhibited by 37 % and 62 %, respectively, in the eutrophic lake treatment. Conversely, in the water spring treatment, the vertical growth of *C. demersum* increased by 53 % and 66 % in the same concentrations, respectively (Fig. 6).

3.8. Biofilm colonization

Large differences were observed in the biofilm colonization rates between the controls of both treatments, with the mean colonization rate in the eutrophic lake and the water spring treatments being 0.27 ± 0.14 mg/d and 0.06 ± 0.04 mg/d (mean ±SD), respectively (Fig. 6). In the eutrophic lake treatment, significant effects of azoxystrobin were found in all concentrations (NOEC < 2 μg/L; Table 4), with the largest colonization inhibition (85 %) corresponding to the highest test concentration (200 μg/L). After the second application, significant effects were only found in the highest test concentration. Significant colonization inhibition was also detected in the water spring treatment from day 28 until the end of the experiment. The NOEC for this treatment was 2 μg/L (Table 4), and the inhibition rate ranged between 59 % and 65 % at 20 μg/L, and between 65 % and 68 % at 200 μg/L over the last 28 days of the experiment (Fig. 6).

3.9. Structural equation modelling

SEM modeling was used to analyze the indirect effects caused by azoxystrobin on the different taxomic groups and functional parameters. The selected model integrated the effects of the first 3 weeks of exposure, and therefore includes the results of samples from D7 and D21. The two factors considered in the model were the azoxystrobin exposure and the treatment, which considers the structural and functional differences between the eutrophic lake and the water spring mesocosms. The model demonstrates a generally acceptable fit to the data, as evidenced by a range of fit indices. The Chi-square test yields a p-value of 0.09, suggesting no significant discrepancies between the model and the observed data. The SRMR value was 0.10 and the RMSEA value was 0.09, which are close to the acceptable ranges proposed by Kline (2023). The GFI (0.954) and CFI (0.953) show values above the threshold of 0.95, which indicate an optimal model fitting, while the AGFI's (0.839) is slighly lower than the established threshold (0.9). For further information regarding the interpretation of these indices, the reader is referred to Kline (2023).

The SEM analysis demonstrated significant direct and indirect effects associated with azoxystrobin exposure. Fig. 7 shows the main results of the final model, where the negative causal relationships are shown in

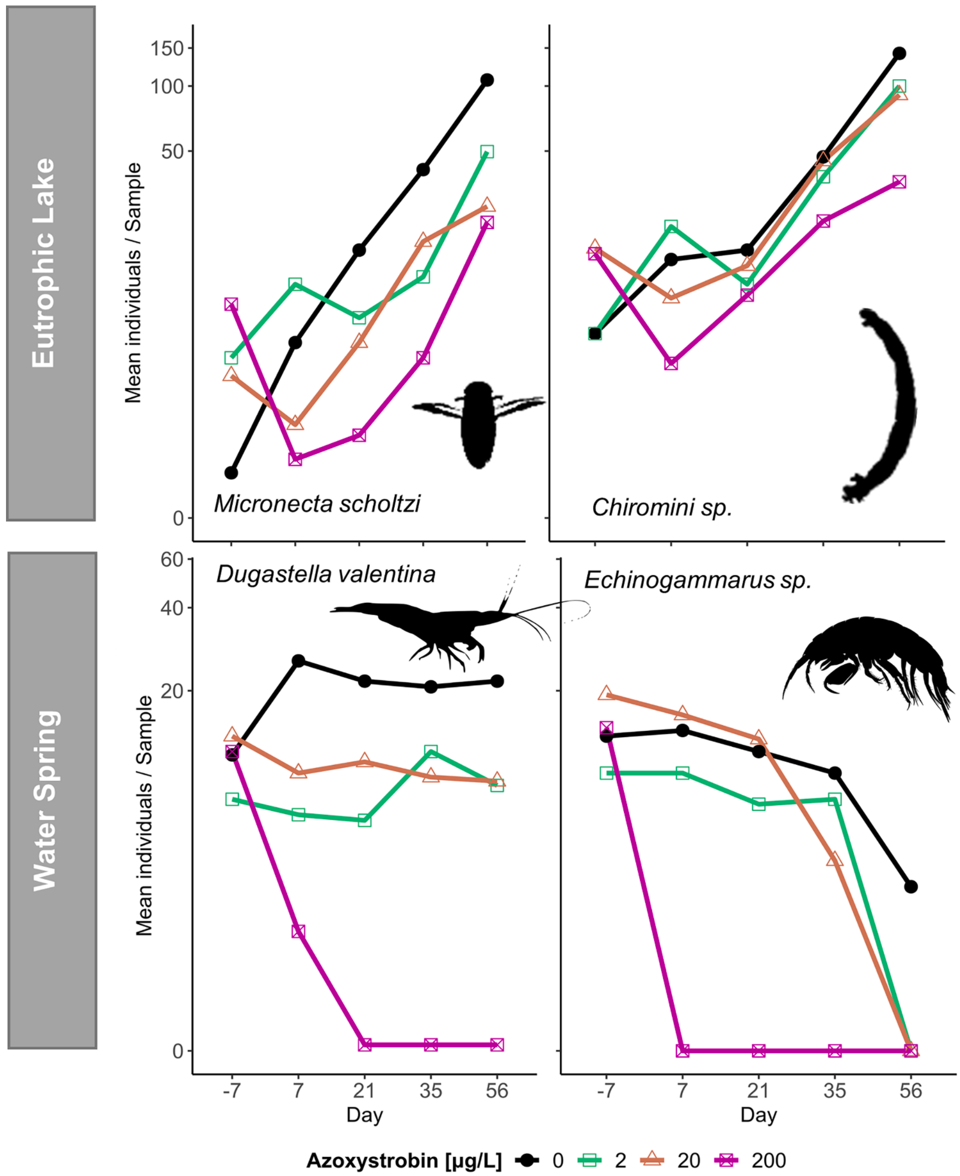

Fig. 5. Response of some macroinvertebrate taxa showing significant abundance declines due to the azoxystrobin application in each treatment.

orange, the positive ones in blue, and significative covariations in gray. The coefficients's strength, listed next to the arrows, confirmed that the most important direct effects of azoxystrobin corresponded to those observed in macrozooplankton abundance and organic matter decomposition, followed by effects on detritivores and on hebivorous macroinvertebrates. The SEM analysis also reflected a negative relationship betweeen the eutrophic lake treatment and the detritivorous abundance and the organic matter decomposition rate, and a positive relationship with the biofilm colonization rate and the macrozopankton abundance. The SEM also showed significant indirect effects (in terms of covariation) between herbivores and predators, microzooplankton and macrozooplankton, and phytoplankton and microzooplankton abundance, showing positive relationships among them.

4. Discussion

The results of this study show that the communities representative of eutrophic lake and water spring habitats of Mediterranean coastal wetlands respond differently to the fungicide azoxystrobin. Such differences can be attributed to the differential dissipation of azoxystrobin (two times faster in the eutrophic lake treatment), the nutrient availability for phytoplankton and macrophyte growth, and structural and functional differences between both aquatic habitats.

Overall, the DT50s obtained in this experiment are very similar to those obtained in previous experiments (between 13 and 26 days according to Jones and Lake, 2000; Zafar Iqbal et al., 2012 and Gustafsson et al., 2010) although the dissipation observed in the eutrophic lake treatment ($DT_{50} = 11$ days) was slightly faster than previously reported. The relationship between nutrient enrichment and increased azoxystrobin dissipation in the eutrophic lake treatment has multiple possible explanations. First, it is likely that the limited availability of P in the water spring treatment resulted in lower microbial activity and thus, slower azoxystrobin biodegradation, as the P concentration in water has been proven to affect decomposition rates of some organic pollutants such as Nitrophenol, 2,4-D or Alachlor (Wang et al., 1984; Jones and Alexander, 1988; Graham et al., 2000). The high N and P loads into the eutrophic lake treatment may also have contributed to a faster dissipation from the water column due to the associated larger primary producer biomass. Besides phytoplankton, much of the primary production in the eutrophic lake treatment mesocosms was carried out by

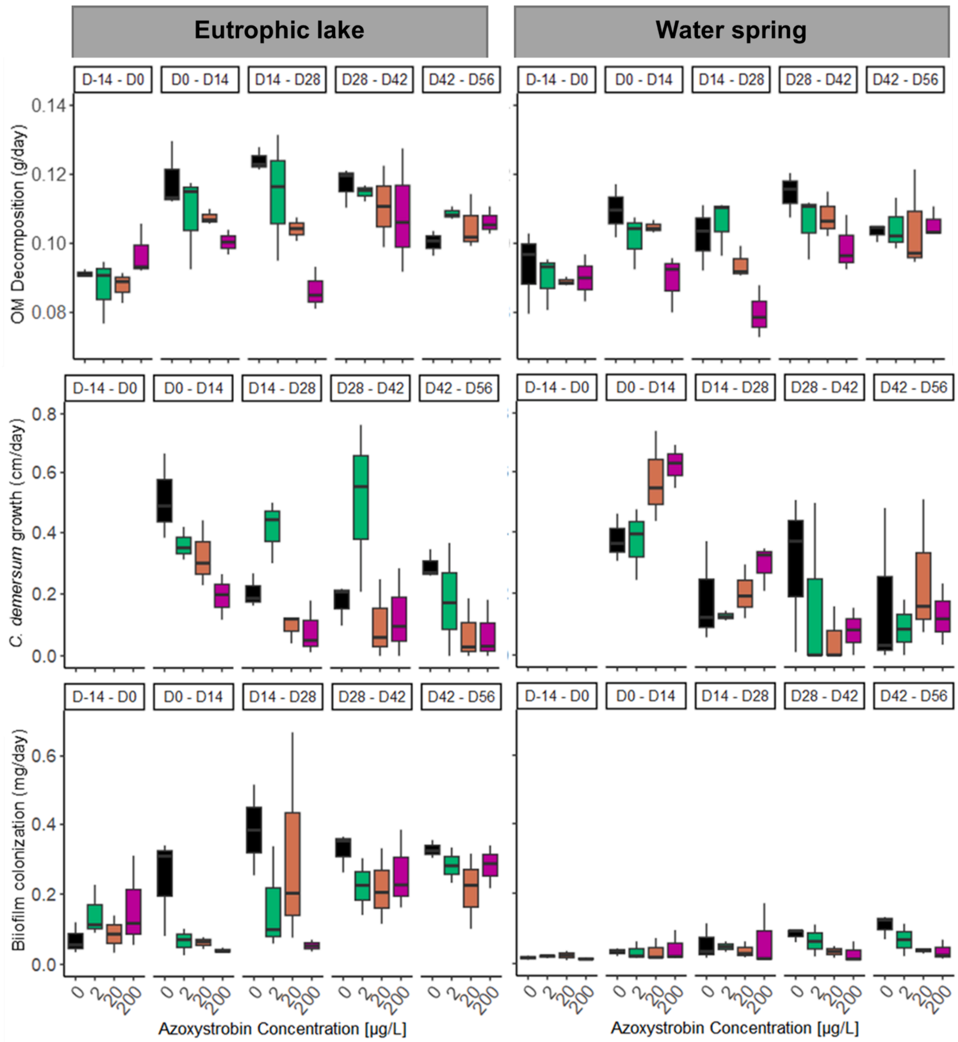

Fig. 6. Effects of azoxystrobin on organic matter (OM) decomposition, *C. demersum* growth and biofilm colonization throughout the different sampling dates. Labels correspond to the 14-day interval in which the endpoint was evaluated.

Table 4
Calculated NOECs for the functional parameters in the different treatments. D: day relative to the first azoxystrobin application. Bold numbers indicate significant azoxystrobin-related effects. ↑: increase; ↓: decrease.

Functional parameters	Eutrophic lake					Water spring				
	D-14 to D0	D0 to D14	D14 to D28	D28 to D42	D42 to D56	D-14 to D0	D0 to D14	D14 to D28	D28 to D42	D42 to D56
Organic matter decomposition	>200	**20↓**	**2↓**	**20↓**	>200	>200	**20↓**	**20↓**	**20↓**	>200
C. demersum growth	–	**2↓**	**20↓**	>200	**2↓**	–	**2↑**	>200	>200	>200
M. spicatum growth	–	>200	>200	>200	>200	–	>200	>200	>200	>200
Biofilm colonization	>200	**<2↓**	**20↓**	>200	>200	>200	>200	>200	**2↓**	**2↓**

periphyton and filamentous algae. Filamentous algae have been studied for its ability to adsorb pesticides in the context of chemical bioremediation (Friesen-Pankratz et al., 2003; González-Barreiro et al., 2006; Dosnon-Olette et al., 2010). It is therefore plausible that the greater abundance of such algae in the eutrophic lake communities played a role in enhancing azoxystrobin uptake from the water column. Furthermore, increased primary production in the eutrophic lake mesocosms resulted in a higher pH, which may facilitate the binding of azoxystrobin to organic matter (Bending et al., 2006).

The clearest effects of azoxystrobin on the phytoplankton community occurred in the water spring treatment towards the end of the experiment (NOEC = 2 µg/L), while in the eutrophic lake treatment effects were only observed at the highest test concentration (NOEC = 20 µg/L). The most sensitive phytoplankton taxon was the diatom *Nitzschia* sp. Diatoms are widely recognized as being among the most sensitive taxonomic groups to fungicides such as azoxystrobin. For example, in the EFSA draft assessment report of azoxystrobin (European Food Safety Authority, 2009) *Navicula pelliculosa* was found to be the most sensitive species, with an EC50 (growth inhibition) of 14 µg/L. Given the great importance of diatoms as primary producers and sediment stabilizers (Santos et al., 2021), it is likely that the effects of azoxystrobin on diatoms cause long-term structural and functional changes on phytoplankton communities under field conditions. According to the literature, desmids also constitute one of the most sensitive groups to azoxystrobin toxicity (van Wijngaarden et al., 2014). In this experiment, however, no significant effects of azoxystrobin on this group were

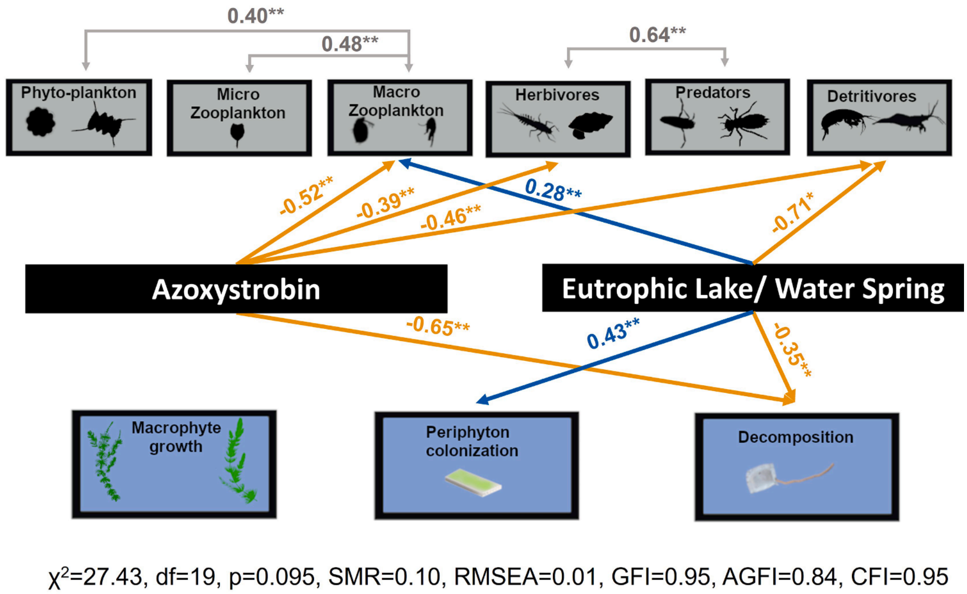

Fig. 7. Scheme showing the outcomes of the final unsaturated SEM model, where only significant causal relationships from the original saturated model were kept. The arrows indicate statistically significant relationships and standardized path coefficients. Negative relationships are drawn in orange, positive ones are drawn in blue, and indirect effects as a result of covariation are drawn in grey. The asterisks indicate significant relationships: one asterisk indicates a p-value between 0.05 and 0.01, and two asterisks indicate a p-value lower than 0.01. The model shows an overall acceptable fit to the data, with a Chi-square p-value of 0.095 indicating no major discrepancies and fit indices like GFI and CFI suggesting a good fit. SRMR and RMSEA values are slightly higher than recommended but still fall within a tolerable range.

detected due to their low and erratic abundances. Other green algae such as *Coelastrum sp* and *Desmodesmus* spp., both belonging to the *Scenedesmaceae* family, did show significant azoxystrobin related declines in the eutrophic lake treatment, although these occurred in isolated sampling dates. We cannot confirm whether these effects are due to direct toxicity or indirect effects, however other studies have pointed at *Desmodesmus* spp. as being highly sensitivity to a related fungicide, difenoconazole (Queirós et al., 2023). Thus, further investigations are recommended to assess the toxicity potential of strobilurin fungicides on different species of the *Scenedesmaceae* family.

Azoxystrobin resulted in long-term effects on the zooplankton community, especially in the water spring treatment, probably due to its higher TWACs. Although previous studies identified copepods as the most sensitive zooplankton endpoint (*Eudiaptomus graciloides* and *Cyclops vicinus* in Lauridsen et al., 2003; *Eurythemora affinis* and *Acartia bifilosa* in Gustafsson et al. (2010) and Calanoida in general in Van Wijngaarden et al., 2014), in our case we found comparable effects in some species of cladocerans and rotifers. The effect on the latter is particularly striking, as it occurred at concentrations an order of magnitude lower than those reported in the literature (44-day NOEC of 32.7 μg/L for *Synchaeta sp*. and *Cephalodella gibba* in Van Wijngaarden, et al., 2014).

In the case of the macroinvertebrate communities, significant effects on the community extended throughout most of the experiment. While the NOEC of the eutrophic lake community was 20 μg/L, that of the water spring treatment dropped to <2 μg/L mostly due to the population decline of the macrocrustaceans *Dugastella valentina* and *Echinogammarus* sp. which were both affected in the lowest test concentration. Crustaceans have been recognized as being particularly sensitive to chronic exposure to azoxystrobin. In fact, the lowest published chronic NOEC for aquatic invertebrates was 9.5 μg/L for the mysid shrimp *Americamysis bahia* (Kent et al., 1993). Gammarids are also among the most sensitive species to azoxystrobin (EC50 of 90.8 μg/L in Zubrod et al., 2014). As detritivorous organisms, both shrimps and gammarids are affected directly and indirectly by fungicides (Zubrod et al., 2011). As they are leaf-associated fungal decomposers, indirect effects can arise from the potential impact of the fungicide on their primary food sources (Bärlocher, 1985). In addition to these two taxa, the effects on the hemiptera *Micronecta* was also found to be significant, with NOECs of 20 μg/L and <2 μg/L in the eutrophic lake and the water spring treatments, respectively. This is consistent with the study by van Wijngaarden et al. (2014), which established a NOEC (42 d) of 3.3 μg/L for the *Corixa* genus, which belongs to this family. The effects on chironomids are also consistent with those found in the literature, e.g. Wei et al. (2021) reported an LC50 of 52.9 μg/l for azoxystrobin. Impacts of azoxystrobin on the beetle species *Colymbetes fuscus*, encompassing both its larval stage and its subsequent influence on adult population size, were not previously reported in the literature. This circumstance likely stems from the nature of an indirect relationship because the effects only occurred in the eutrophic lake treatment (which had a lower TWAC, as opposed to the water spring treatment). The SEM modelling subsequently helped to confirm this, as the results showed that the effects on predators were mostly indirect and a consequence of direct impacts on herbivores, as has been described in other studies (Sánchez-Bayo 2021). The fact that chronic effects were found on numerous macroinvertebrate taxa at relatively low concentrations (2 μg/L) differs from the bulk of the literature, which established zooplankton, as the most sensitive endpoint (Lauridsen et al., 2003; Gustafsson et al., 2010; Van Wijngaarden et al., 2014). This highlights the importance of testing a compound in different environments, as the effects may vary locally depending on the specific characteristics of the ecological scenario.

Effects on organic matter decomposition were likely driven by azoxystrobin impacts on non-target microorganisms and fungi. This could potentially instigate alterations in the ecosystem energy balance (Baron et al., 2002; Dudgeon et al., 2006, McMahon et al., 2012). Although eutrophication has been shown to have positive effects on organic matter decomposition (Pascoal et al., 2003; Hagen et al., 2006; Magbanua et al., 2010) the results of this work are in line with those obtained by Rasmussen et al. (2012), who documented how the negative effects of agricultural pesticides prevail over the stimulatory effects of eutrophication. The effects of strobilurins in general, and more specifically azoxystrobin, on non-target fungi have been shown to occur at lower concentrations than initially expected (such as the HC_5 of 42 μg/L derived by Maltby et al., 2009). For example, NOEC values estimated by

Dijksterhuis et al. (2011) for *Pythium* spp. were 2 μg/L. Since aquatic fungi, especially hyphomycetes, dominate the early stages of decomposition of plant matter (Duarte et al., 2008), it is possible that the direct toxicity of azoxystrobin on these organisms caused the observed slowdown in organic matter decomposition. Moreover, the fact that azoxystrobin affects the organic matter breakdown pathway both through an effect on shredder invertebrates and trough non-target fungi and microorganisms is of particular concern and it is likely to lead to alterations in organic carbon mineralization throughout the whole ecosystem.

In addition to its impact on phytoplankton communities, in this study azoxystrobin exerted negative effects on biofilm biomass and macrophyte growth. While the outcomes concerning macrophytes appear inconsistent between treatments, potentially attributed to competitive interactions with algae under nutrient-rich conditions, the broader implication is that azoxystrobin perturbs various primary production pathways. Coupled with its influence on decomposition processes, this points toward substantial perturbations in the organic matter and energy dynamics within the ecosystem.

The combination of SEM with the PRC method allowed for more robust conclusions regarding the causal mechanisms that drive the ecosystem's response, contributing to a better understanding of the effects of azoxystrobin on the test mesocosms. On the one hand, the results derived from the implementation of SEM modelling reaffirm the existence of direct impacts of azoxystrobin on some taxonomic groups and highlight the structural effects caused by the different habitat on the abundance of some species. On the other hand, it also provides insights into the indirect effects caused by azoxystrobin, that were not identified by the PRC approach. According to the SEM results, the effects of azoxystrobin on the phytoplankton are mostly indirect and occur as a consequence of the direct effect of azoxystrobin on macrozooplankton abundance. This is also the case for microzooplankton. The abundance declines of cladocerans and copepods coincided with an increase in the abundance of *cryptophyceae*; a phenomenon already described in the literature (Perbiche-Neves et al., 2016; Solis et al., 2018). This likely prompted a shift in competitive interactions within phytoplankton communities, leading to a decline in the overall number of phytoplankton cells. The effects on microzooplankton may stem from a relative decrease of green algae, which constitute a fundamental component of the rotifers' diet (Flores-Burgos et al., 2005). In this case one could argue for a top-down propagation of the effects of azoxystrobin, where the disturbance occurs on the macrozooplankton and consequently affects the lower trophic levels. The opposite pattern was observed between herbivorous macroinvertebrates and predators, with herbivores being directly affected by azoxystrobin, and determining an abundance decline of predators by starvation. In this case, it could be described as a bottom-up effect of azoxystrobin, which spread from a lower to a higher trophic level. This positive covariation between herbivores and predators helps to explain why *Colymbetes fuscus* was affected in the eutrophic lake treatment and highlights the existence of trophic cascading effects. Overall, the SEM model results suggest that azoxystrobin's effects on the freshwater food web are complex, multifaceted and habitat-dependant. The disruption of trophic interactions and potential trophic cascading effects could further influence the structure and functioning of the ecosystem. However, the specific outcomes would depend on the ecological characteristics of the affected ecosystem. In the case of the ANP, azoxystrobin effects could extend to higher trophic levels, ultimately influencing the provision of essential ecosystem services. These effects may encompass both structural changes and potential bioaccumulation, thereby affecting key aspects such as fisheries (Azevedo et al., 2022). The altered dynamics within the food web could also result in reduced availability of invertebrates for bird feeding, thereby impacting avian populations dependent on these resources (Hanazato, 2001).

Measured environmental concentrations in the Albufera lake often exceed 2 μg/L. For example, in September 2019 (one month after the first fungicide application in the ANP), the mean concentration in the Albufera lake was 3.8 μg/L (Servei de Vida Silvestre i Red Natura 2000, 2021) . Furthermore, azoxystrobin sampling in the constructed wetlands of the ANP revealed concentrations between 0.5 and 1 μg/L (Rodrigo et al., 2022). This range of concentrations corresponds to the lowest concentration level in this experiment. If we compare these concentrations with the results obtained for the eutrophic lake treatment, we observe that in this concentration range negative effects on the green algae communities, cladocerans and rotifers, and some top predators, such as the coleopteran *Colymbetes fuscus* can be expected. In addition to these structural effects, the possible effects on the energetic balance of the system are particularly serious, as both primary production in terms of periphyton and macrophytes, as well as the decomposition of organic matter, are likely to be affected. It should be noted that much higher water concentrations are expected in the rice fields of the study area (Martínez-Megías et al., 2023), and in different lentic habitats outside the ANP (e.g. 29.7 μg/L in Berenzen et al., 2005; close to 20 μg/L in Edwards et al., 2016). Therefore, it is expected that current use of azoxystrobin in agriculture, especially near water bodies, results in environmental exposure concentrations that affect structural and functional parameters of aquatic ecosystems, and this may include cascading effects throughout the foodweb, as has been observed for neonicotinoid insecticides (Yamamuro et al., 2019). Further studies should be dedicated to assess how such effects can propagate beyond the model ecosystems studied here, for example by affecting bird and fish species depending on macrocrustacea and some insects as food source, or how azoxystrobin can contribute to the population decline of autochthonous species such as *Dugastella valentina*, which is endangered according to the IUCN (International Union for Conservation of Nature).

To sum up, this study highlights the importance of considering the unique characteristics of different ecosystems when assessing the potential risks of pesticides. The eutrophic lake and the water spring model ecosystems investigated here, which are characteristic of coastal Mediterranean wetlands, seemed to show similar overall sensitivity towards azoxystrobin (lowest NOEC < 2 μg/L, with slight effects at 2 μg/L), but there were notable differences in the endpoints affected and the intensity of the effects in each of them. This may be attributed to variations in environmental conditions, which in turn affect pesticide availability, and community composition. Our study shows that azoxystrobin could significantly affect aquatic populations and communities in Mediterranean wetlands. Such disturbances do not only pose a risk to aquatic life but also have the potential to impact ecosystem funcitons. Therefore, there is an urgent need for the development of strategies to mitigate these ecological consequences, safeguarding both natural biodiversity and human well-being.

CRediT authorship contribution statement

Pablo Amador: Conceptualization, Investigation, Methodology, Data curation, Writing – review & editing. **Constanza Vega:** Investigation, Methodology, Writing – review & editing. **Natividad Isael Navarro Pacheco:** Investigation, Methodology, Writing – review & editing. **Jesús Moratalla-López:** Investigation, Methodology, Writing – review & editing. **Jose Palacios:** Investigation, Methodology, Writing – review & editing. **Melina Celeste Crettaz Minaglia:** Investigation, Methodology, Writing – review & editing. **Isabel López:** Investigation, Methodology, Writing – review & editing. **Mónica Díaz:** Investigation, Methodology, Writing – review & editing. **Andreu Rico:** Conceptualization, Investigation, Funding acquisition, Methodology, Data curation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2023.106828.

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