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ZOOPLANKTON UNDER MULTIPLE STRESSORS

Effects of the herbicide bentazone on the structure of plankton and benthic communities representative of Mediterranean coastal wetlands: a mesocosm experiment

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Abstract Pesticide pollution poses one of the most important threats for the ecological status of coastal wetland ecosystems. In this study, we evaluated the effects of the herbicide bentazone on aquatic communities characteristic of Mediterranean coastal wetlands using outdoor mesocosms. The herbicide was applied weekly for four weeks at concentrations of 0 (control), 25, 250, and 2500 µg/L in mesocosms representing two different ecological conditions: one with submerged macrophytes and one without macrophytes. The impact of bentazone on diverse taxonomic groups, including benthic diatoms, phytoplankton, submerged macrophytes, zooplankton, and aquatic macroinvertebrates, was examined before the first bentazone application, after the last application and sixty days after the last application. The results show that benthic diatoms were the most affected group in terms of community structure. Zooplankton was the second most affected group, mainly driven by indirect effects that resulted in the replacement of large filter feeders by small filter feeders. This suggests strong bottom-up effects caused by the herbicide, altering the structure of the primary producers' community which, in turn, indirectly affected key zooplankton taxa. Our study shows that the exposure levels of bentazone measured in Mediterranean coastal wetlands could result in long-term direct and indirect impacts on the structure of aquatic communities.

Keywords Pesticides · Wetlands · Mesocosms · Freshwater ecosystems · Indirect effects

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Introduction

Mediterranean wetlands harbor a high diversity of aquatic organisms (Bagella & Maria, 2012; Antón-Pardo & Armengol, 2014 Boix et al., 2010) and are considered key habitats for the conservation and protection of rare and endemic species of crustaceans and insects (Alonso, 1998; Millán et al., 2014), as well as for endangered fish and waterbirds (Armengol

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et al., 2008; Doadrio, 2001). Although some types of Mediterranean wetlands are considered priority habitats for conservation in the EU (e.g., Habitats Directive 92/43/CEE), they are subjected to an increasing number of impacts that threaten their biodiversity, functioning and ecosystem services delivery (Camacho et al., 2019; Taylor et al., 2021). Among these impacts, chemical pollution has been identified as one of the main drivers of biodiversity loss in Mediterranean wetlands (Martínez-Megías & Rico, 2022). Among the wide range of contaminants found in Mediterranean wetlands, agricultural pesticides and pharmaceuticals have been highlighted as posing the greatest ecotoxicological hazard (Barbieri et al., 2021; Sadutto et al., 2021; Martínez-Megías et al., 2024). Herbicides are frequently used in intensive agriculture surrounding Mediterranean wetlands to eliminate unwanted weeds (Gómez de Barreda et al., 2021). Due to the intensive cultivation of rice in some Mediterranean wetlands, herbicides are one of the most frequently detected compounds in these habitats (Rodrigo et al., 2022; Martínez-Megías et al., 2024). Among them, the herbicide bentazone is widely used in rice farming to treat and prevent the grassweed (*Echinochloa* sp.). Previous monitoring studies have found that this compound occurs in a large number of Mediterranean wetlands where rice is also cultivated. For example, Barbieri et al. (2021) found residues of bentazone in all samples taken along different freshwater ecosystems of the Ebro delta (Catalonia, Spain), in concentrations that ranged from 0.15 to 180 µg/L. On the other hand, studies carried out in the Albufera Natural Park (Valencia, Spain) in 2016 found bentazone in 57% of the samples ($N=75$), with concentrations that ranged from 0.03 to 13 µg/L (Calvo et al., 2021). However, samples obtained through the regular monitoring programs of the Valencian governmental agencies in the same natural park have found concentration peaks up to 21 µg/L in the Albufera Lake (GVA, 2023).

Agricultural herbicides have been found to pose harmful effects on a wide range of non-target aquatic organisms (Leboulanger et al., 2011). Primary producers, such as phytoplankton, benthic diatoms and some aquatic macrophytes, are the most sensitive groups to herbicides (Wood et al., 2014). Bentazone belongs to the benzothiazinone group and inhibits the photosystem II (PS II) of primary producers, such as microalgae, leading to reduced growth and productivity. Effects on zooplankton have been reported at concentrations that are about two orders of magnitude larger than those found in the environment (*Daphnia magna* Straus, 1820, $LC_{50}=64{,}000$ µg/L; Barata et al., 2007). However, previous studies indicate that indirect effects of herbicides on zooplankton and larger invertebrates may be expected due to the change in quantity and quality of food resources (Hasenbein et al., 2017a, b). Plankton and benthic invertebrates are integral components of aquatic food webs and play crucial roles in nutrient cycling and energy transfer, so changes in their community structure can have effects that may span throughout the overall ecosystem dynamics (Carpenter et al., 2011).

Some studies show that the ecological status of wetlands could be crucial for mitigating the effects of stressors and promoting ecosystem resilience (Turner et al., 2000). In this context, submerged macrophytes can play a particularly important role. Macrophytes provide habitat and food resources for other aquatic taxa, reduce nutrient levels in the water, and stabilize sediments, which can significantly influence the ecological status of these habitats (Carpenter & Lodge, 1986; Rodrigo et al., 2015, Scheffer, 2004; Camacho et al., 2016). Submerged macrophytes can mitigate the harmful effects of pesticides on aquatic communities by providing a buffering effect against both direct and indirect effects, through the provision of a physical barrier and the absorption of pollutants (Brogan & Relyea, 2015; Stang et al., 2016). Thus, macrophytes have been used as a valuable tool in the management of pesticide pollution in aquatic environments (Fletcher et al., 2022). At the same time, macrophytes act as an environmental filter, influencing the dominance of certain aquatic species and selecting species assemblages that may be more or less sensitive to chemical pollution. In this context, several studies have shown that the presence of macrophytes can alter the trophic status of the ecosystem and the resilience of specific aquatic groups to potentially toxic chemicals (Brogan & Relyea, 2015). For example, Amador et al. (2024) found that freshwater mesocosms dominated by macrophytes (*Myriophyllum spicatum* L.) promoted the establishment of macrocrustacean populations of *Dugastella valentina* (Ferrer Galdiano, 1924), and *Echinogammarus* sp., which are more sensitive to the fungicide azoxystrobin than the insect taxa that prevail in phytoplankton dominated systems. Brock et al. (1992) also described significantly different secondary effects on macroinvertebrates exposed to the insecticide chlorpyrifos in model ecosystems that were either dominated by *Elodea* sp. or macrophyte-free. Thus, the capacity of macrophytes to improve water quality in coastal wetlands and their contribution to ecosystem resilience against chemical stressors should be further investigated, particularly for chemicals with herbicidal mode of action.

The aim of this study was to assess the direct and indirect effects of the herbicide bentazone on aquatic communities representative of Mediterranean coastal wetlands under two different ecological conditions: one with aquatic vegetation (dominated by *Myriophyllum spicatum*) and the other one without. This was done using freshwater mesocosms stocked with aquatic organisms representative of the remaining well-preserved areas of the Albufera Natural Park, a Mediterranean coastal lagoon located in eastern Spain. Aquatic organisms were chronically exposed to a range of bentazone concentrations similar to those that have been measured in Mediterranean coastal wetlands. The direct and indirect effects of bentazone on planktonic and benthic communities were evaluated over a period of 90 days. Our investigation elucidates the mechanisms by which bentazone may alter community structure and population dynamics in different ecological scenarios, thus providing valuable insights for the conservation and management of Mediterranean coastal wetlands affected by this pollutant.

Materials and methods

Experimental design

The experiment was performed in 24 mesocosms, installed at the Albufera Biological Station in El Palmar (Valencia, Spain) (Lat.: 39.315572; Long.: -0.319642). Each mesocosm consisted of a PVC coated glass fiber tank (diameter: 140 cm, depth: 95 cm). The bottom of the mesocosms was originally covered with 10 cm of silty-clay sediment (3–4% organic matter), and then filled with 1154 L of dechlorinated tap water. Each mesocosm contained two pebble baskets and two plastic traps filled with *Populus* sp. leaves and pebbles, which were used as substrates for macroinvertebrate sampling. The mesocosms were inoculated with phytoplankton, zooplankton and macroinvertebrates taken from nearby ponds and limnocrene springs (locally termed ´ullals´) on two occasions during february 2023. All these sites were shallow and presented oligohaline (1000–2000 µS/cm) and alkaline (pH 7,2–8,4) water conditions (Bisquert-Ribes et al., 2025; Rodrigo & Colom, 1999; Soria, 1993). The inoculum samples were homogenized in 30 L of dechlorinated water, and then added to each mesocosm (1 L per mesocosm). The inoculation took place 30 days prior to the start of the experiment (pre-experimental period). In half of the mesocosms ($N=12$), 40 shoots (10 bunches of 4 shoots, 20 cm height) of the hydrophyte *Myriophyllum spicatum* were planted (treatment with macrophytes: M), while in the other half, no macrophytes were planted (NM: no macrophytes treatment). Macrophytes were collected from a limnocrene spring (similar to those previously mentioned) with limited contamination and washed with tap water, maintained for 7 days in a climate chamber, and washed again prior to introduction into the mesocosms. During the pre-experimental period, the biological communities were allowed to establish and were regularly homogenized by exchanging water among the mesocosms corresponding to the M and NM groups, separately, using a water pump or buckets. Water losses by evaporation were weekly compensated by adding dechlorinated tap water previously filtered through a 20 µm net.

In each of the two different ecological conditions (M and NM), four exposure levels of bentazone were tested: *C* control (0 µg/L); low (25 µg/L); medium (250 µg/L); and high (2500 µg/L), with three replicates per exposure level. Bentazone was applied weekly for four weeks (i.e., four applications). Herbicide applications were done to maintain a pseudo-constant exposure regime, starting on the 28th March 2023 (D0), as it is expected to occur in wetlands receiving drainage waters from rice fields during spring. The experiment had a duration of three months since the first application of the test substance. Three biological sampling campaigns were performed: at D-7 (7 days before the first bentazone application); at D30 (one month after the first bentazone application); and at D90 (three months after the start of the exposure period, and two months after the last bentazone application; see Fig. 1). The application and analysis of the test substance as well as the

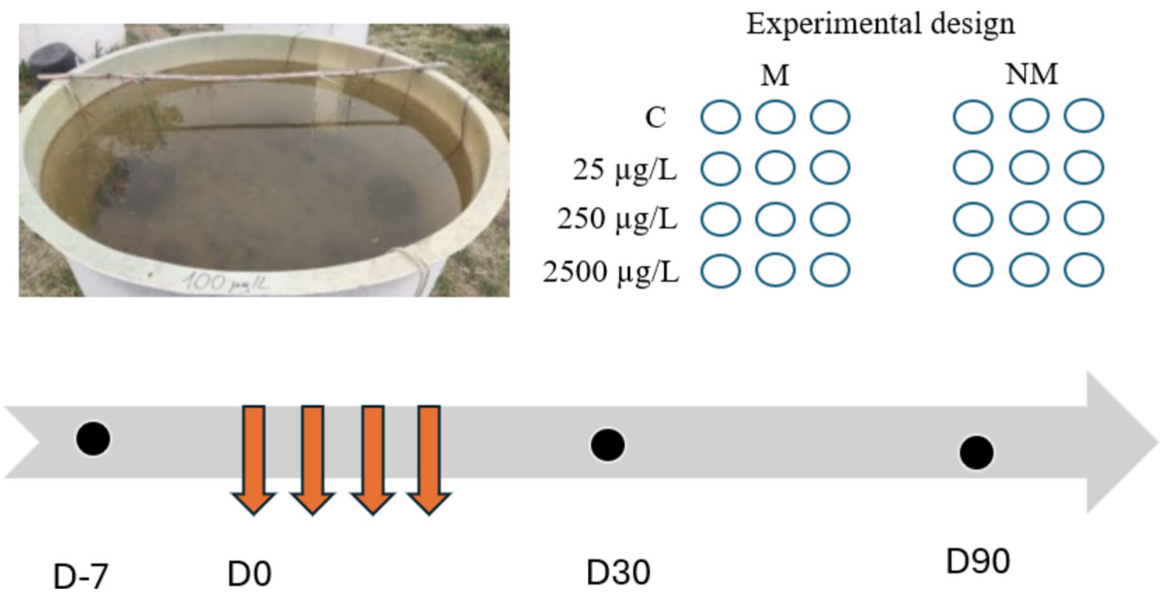

Fig. 1 Diagram of the experimental design and timeline showing the sampling dates (dots) and weekly applications of bentazone (arrows)

methods used to monitor the response of the different structural and functional ecosystem parameters are described in the following sections.

Chemical application, sampling and analytical verification

A bentazone stock solution was prepared by dissolving 65.568 g of the commercial product Basagran SL (BASF), which contained 44% of bentazone w/w (active ingredient, a.i.) in 1 L of distilled water and stirring it at 60 ºC over 30 min, producing a final concentration of 28.85 g a.i. /L. Dosing solutions were prepared by diluting 1, 10 and 100 mL of the stock solution into 1 L of distilled water. Next, they were poured over the mesocosms (1154 L) to produce concentrations of 25, 250 and 2500 µg/L, respectively. After addition, the mesocosms water was gently stirred with a wooden stick to allow a rapid and complete mixing of the dosing solutions within the water column. The stock and dosing solutions of the second, third and fourth applications were prepared considering a 30% dissipation rate of the test compound in one week. A pre-test was performed before the experiment to assess the dissipation rate in two additional mesocosms by adding a concentration of 25 µg/L, and evaluating the concentration on D0 (before and 2 h after the bentazone application), on D7 and D14.

Sampling for the determination of bentazone concentrations in the 25 µg/L treatment was done on D0 (2 h after the first application), on D7, D14, and D21 before and after the weekly application to assess the dissipation and accuracy of the dosing, and on D30, D60, D90 to assess the dissipation of the test compound after the last application. In the other chemical treatments, bentazone sampling was done only on D0, D7, D14, D21 (all after the application), D30 and D90 to assess the accuracy of the dosing and dissipation. Sampling of the control mesocosms was done on D0, D30 and D90 to assess possible (cross-) contamination. Bentazone sampling in water was done by taking 5 mL of water from a 5 L sample taken from depth-integrated subsamples. The water samples were stored in amber glass vials at − 20 °C until further analysis.

Bentazone analysis was done by direct injection of water samples into a liquid chromatograph (LC) coupled to a triple quadrupole mass spectrometer (MS/MS) equipped with an electrospray ionization interface. The analytical standards and reagents used for the analysis, as well as the conditions and efficiency of the analytical method are described in the Supporting Information (Annex S1 and

Table S1). The Limit of Quantification (LOQ) of the method was 0.25 µg/L, and the Limit of Detection (LOD) was 0.07 µg/L. The percentage of the intended concentration corresponding to the measured concentration was calculated, as well as the dissipation coefficient (*k*) of the low concentration treatments (25 µg/L) based on the exposure concentrations measured after the last application assuming first-order kinetics. Finally, the half live (DT50) of the compound was calculated as Ln (2) divided by *k*.

Physical and chemical parameters

Water physical and chemical parameters, including water temperature (°C), conductivity (µS/cm), and dissolved oxygen (DO, % saturation) were determined in situ using a multiparameter probe WTW Multi 3410 logger. The pH level was determined using a Crison Basic-20 pH-meter. Nutrient concentrations were determined in the laboratory, namely the concentrations of soluble orthophosphate (SRP, µM), total phosphorous (TP, µM), nitrate ($NO3^-$, µM), and ammonia ($NH4^+$, µM). The laboratory analyses were done in accordance with the protocols established by APHA (2005). The chlorophyll-a (Chl-*a,* µg/L) concentration in the mesocosm water was determined following Picazo et al (2013). All these parameters were measured on D-7, D30 and D90 relative to the first herbicide application.

Phytoplankton

Phytoplankton samples were taken on D-7 (7 days prior to the initial application of the compound -D0-), D30 and D90 relative to the first herbicide application. Depth-integrated water samples were taken with a PVC tube (diameter: 9 cm, length: 95 cm) (six sub-samples per mesocosm mixed in a bucket). A sample of 250 mL was introduced into amber glass bottles and 10% Lugol's iodine was added for preservation. Identification and counting were done to the lowest taxonomic resolution possible with an optical microscope (Olympus CX41) and a Sedgewick-Rafter chamber. Ciliates, albeit not being phytoplankton, were introduced as part of this dataset. Results were expressed as the number of cells per mL of water.

Benthic diatoms

Benthic diatom samples were taken on D-7, D30 and D90 relative to the first herbicide application. A sample of 50 gr of surface sediment was taken from each mesocosm and a subsample of 50 mg of fresh sediment was examined under light microscopy with a Nikon Eclipse E600 equipped with a 40 × objective (N.A. 0.65) with differential interference contrast (Nomarski) optics. The health status of each identified cell was recorded as either `healthy´ or `unhealthy´ by visual inspection of the cells according to Wood et al. (2014). Only intact cells with chloroplast and normal cell contents were considered healthy. We also added 250 µl of a solution of polystyrene beads at a concentration of 10^6 beads/mL to correct the diatom concentration estimate per gram. Polystyrene beads of 5 µm particle size for analytical standard (Sigma-Aldrich) were used as external marker, using a stock of $1 \cdot 10^{10}$ particles/mL in a NaOH (2% v/v) solution. Microparticle concentration was tested in a flow cytometer Epics XL (Coulter Corporation, USA) using a 15-mW argon laser at a wavelength of 488 nm at the Faculty of Veterinary Science of the University of Santiago de Compostela. Benthic diatoms were identified and quantified in 10^3 valves per gram of sediment.

Zooplankton

Zooplankton samples were taken on the same sampling days (D-7, D30 and D90) and using the same depth-integrated sampling method as for phytoplankton. Then, 5 L of the water sample were filtered through a plankton net of 55 µm mesh size, and the retained organisms were fixed with Lugol´s iodine solution. Cladocerans, rotifers and copepods were identified up to the lowest possible taxonomic level and counted in the entire zooplankton sample using an inverted microscope (Leica DM IL LED) with a magnification of 40-100x. For copepods, a distinction was made among adults, nauplius and copepodite stages. The number of individuals of each species was expressed in individuals per liter of mesocosm water.

Macroinvertebrates

Macroinvertebrate samples were taken on D-7, D30 and D90 relative to the first herbicide application. To collect pelagic and benthic individuals, three different sampling methods were used. First, a net (mesh size: 500 µm) was dragged twice through the lateral walls of the mesocosms (in both directions) to catch the animals that were swimming or resting on the mesocosm's wall. Second, the two pebble baskets positioned over the sediment surface were collected. Third, the two plastic traps were collected from the sediment's surface using a net. The macroinvertebrates collected through each method from each mesocosm were pooled together into a plastic tray, identified to the lowest taxonomic resolution level possible, and counted in situ. Afterwards, the colonizing pebble baskets and traps, and the macroinvertebrates were returned to their original mesocosm. Results were expressed as number of individuals per sample.

Submerged macrophytes

The state of submerged vegetation was assessed at D0, D30 and D90, and the extent of vegetation cover in each mesocosm was quantified. At the end of the experiment (D90), all the vegetal biomass was collected in each mesocosm by means of a hook and was stored in plastic bags in the laboratory. It is remarkable that there was a considerable growth of filamentous algae both in the treatments with and without planted macrophytes. In the treatments with planted macrophytes, it was unviable to separate the biomass of filamentous algae from that of the submerged phanerogams (*M. spicatum*) and, consequently, the macrophyte biomass is referred hereafter to the sum of both types of aquatic vegetation. Once in the laboratory, the samples were pressed carefully to remove excess water, and they were air dried for 8–10 days. After this period, each sample was weighed in a balance (Mettler PJ3000) to obtain the macrophyte dry weight (DW) per mesocosm. The relationship between the vegetation dry weight and the vegetation cover percentage was established by means of a linear regression, for the two distinct ecological conditions separately. The calculated regression equation was used to estimate the aquatic plant biomass corresponding to D30 in the different mesocosms.

Data analyses

Biological indices including species richness, abundance, and Shannon diversity were calculated for phytoplankton, diatoms, zooplankton and macroinvertebrates on each sampling date. A two-way ANOVA was used to determine statistically significant differences in the calculated values related to the different levels of bentazone contamination and the two different ecological conditions (M or NM) on all the sampling dates. Similarly, two-way ANOVAs were used to determine the differences between the plant biomass of the two ecological conditions and the effects of the herbicide, as well as the differences in the physical and chemical variables.

A permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) was performed for each sampling day to test for differences in the community structure caused by the different exposure levels of the herbicide and the two different ecological conditions (M or NM). The PERMANOVA analysis was done through a Bray–Curtis similarity matrix with log $(x+1)$ transformed taxa abundances and using 999 permutations. When statistically significant effects by the chemical pollutant were found, a pair-wise test was used to determine the No Observed Effect Concentrations (NOECs) for the community, defined as the highest test concentration that does not result in statistically significant effects as compared to the chemical control. A Principal Coordinates Analysis (PCoA) was used to visualize differences in the community structure of each group, considering only those sampling days and taxonomic groups that showed significant effects by the treatment as result of the PERMANOVA test ($P < 0.05$). All these statistical tests were performed with the software Primer Version 7 (PRIMER-E Ltd, Plymouth, UK). Finally, a bentazone NOEC for each population was calculated for the two ecological conditions separately, using the Williams test (Williams, 1972). The Williams test was calculated with the Community Analysis computer program, version 4.3 (Hommen et al., 1994).

The R *lavaan* package (Rosseel, 2012) was used to conduct a Structural Equation Modeling (SEM), which includes a matrix of both structural and functional data. The different taxa (primary producers and zooplankton) were divided into trophic groups, including benthic and planktonic primary producers (diatoms and phytoplankton, respectively), small filter-feeders (rotifers, copepod nauplii and copepodites), large filter-feeders (cladocerans and adult *C. numidicus* copepods), and predators (adult *Acanthocyclops americanus* (Marsh, 1893) (García et al., 2007; Geraldes & Boavida, 2004)), and the total abundance of each group was calculated by summing the initial counts of the different species in each group. The data were then log-transformed to meet SEM´s assumptions of linearity and normality. The SEM was constructed using data from days 30 and 90 after the first herbicide application. The model was fitted to account for the effect of bentazone concentrations and the ecological conditions in all trophic group abundances. A covariation between all trophic groups was assumed, which may suggest the spread of toxic effects throughout the entire food web. 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. To evaluate the model fitting, several indices were used. These included 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 of the model (Kline, 2023). The indices collectively provided a comprehensive understanding of the model performance, enabling the identification of both its strengths and weaknesses.

Results

Exposure concentrations of bentazone

The results of the bentazone analysis for the first application in all the treatments showed that the measured concentrations were, on average, 89% of the intended concentration in the treatment without macrophytes, and 85% in the treatment with macrophytes. After the last application, these values were 125% and 118%, respectively, indicating that there was a slight concentration build-up. Considering the four applications, the average percentage of the intended dose was 110 ± 20% and 108 ± 18% in the treatment without macrophytes and with macrophytes (mean ± SD), respectively, indicating that the mesocosms received the appropriate dosing. No residues of the test compound were found in the control mesocosms.

The dissipation coefficient (k) of bentazone in the 25 µg/L treatment without macrophytes was 0.054 d^{-1}, and in the treatment with macrophytes was 0.059 d^{-1}. These dissipation coefficients corresponded to half-lives (DT50) of 12.8 days and 11.6 days in the treatments without macrophytes and with macrophytes, respectively, showing that the dissipation was similar in both treatments. The measured concentrations in the mesocosms with 25 µg/L of bentazone are shown in Fig. 2, while a complete overview of the exposure concentrations is provided in Table S2.

Physical and chemical parameters

The mean water temperature was similar in both ecological conditions (20.45 ± 5.14 ºC), with minimum values in D-7 (14.38 ± 0.15 ºC) and maximum in D90 (26.45 ± 0.16 ºC; Table S3). The treatment with macrophytes showed lower mean conductivity (1057 ± 41 µS/cm) and nitrate concentrations (22.76 ± 14.12 µM) than the mesocosms without macrophytes (1094 ± 43 µS/cm and 24.94 ± 12.04 µM, respectively). Furthermore, it was observed that the mesocosms with macrophytes exhibited higher pH (9.76 ± 0.47) and DO (130 ± 15%) values than those observed in the mesocosms without macrophytes (9.27 ± 0.47 and 117 ± 20%, respectively). A significant decrease in nitrates was observed for the highest herbicide concentration in both ecological conditions at D30, decreasing from 15.3 to 5.7 µM, and from 17.9 to 7.9 µM, in the mesocosms with and without macrophytes, respectively (Table S3). No effects related to the herbicide treatment were observed in the rest of physico-chemical variables (Table S4).

Phytoplankton

A total of 33 taxa were identified in the phytoplankton samples, belonging to the following taxonomic groups: Bacillariophyta (12 taxa), Chlorophyta (10), Chroococcales (2), Cryptophyta (2), Dinophyta (2), Charophyta (2), Euglenophyta (1), Cyanobacteria (1) and Ciliophora (1). The most abundant phytoplankton genera were *Cryptomonas* and *Rhodomonas*. The rest occurred in very low densities (< 1 cell/mL) and were only observed in a limited number of samples and sampling dates.

Considering the abundance, species richness and the Shannon diversity index, significant differences were observed between the two ecological conditions on D30. The abundance of organisms was higher in

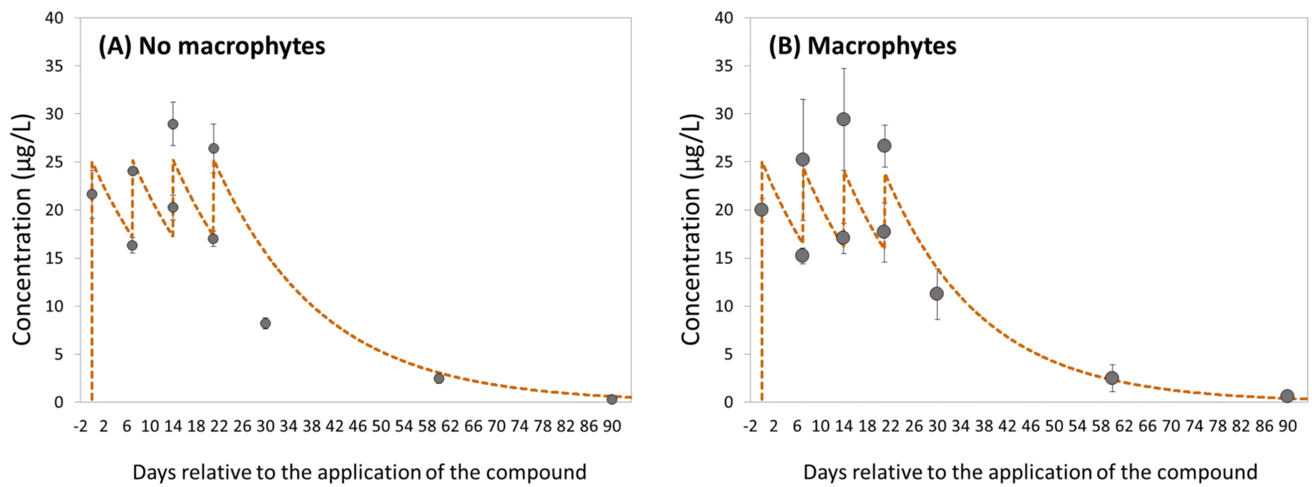

Fig. 2 Measured concentrations of bentazone in the treatments exposed to 25 µg/L of bentazone with (A) and (B) without macrophytes. The circles show the measured concentrations of bentazone (mean ± SD), while the dashed line indicates the modeled concentration (assuming first order kinetics) based on a dissipation coefficient of 0.054 and 0.059 d^{-1} in the treatment without and with macrophytes, respectively

the mesocosms with macrophytes, while the species richness and Shannon index were higher in the mesocosms without macrophytes. No significant differences were found for the four levels of chemical pollution and the analyzed biological indices (Tables S5 and S6).

The PERMANOVA did not show a significant effect of the bentazone treatment on the phytoplankton community for any sampling date, however, the two different ecological conditions (M and NM) showed significant differences in the structure of the phytoplankton community on D30 and D90 (Table 1). The principal distinction between the two treatments was the higher abundance of *Rhodomonas* in the mesocosms with planted macrophytes.

Benthic diatoms

A total of seven taxa were found to dominate the diatom assemblage throughout the experimental period. Significant discrepancies in abundance, species richness, and Shannon diversity were identified between the two ecological conditions on all sampling dates. The three calculated indices exhibited higher values in the mesocosms with macrophytes. Significant differences were also observed in these indices for the various contaminant levels on D30 and D90, with the value of these indices significantly reduced in the 250 µg/L and 2500 µg/L concentrations in comparison to the control and the 25 µg/L concentration. A greater difference in these indices was observed between the two ecological conditions in the control treatment and the lower concentration, with smaller differences in the two higher concentrations (Tables S5 and S6).

The PERMANOVA showed significant differences in the two ecological conditions for all the sampling dates. *Achnanthidium minutisimum* and *Cocconeis placentula* were identified only in the mesocosms with macrophytes, while the rest were found in both ecological conditions. The PERMANOVA test also showed a significant effect of the bentazone treatment on day 30 and 90 (Table 1). The groupings of the diatom community assemblages from day 30 and 90 can be visualized in the PCoA plots (Fig. 3). The Williams test showed significant population declines linked to all bentazone concentrations in the treatment with macrophytes on *Acanthidium minutissimum (Kütz.) Czarnecki.*, *Cocconeis placentula Ehr.*, *Gomphonema parvulum (Kützing) Kützing*, and *Nitzschia palea (Kützing) W. Smith.* on day 30 and/or day 90 (i.e., calculated NOECs < 25 µ/L; Table 2). As for the treatment without macrophytes, *N. palea* and *Encyonema minutum Krammer.* showed the highest sensitivity, with a calculated NOEC < 25 µg/L on day 30 and 90 (Table 2; Fig. 4).

Table 1 Results of the PERMANOVA for all taxonomic groups (*P*-values shown)

Taxonomic group	Factors	D-7	D30	D90
Phytoplankton	Macrophytes	0.537	0.007**	0.003**
	Bentazone	0.764	0.514	0.875
	Interaction	0.955	0.383	0.626
Benthic diatoms	Macrophytes	0.002**	0.022*	0.033*
	Bentazone	0.875	0.002**	0.002**
	Interaction	0.558	0.139	0.284
Zooplankton	Macrophytes	0.071	0.017*	0.001**
	Bentazone	0.818	0.059	0.002**
	Interaction	0.988	0.241	0.047*
Macroinvertebrates	Macrophytes	0.067	0.119	0.005**
	Bentazone	0.870	0.136	0.812
	Interaction	0.476	0.385	0.752

The table shows the effect of the two ecological conditions (Macrophytes), the effect of the chemical concentrations (Bentazone), and the interaction of both (Interaction)

D day relative to the first bentazone application

*indicates statistically significant effects by the evaluated factor (*P*-value < 0.05)

**P*-value < 0.05; ***P*-value < 0.01

Zooplankton community

The zooplankton community was composed of 17 Rotifera, nine Cladocera and four Copepoda taxa. The most abundant organisms were juvenile Copepoda stages (nauplii and copepodites) and Rotifera (*Keratella quadrata* (Müller, 1786)*, Polyarthra* cf. *dolichoptera* (Idelson, 1925)*, Hexarthra* cf. *fennica* (Levander, 1892)*, Keratella cochlearis* (Gosse, 1851) and *Notholca acuminata* (Ehrenberg, 1832). Significant differences were observed in the abundance of zooplankton organisms (D-7 and D30) between the two ecological conditions, and in the Shannon diversity index (D-7 and D90). In all cases, these indices exhibited a higher value in mesocosms with macrophytes. Additionally, notable discrepancies were observed in the abundance and Shannon diversity

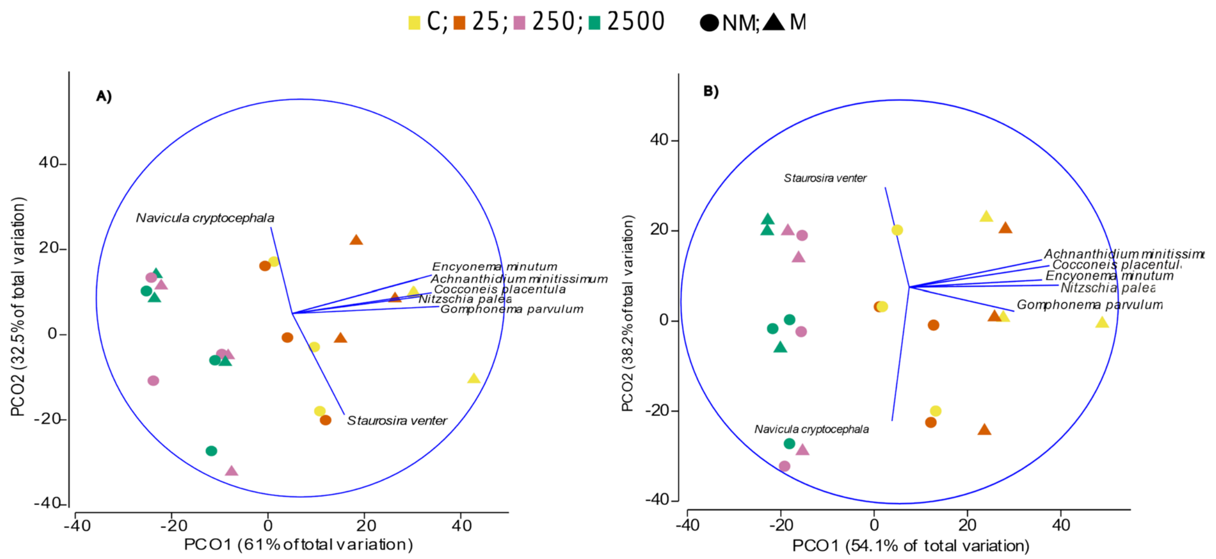

Fig. 3 Representation of the firsts two axes of the Principal Coordinates Analysis (PCoA) for diatoms sampled on D30 (A) and D90 (B). The points represent all the mesocosms, the concentration of pollutant is classified by colors (■ C; ■ 25; ■ 250; ■ 2500) and the two ecological conditions are represented by different symbols (●NM; ▲M)

indices across the various contaminant concentrations at D90. The zooplankton abundance increased at the 250 µg/L and 2500 µg/L concentrations, whereas the Shannon diversity declined at these same concentrations in comparison to the control and the 25 µg/L concentration (Tables S5 and S6).

The PERMANOVA showed significant differences between the two ecological conditions. Copepodites and adult calanoids were more abundant in mesocosms without macrophytes, while species such as *Chydorus sphaericus* (O.F.Müller, 1776) were more abundant in the mesocosms with macrophytes. The analysis also revealed a significant effect of bentazone on zooplankton community composition on D90, and a marginal effect on D30 (Table 1). The grouping of the zooplankton community for D90 can be visualized in the PCoA plot (Figs. 5, and S1). The PCoA shows that *K. cochlearis* and *P.* cf. *dolichoptera* were dominant in the highest bentazone concentration and calanoid copepods dominated in the mesocosmos exposed to the lower concentrations. The Williams test for each species revealed significant differences related to the bentazone concentrations. The abundance of copepod nauplii (NOEC < 250 µg/L in the M treatment and NOEC < 25 µg/L in NM treatment for D90) and *K. quadrata* (NOEC < 25 µg/L in the NM treatment and > 2500 µg/L in M treatment for D90) increased (Table 2). Adults of the calanoid *Copidodiaptomus numidicus* (Gurney, 1909) reduced its abundance in the NM treatment on D30 (NOEC < 25 µg/L) and recovered for D90 (NOEC > 2500 µg/L), while in the M treatment no effects were observed. Calanoid copepodites reduced their abundance in the M treatment on D90 (NOEC < 250 µg/L), and in the NM treatment they also reduced their abundance on D30 (NOEC < 25 µg/L) at much lower concentrations, and on D90 (NOEC < 250 µg/L) as shown in (Table 2).

Macroinvertebrate community

During the experimental period, up to 24 different macroinvertebrate taxa were identified, most of them belonged to Insecta (14 taxa), followed by Crustacea (4) and Mollusca (3). The most abundant species was the snail *Physella acuta* (Draparnaud, 1805), followed by Chironomini (Insecta), Lumbriculidae (Annelida) and the Decapoda (Crustacea) *D. valentina.* Significant differences were observed in the abundance and Shannon diversity in D90 for the two ecological conditions, with an increase in both cases in the mesocosms without macrophytes. No significant differences were observed in the abundance, species richness, and Shannon diversity index across the four levels of bentazone pollution (Tables S5 and S6).

Table 2 Calculated NOECs for the phytoplankton, benthic diatoms, zooplankton and macroinvertebrate communities and taxa populations

Taxonomic groups/taxa	M			NM		
	D-7	D30	D90	D-7	D30	D90
Phytoplankton						
Community	> 2500	> 2500	> 2500	> 2500	> 2500	> 2500
Aphanocapsa sp.					250↓	> 2500
Cryptomonas sp.	> 2500	250↑	> 2500	> 2500	> 2500	> 2500
Desmodesmus spp,					> 2500	25↓
Peridinium sp.				> 2500		250↓
Benthic diatoms						
Community	> 2500	25	25	> 2500	250	> 2500
Achnanthidium minutissimum (Kützing) Czarnecki	> 2500	< 25↓	< 25↓			
Cocconeis placentula Ehrenberg	> 2500	25↓	< 25↓			
Encyonema minutum (Hilse) D.G.Mann	> 2500	25↓	25↓	> 2500	25↓	25↓
Gomphonema parvulum (Kützing) Kützing	> 2500	> 2500	< 25↓			
Nitzschia palea (Kützing) W.Smith	> 2500	< 25↓	< 25↓	> 2500	< 25↓	< 25↓
Zooplankton						
Community	> 2500	> 2500	> 2500	> 2500	> 2500	25
Polyarthra cf. *dolichoptera* (Idelson, 1925)	> 2500	> 2500	250↑	> 2500	> 2500	> 2500
Notholca cf. *acuminata* (Ehrenberg, 1832)	> 2500	25↑		> 2500	> 2500	
Keratella cochlearis (Gosse, 1851)	> 2500	> 2500		> 2500	250↑	> 2500
Keratella quadrata (Müller, 1786)	> 2500	> 2500	> 2500	> 2500	250↑	< 25↑
Daphnia magna Straus, 1820	> 2500	250↓		> 2500	> 2500	
Daphnia pulicaria Forbes, 1893	> 2500	> 2500		> 2500	25↓	
Nauplii	> 2500	> 2500	250↑	> 2500	> 2500	25↑
Calanoid copepodites	> 2500	> 2500	250↓	> 2500	25↓	250↓
Copidodiaptomus numidicus (Gurney, 1909)		> 2500	> 2500	> 2500	< 25↓	2500
Macroinvertebrates						
Community	> 2500	> 2500	> 2500	> 2500	> 2500	> 2500

Only the taxa that showed significant bentazone-related effects in one sampling date or more are displayed, while the rest are presented in the supplementary information (Table S7)

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

D day relative to the first bentazone application, *M* macrophytes treatment, *NM* non-macrophytes treatment

↑: abundance increase; ↓: abundance decrease relative to the control

The PERMANOVA did not show a significant effect of bentazone on the macroinvertebrate community for any sampling date. However, significant differences in the two different ecological conditions (M and NM) were found on D90 (Table 1). The abundance of *D. valentina*, *Caenis* sp. (Insecta, Ephemeroptera), and *Echinogammarus* sp. (Amphipoda, Crustacea) was higher in the treatments without macrophytes, while Anisoptera (Insecta) was more abundant in the mesocosms with macrophytes (Fig. S2).

Submerged macrophytes

As mentioned above, spontaneous growth of filamentous algae was found in the mesocosms without macrophytes, however on D30 there were statistically significant differences between the macrophyte biomass in the mesocosms with planted macrophytes and without, being higher in the mesocosms with planted macrophytes. On D30, statistically significant effects of bentazone were not

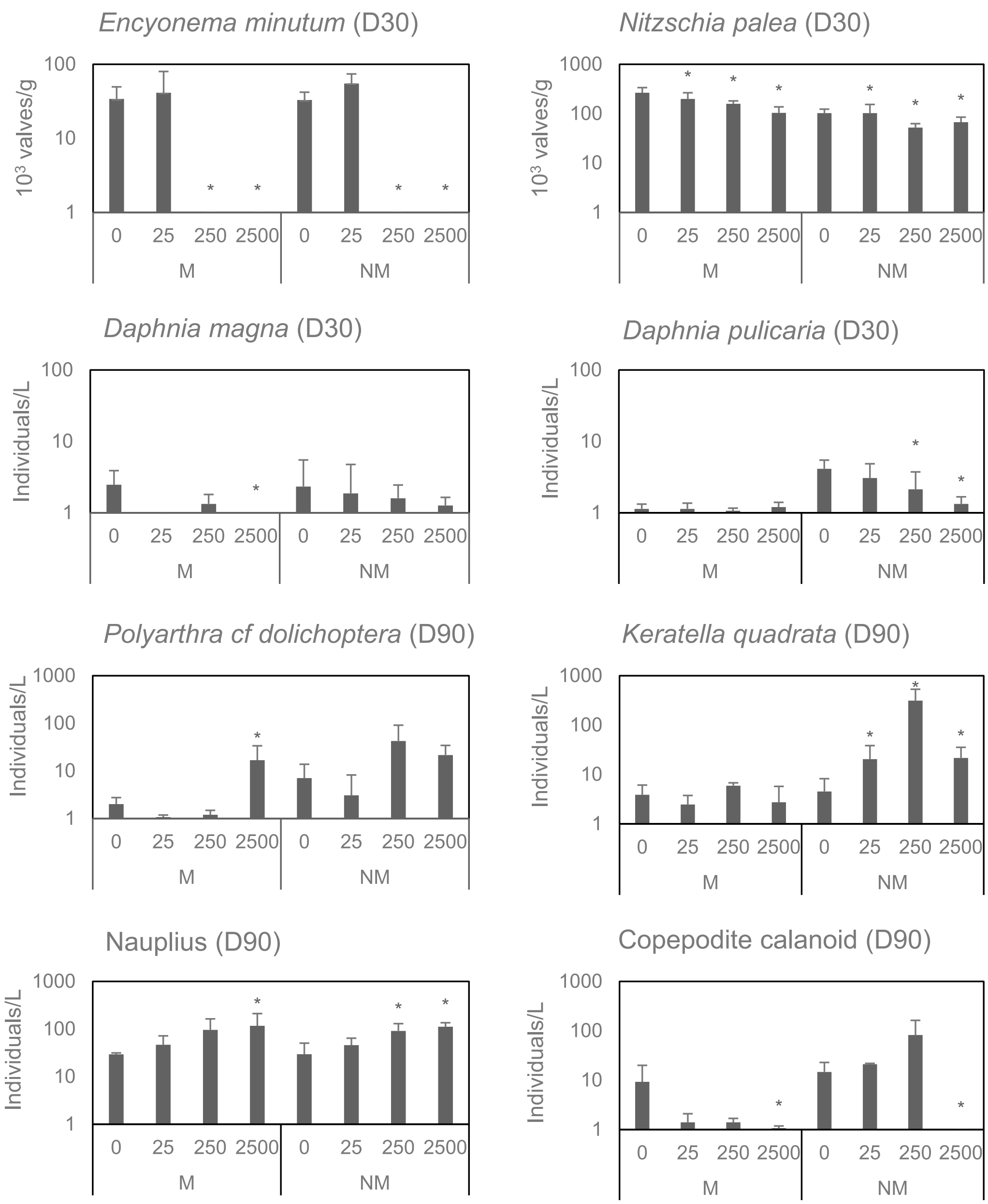

Fig. 4 Population abundance of the most responding taxa in the mesocosms exposed to 0, 25, 250 and 2500 µg/L of bentazone under the different ecological conditions (M and NM), *represents significant differences with the controls as indicated by the Williams test

observed on the total macrophyte biomass of the mesocosms that contained planted macrophytes (M); however, the growth of filamentous algae was statistically reduced in the mesocosms without macrophytes exposed to the highest bentazone concentration (2500 µg/L; Fig. S3). On D90, the biomass differences between the mesocosms with and without macrophytes were lower due to the continued growth of filamentous algae in both types of mesocosms. No effects of bentazone were found on macrophyte biomass on D90 for any of the two ecological conditions (Fig. S3).

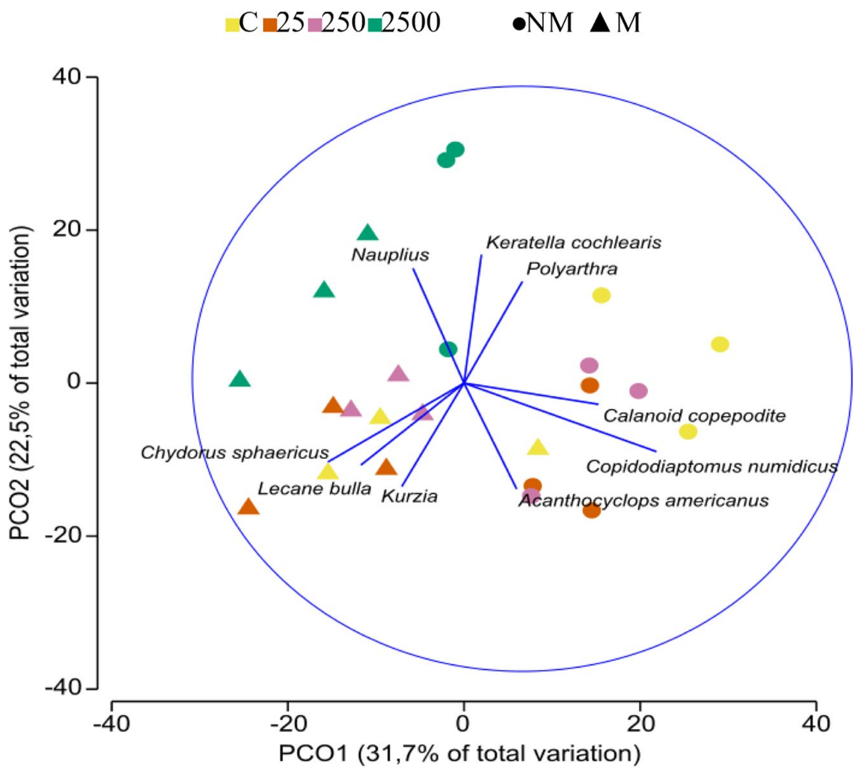

Fig. 5 Principal Coordinates Analysis (PCoA) for zooplankton on D90. The points represent all mesocosms, the concentration of bentazone (µg/L) is shown by colors (■ C; ■ 25; ■ 250; ■ 2500) and the two ecological conditions are represented by different symbols (●NM; ▲M). Distances between points are proportional to similarities in community composition. The species are represented in correlation with the abundance in the different mesocosms

Structural equation modeling (SEM)

A SEM approach was used to analyze the direct and indirect effects caused by bentazone on the trophic (functional) groups (Fig. 6). The process began with the construction of a saturated model that assumed a direct negative effect of bentazone on all functional groups. Following this, non-significant relationships were removed, resulting in the final unsaturated model. The model demonstrates a generally acceptable fit to the data, as evidenced by the ranges of fit indices: the Chi-square test yields a *P*-value of 0.230, suggesting no significant discrepancies between the model and the observed data; the SRMR value was 0.096; and the RMSEA value was 0.086 which are close to the acceptable ranges proposed by Kline (2023). The CFI (0.98) shows a value above the threshold of 0.95, which indicates an optimal fitting of the model. The SEM analysis demonstrated significant direct and indirect effects associated with bentazone exposure. The pathway coefficient strength confirmed that the most important direct negative effects of bentazone corresponded to those observed on the benthic diatom abundance. Additionally, the data indicated a negative correlation between the non-macrophytes treatment and the composition of benthic diatom species. In addition, the SEM showed significant negative indirect effects (in terms of covariation) between benthic diatoms and small filter-feeders, and between phytoplankton and benthic diatoms. A positive relationship was observed among phytoplankton and small filter-feeders, and predators and small filter feeders (Fig. 6).

Discussion

This study shows that the herbicide bentazone significantly affects the structure of aquatic communities and that such effects differed under the two tested ecological conditions, with and without macrophytes. Bentazone exhibited a similar exposure regime in both ecological conditions, with equivalent dissipation coefficients, and with similar DT50s to those obtained in previous experiments (Ross et al., 1989; Zanella et al., 2011). This suggests that the differences in the response of aquatic communities to bentazone under these two ecological conditions were

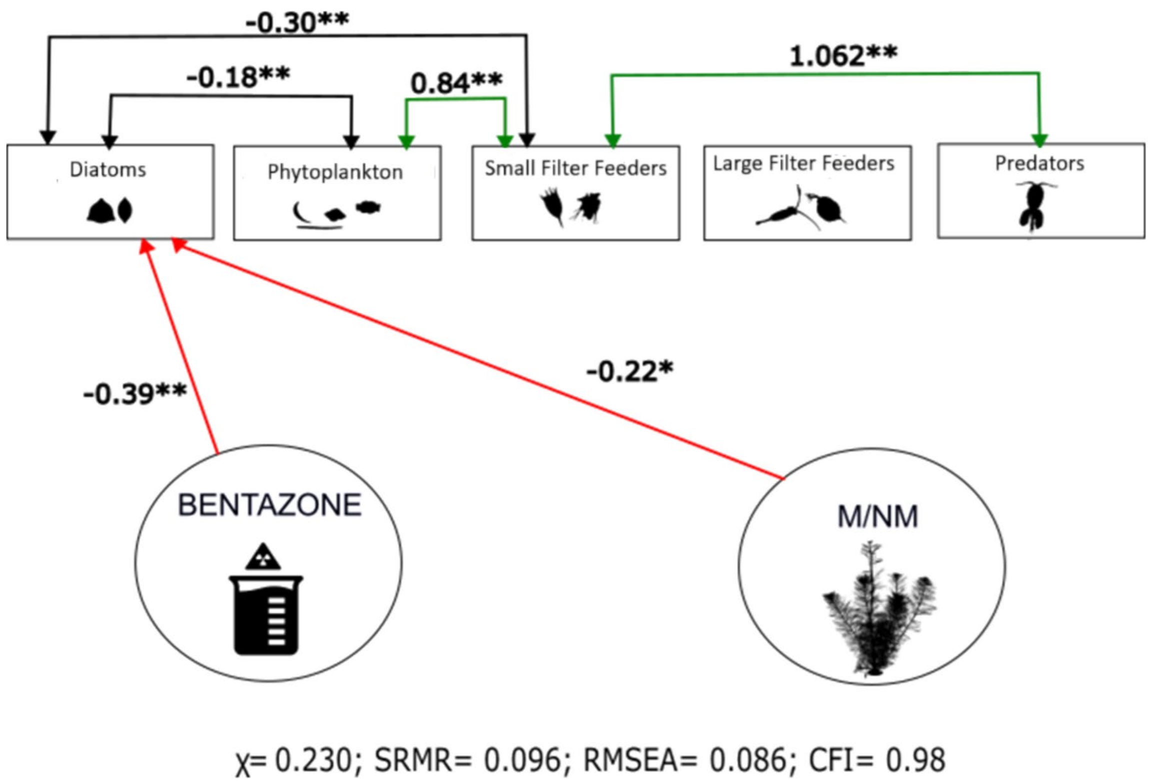

Fig. 6 Scheme showing the outcomes of the final unsaturated SEM model, where only significant causal relationships from the original saturated model were kept. The arrows indicate statistically significant relationships among trophic groups and standardized path coefficients. Negative direct relationships are drawn in red. The indirect effects as a result of covariation are drawn in black for the negative relationship and in green for the positive relationship. The asterisks indicate significant relationships: **P*-value < 0.05; ***P*-value < 0.01. *M* macrophytes/*NM* without macrophytes

related to differences in the structure of the aquatic communities and species interactions, rather than to different herbicide bioavailability or effects on macrophyte growth.

The disparity in the structure of the aquatic communities in the mesocosms with and without macrophytes can be attributed to moderate differences in physical and chemical parameters (pH, DO, nitrate) and to differences in habitat characteristics (i.e., surface cover, presence of refugees). These discrepancies were reduced towards the end of the experiment (D90) due to the accelerated growth of filamentous algae with increasing sunlight and water temperature. The growth of filamentous algae was negatively affected by bentazone in the highest test concentration in the mesocosms that were not planted with *M. spicatum*, however, an effect on the macrophyte growth could not be demonstrated in the mesocosms that contained *M. spicatum*. Hanfland et al. (2024) determined an EC20-14d of 957 μg/L for *M. spicatum* exposed to bentazone under laboratory conditions. Based on their results, we expected some effects on the development of *M. spicatum* in the highest test concentration. However, the growth rate of *M. spicatum* in our mesocosms was considerably low (*personal observation*) and such slow pace was probably related to the rapid growth of filamentous algae, which competed for light and nutrients with them.

The most sensitive taxonomic group to bentazone were benthic diatoms, which were affected by bentazone in both ecological conditions, decreasing their abundance, species richness and diversity. The herbicide showed a larger and longer lasting impact on the diatom community in the mesocosms with macrophytes (NOEC: 25 μg/L), which did not recover within the experimental period; while in the mesocosms without macrophytes, the sensitivity was lower (NOEC: 250 μg/L) and there was community recovery at the end of the experiment. The observed differences in the effects of the herbicide are attributed to the different species composition of the two ecological conditions. Notably, the species *A. minutissimum*, *C. placentula* and *G. parvulum*, found exclusively in the macrophyte treatment, along with *N. palea* and *E. minutum*, found in both treatments, were the most sensitive species to the herbicide; while *Navicula cryptocephala Kützing*. and *Staurosira venter (Ehrenberg) P.B. Hamilton.*, found in both treatments, were the most tolerant species. The high sensitivity of some benthic diatom species to herbicides has been observed in other studies performed with other photosystem II inhibitors, suggesting these taxa are good bioindicators for this kind of compounds in surface waters (Wood et al., 2014, 2016). In line with our study, Wood et al. (2016) concluded that species in the genus *Gomphohonema* and *Encyonema* are highly sensitive to herbicides, while *Navicula* are among the most tolerant taxa.

The phytoplankton community displayed low species richness and diversity, and was dominated by *Rhodomonas* and *Cryptomonas* in both treatments, which did not show a significant treatment-related decline. In fact, *Cryptomonas* was found to increase at the highest treatment level on D30 in the macrophyte-dominated mesocosms. Previous studies suggest that similar Cryptophyta taxa can tolerate high exposure levels of herbicides, and may even be stimulated by their presence, due to lower competition (Devilla et al., 2005; Huertas et al., 2010).

The varying tolerance of microalgae (phytoplankton and benthic diatoms) to herbicides that inhibit the photosystem II has been related to the trophic mode of their metabolism (Hellebust & Lewin, 1977; Hamilton et al., 1988). While most strict autotrophs are highly sensitive to this compound, Larras et al. (2012) showed that some mixotrophic diatom species might be more tolerant because they can change their trophic mode from autotrophy to heterotrophy under light limitations or due to the presence of other stressors acting similarly. The existence of a heterotrophic accessory metabolism is common in many *Navicula* species (Goldsborough & Robinson, 1986; Peres et al., 1996; Wood et al., 2016; Villanova and Spetea, 2021), and Cryptophyta are also considered a mixotrophic group (Camacho et al., 2001; Kim et al., 2018), which may explain their high tolerance to the tested herbicide.

Although a notable decline in nitrate levels was observed at D30 for the highest test concentration (2500 µg/L) in both ecological conditions, it was determined that this phenomenon was unlikely to be the primary driver of the observed impact on the diatom community. This is because at the low and intermediate concentrations (25 and 250 µg/L), the effects on the diatom community were already evident, while the reduction in nitrate levels was not observed. The nitrate concentration decline may be attributed to an inhibitory impact on nitrification, as previously documented by Allievi et al. (1996), which described chemical related effects on nitrification that were modulated by the type of substrate and the microbial community established on it.

The strongest effects of bentazone on the zooplankton community occurred in the treatment without macrophytes in the last sampling day (D90) (NOEC = 25 µg/L), while in the macrophytes treatment no significant effects were observed at the community level (NOEC > 2500 µg/L), although some species displayed significant effects. On day 30, the highest herbicide concentration reduced the population abundance of some large filter feeders (i.e., *D. magna* in the mesocosms with macrophyte, and *Daphnia pulicaria Forbes, 1893* in the mesocosms without macrophytes). By day 90, these groups were not found in any of the mesocosms, so that population recovery could not be evaluated. Such decline may be related to the seasonal succession of large cladocerans, which tend to decline with increasing water temperatures in the beginning of summer (Menéndez-López & Comín, 2021) (Table S3).

The adults and copepodite stages of the calanoid copepod *C. nummidicus*, another large filter feeder (Caramujo & Boavida, 2000, Geraldes & George, 2004, Geraldes & Boavida, 2012), were the most sensitive zooplankton species (NOEC < 25 µg/L), reducing their abundance, particularly in the mesocosms without macrophytes. The decrease in large filter feeder species, such as Cladocera and Copepoda, seemed to favor the increase of certain rotifer species, such as *P.* cf. *dolichoptera* in the mesocosms dominated by macrophytes, or *K. cochlearis* and *K. quadrata* in the mesocosm without planted macrophytes. Similar changes in the zooplankton community composition have been reported in previous studies evaluating the long-term impacts of agricultural pesticides (e.g., Hanazato, 2001; Polazzo et al., 2022; Rico et al., 2018; Vera et al., 2012). Generally, larger filter feeder species are more susceptible to food limitation than smaller species (Friberg-Jensen et al., 2003; Macisaac and Gilbert 1991; Wendt-Rasch et al., 2003), which may explain the change in the dominance pattern observed here. Our study revealed that the most significant effects were observed on D90 (60 days after the last application), suggesting a long-term impact and indirect effects of bentazone on the zooplankton community. In this case, since the amount of phytoplankton in the water column was very low, it is expected that these indirect effects were mediated by the reduction of primary producers in the biofilms set in the mesocosm´s wall and in the sediment, which likely were the main food resource of the Cladocera and herbivorous Cyclopoids in the test mesocosms.

As for macroinvertebrates, no statistically significant effects of the pollutant were observed neither for the whole community nor on any of the species found. However, other studies have observed indirect effects of some herbicides on the aquatic macroinvertebrate community, causing changes in species interactions and dominance patterns (Rumschlag et al., 2020).

The complementary use of both PERMANOVA and SEM analyses enhanced our understanding of the direct and indirect effects of bentazone in the mesocosm experiment. The SEM analysis confirmed the direct effects of the herbicide on diatoms and shed light on some of the indirect effects, which were only intuited in previous analyses. Based on the SEM results, the impact of bentazone on zooplankton is primarily indirect as it occurred as a result of the direct effect of bentazone on primary producers. The SEM also indicated that the increase in small filter feeders had a positive impact on predators (here mainly represented by the copepod *A. americanus*), a phenomenon that has also been described in other studies (Chang et al., 2005; García et al., 2011). Overall, the SEM analysis confirms that Photosystem II inhibitors directly impact the composition of primary producers, benthic diatoms in this case, resulting in a reduction in both their abundance and species diversity. Such reduction led to a change in food availability and competitive interactions within the zooplankton community, changing the equilibrium between small and large filter feeders, as indicated in previous studies (Rico-Martinez et al., 2012; Zhao et al., 2021).

Our study shows that long term effects may be expected in some benthic diatom taxa at concentrations of 25 µg/L, as well as on the overall structure of the whole benthic diatom assemblages at concentrations above 25 µg/L. This suggests that the current exposure levels of bentazone measured in Mediterranean coastal wetlands impacted by rice farming (Barbieri et al., 2021; Calvo et al., 2021; Martínez-Megías et al., 2023) are very likely to pose direct toxic effects to these communities and may result in significant side-effects on large filter feeders. Large filter feeders have an important role as phytoplankton grazers and as food source for fish and other vertebrate species (Hanazato, 1998) and can thus be considered keystone species in Mediterranean coastal wetlands. Under certain conditions, such as those of many eutrophic Mediterranean wetlands, a significant decrease of zooplankton grazing could yield to an increase of phytoplankton biomass and the proliferation of certain algae species, including cyanobacterial blooms (Ger et al., 2016).

In conclusion, this study describes the impacts that the herbicide bentazone may have on aquatic organisms from Mediterranean coastal wetlands. The potential long-term effects on benthic diatom communities, as well as the bottom-up effects on the food web, emphasize the urgency of addressing this environmental issue. It is also important to consider the potential for this herbicide to interact with other pesticides present in the environment, and the possibility that synergistic ecological effects occur. Further monitoring and modeling studies are needed to fully comprehend the extent of the risks caused by bentazone and other similar pesticides at the ecosystem level, and to develop exposure mitigation strategies to safeguard the ecological integrity of Mediterranean coastal wetlands.

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Data availability Data will be made available on request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Not applicable.

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