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Glyphosate: A Terrestrial Threat to Marine Plants? A Study on the Seagrass *Zostera marina*

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Abstract

Glyphosate-based herbicides (GBHs) are extensively used worldwide, raising concerns about their potential effect on non-target aquatic ecosystems. This study investigated the short-term physiological effects of a commercially available GBH on the seagrass *Zostera marina* under controlled mesocosm conditions. *Z. marina* individuals were exposed to three concentrations of glyphosate (0.165, 51, and 5100 mg L^{-1}) for 4 days, and the impacts on photosynthetic performance, growth rate, photosynthetic pigments content and energy metabolism were assessed. Exposure to 5100 mg L^{-1} of glyphosate caused rapid water acidification and complete plant mortality within 24 h. Exposure to 51 mg L^{-1} of glyphosate significantly impaired photosynthetic efficiency and foliar growth rate. Energy availability, photosynthesis and photosynthetic pigments content were highly disrupted at both higher concentrations. Exposure to 0.165 mg L^{-1} of glyphosate decreased the foliar chlorophyll *a/b* ratio. These findings show that *Z. marina* can potentially be threatened by the presence of GBHs even at lower concentrations and underscore the necessity for monitoring herbicide pollution in coastal waters to protect seagrass habitats and associated ecosystems. Further research is needed to assess long-term effects and the role of herbicide formulations in mediating toxicity.

Keywords: seagrass; *Zostera marina*; glyphosate; herbicide contamination; marine pollution; marine plant physiology; agricultural runoff

Academic Editors: Diego Macías and Beatriz Morales-Nin

Received: 27 June 2025
Revised: 24 July 2025
Accepted: 7 August 2025
Published: 18 August 2025

Citation: Deguette, A.; Pes, K.; Vasconcellos, B.; Costa, M.; Silva, J.; Barrote, I. Glyphosate: A Terrestrial Threat to Marine Plants? A Study on the Seagrass *Zostera marina*. *Oceans* **2025**, *6*, 51. https://doi.org/10.3390/oceans6030051

1. Introduction

Synthetic agrochemicals started to be largely used after World War II to increase crop productivity and overcome human malnutrition [1]. Among all herbicides, glyphosate (*N*-(phosphonomethyl)glycine) remains one of the most widely used to control unwanted weeds and algae [2]. It is a non-selective, post-emergent, and systemic herbicide that was first commercialized by Monsanto in 1974 [3]. It is commonly sold as salt and mixed with surfactants such as polyoxymethylene amine (POEA), as in the popular commercial product RoundUp®, to increase uptake and translocation into the growing parts of the

plants [1]. Besides the controversy around the risk of glyphosate and its surfactants being environmental and health hazards, in 2023 the European Commission renewed for 10 more years the authorization of its use, with some restrictions [4–8] (see Commission Implementing Regulation (EU) 2023/2660 of 28 November 2023 [9]). In Portugal, Decree-Law No. 35/2017 of March 24 prohibits the application of herbicides in some urban areas, such as gardens and parks, schools and hospitals [10]. Nonetheless, glyphosate-based herbicides are extensively used in orange groves and greenhouse cultivation in southern Portugal. Their proximity to the coast or waterways provides a potential source of herbicide to the marine environment. Although no public information is available regarding the amount of glyphosate used by the agricultural sector in the EU, Antier et al. (2020) [11] found that, in 2017, more than 50% of the total herbicide formulations sold in Portugal contained glyphosate.

Glyphosate acts through the inhibition of the chloroplastic enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is an intermediate of the aromatic amino acid synthesis in the shikimate pathway [1,12]. Consequently, it causes a decline in protein synthesis and blocks plant growth, which is usually followed by chlorosis and necrosis within two to four days after application of the herbicide [13]. Once glyphosate reaches the soil, it normally becomes immobilized by absorption or binds to soil particles. Still, some free molecules can be transformed into aminomethylphosphonic acid (AMPA) and CO_2 by microbial metabolization (reviewed by Vereecken, 2005 [14]). Furthermore, episodes of runoff may transport this herbicide to groundwater, surface water and aquatic environments [15–20], and potentially end up in seawater [21]. While the presence of glyphosate and its metabolite AMPA in European freshwater environments has been extensively reported [21–28] only a few studies measured the presence of glyphosate in the marine environment, ranging from 0.42 ng L^{-1} to 1.2 µg L^{-1} in European seawaters [29–31]. Glyphosate was reported to be persistent in seawater for 47 to 315 days [32]. In small, enclosed water systems near agricultural lands and following runoff episodes, we might expect to observe glyphosate concentrations on the mg L^{-1} scale.

The proximity of agricultural land to coastal regions can facilitate the influx of nutrients and herbicides into aquatic environments, thereby generating water contamination in the adjacent coastal systems. The Ria Formosa is a barrier lagoon located in southern Portugal, extending to approximately 55 km in length and 6 km in width [33]. The lagoon hosts 99% of total seagrass meadows of the Algarve region [34]. Owing to its seagrass meadows, which provide a plethora of ecosystem services and high-value economic benefits [35], the lagoon plays a pivotal role in the region's ecosystem, serving as a vital habitat, breeding ground, and nursery for numerous species [36–39], which justifies its status as a National Park, Natural Reserve, Natura 2000 and Ramsar site. Seagrasses are endangered worldwide [40,41] and their protection is a priority in marine ecosystem conservation. Among the three seagrass species of Ria Formosa, the ubiquitous *Zostera marina*, classified as a *Vulnerable* species in the Red List of the Vascular Flora of Continental Portugal [42], is one of the most endangered, with reported decreases since 2010 [34,43]. The lagoon is subjected to various anthropogenic pressures, including urbanization, livestock husbandry, and intensive agriculture and aquaculture [44–46]. These activities introduce a variety of sources of pollution, including sewage discharge, aquaculture effluents and agricultural runoff [46–49] that may be a threat to seagrass meadows. Due to the limited hydrodynamic exchange with the Atlantic Ocean, only 50 to 70% of the lagoon's water is renewed daily [50], consequently enhancing the potential for contaminant accumulation.

A review of the literature on macroalgae's exposure to glyphosate reveals several effects, including alterations in growth and biomass, gene expression, photosystem II (PSII) function, reductions in pigment levels, increase in reactive oxygen species (ROS) content

and oxidative stress [51–61]. Among the few studies on seagrasses, it was reported that exposure to glyphosate concentrations above 0.25 to 225 mg L^{-1}, depending on the species, induces reductions in foliar chl *a*, lower biomass, reduction in PSII's activity and higher mortality, with enhanced consequences as concentration increases [58,62–66]. The variety of responses to diverse concentrations suggests species-dependent responses to glyphosate exposure.

In this study, we investigated the impact of three different concentrations of a commonly sold glyphosate formulation on growth, photosynthetic activity, photosynthetic pigments content and adenylate compounds content of *Z. marina* in a controlled, closed-system, mesocosm experiment.

2. Materials and Methods

2.1. Experimental Design

Z. marina individuals with at least three shoots were collected in Ria Formosa coastal lagoon near Culatra island (South Portugal, 37° N, 7°49′ W) on 7 March 2019. Following collection, plants were cleaned from epiphytes by hand and using a scalpel and transported in dark containers filled with local water collected near Ramalhete Field Station (CCMAR, University of Algarve). On the following day, seagrasses were distributed and planted in 20 tanks (26 shoots per tank), each filled with 20 L of artificial seawater. Water was first treated by reverse osmosis to remove potential glyphosate residues and brought to a salinity of 35 ppt (Coral Pro Salt, Red Sea Fish Pharm Ltd., Herzliya, Israel). Plants were acclimated for 6 days at 13.76 ± 0.28 °C, 8.35 ± 0.03 pH, with a light intensity of 59.55 ± 1.28 µmol photons m^{-2} s^{-1} and a photoperiod of 12-12 light-dark cycle.

Subsequently, five aquaria were randomly assigned to each of the four treatments (control and three herbicide dilutions). An herbicide formulation purchased in a common retail store was used for this study (composition: active substance glyphosate potassium salt 35.5% equivalent 360 g glyphosate L^{-1}, etheralkylamine ethoxylate 6%, water and other ingredients in minor proportion 58.5%). Three different concentrations of glyphosate were obtained by diluting the herbicide: (i) 0.165 mg L^{-1}, corresponding to the highest concentration of glyphosate found in streams and rivers in France in 2003–2004 [28] (ii) 51 mg L^{-1}, an intermediate concentration and (iii) 5100 mg L^{-1}, which was, by the time this work was completed, one of the lowest concentrations freely sold in the market for direct application (i.e., without dilution). The control aquaria contained only artificial seawater (0 mg glyphosate L^{-1}). These concentrations are to be considered as "glyphosate equivalent of the glyphosate-based herbicide". All aquaria were maintained in a closed circuit with aeration. At the end of the experiment, seawater contaminated with herbicide was disposed of as chemical waste through the appropriate laboratory channels.

2.2. Water Physical–Chemical Parameters

During the entire experiment, both in the acclimation and the experimental periods, water physical–chemical conditions were measured daily using an Orion Star™ A221 Portable pH Meter (Thermo Fisher Scientific Inc., Ayer Rajah Crescent, Singapore) for pH and temperature and an STX-3 refractometer (VEE GEE Scientific LLC, Vernon Hills, IL, USA) for salinity.

2.3. Sampling Procedures

During the mesocosm experiment, samples from each treatment (n = 5) were collected on different days because of the sudden and visible effect of the herbicide at the higher concentration: one day after exposure for the higher concentration, 4 days for the intermediate and lower concentrations, and 5 days for control condition. Leaves were cleaned

from epiphytes, rinsed in distilled water, blotted dry, frozen in liquid nitrogen and stored at −80 °C until analysis.

2.4. Growth Rate

The growth of the plants was evaluated using the punching method, as described by Worm and Reusch (2000) [67]. One day before the introduction of glyphosate into the water, the plants were identified by marking a specific spot on the lower outer leaf sheath using a syringe needle. The growth of the 3 younger leaves was quantified by measuring the distance from the mark on the oldest leaf, designated as the reference point. Subsequently, the total growth of each plant was measured by the addition of the growth of the 3 younger leaves divided by the number of days of treatment (5 for the control, 4 for the intermediate and lower concentration) to obtain the growth rate (cm day^{-1}). The growth calculation for plants exposed to the higher glyphosate concentration was omitted due to their mortality within a single day of exposure.

2.5. Photosynthetic Efficiency

A Diving-PAM (Underwater Chlorophyll Fluorometer Pulse-Amplitude-Modulated (PAM), Heinz Walz GmbH, Effeltrich, Germany) was employed to assess photosynthetic efficiency, to measure the effective photosynthetic efficiency of PSII in the light (effective quantum yield, $\Delta F/F_m'$) and the potential photosynthetic efficiency following a 30 min dark-acclimation period (maximum quantum yield, F_v/F_m). All fluorescence measurements were conducted on the second or third leaves in the middle of the adaxial surface. Dark-acclimation leaf clips were used to ensure a constant distance between the optic fiber tip and the leaf sample. The effective quantum yield was determined using the same methodology but omitting the dark acclimation period. Fluorescence measurements were conducted daily throughout the experiment. The same two plants per aquarium, previously marked and identified, were used for all measurements.

2.6. Photosynthesis-Irradiance (P-I) Curves and Dark Respiration Rate

Photosynthesis-irradiance (*P-I*) curves were made on the same days the tissue samples for biochemical analysis were collected. Segments of the 2nd or 3rd youngest leaf of a shoot taken from each tank were incubated in chambers with 0.07 L of the same water in which plants were growing. Incubation water was continuously homogenized by a magnetic stirrer, and the temperature was maintained at 14 °C (approx. water temperature in the aquaria) by a Julabo F10 thermostatic circulator (Julabo GmbH, Seelbach, Germany) [68]. The light was provided by a system of LED lamps (LEXMAN, 20W-2452 Lumens, ADEO Services, Ronchin, France). Each chamber was exposed to 10 increasing light intensities (7 ± 0.71; 19.4 ± 1.52; 35 ± 2.55; 69 ± 2.55; 119.8 ± 9.47; 223.6 ± 7.09; 310.6 ± 11.06; 450.6 ± 10.24; 936.2 ± 20.73 and 1325.4 ± 39.18 photosynthetic photon flux density (PPFD), μmol m^{-2} s^{-1}) by using a series of neutral density filters of variable transmittance. Light intensity was measured using a LI-COR LI-190 quantum sensor (LI-COR Biosciences, Lincoln, NE, USA). Each PPFD was maintained between 5 and 10 min, depending on the plants' oxygen production rate. Oxygen concentration was read at the beginning and the end of each exposure to PPFDs using a Microx 4 optical oxygen meter (PreSens Precision Sensing GmbH, Regensburg, Germany). Dark respiration was measured in the same leaf segments at the beginning and the end of each *P-I* curve after a dark period of ~30 min.

After photosynthesis (*P*) and dark-respiration (*DR*) measurements, each leaf segment was dried at 60 °C for 48 h for dry weight.

Photosynthetic/dark-respiration (*P/DR*)rates were calculated as follows:

$$P/DR = \frac{[O_2]_f - [O_2]_i}{t \times 60} \times \frac{v}{DW} \quad (1)$$

where *P/DR*—Photosynthetic/dark-respiration rate (µmol O_2 gDW^{-1} h^{-1}); $[O_2]_f$—final oxygen concentration (µmol/L); $[O_2]_i$—initial oxygen concentration (µmol L^{-1}); *t*—incubation time (min); *v*—volume of water (L); *DW*—sample dry weight (g).

P-I curves were fitted with the Jassby and Platt (1976) [69] equation model using SigmaPlot (SigmaPlot for Windows Version 11.0, 2008, Systat Software Inc., San Jose, CA, USA) to derive P_{max} (maximal photosynthetic rate) and α (photosynthetic quantum efficiency) parameters. The standard error was estimated, and the saturation irradiance (I_k) was calculated as the ratio between P_{max} and α for each treatment, incorporating error propagation.

2.7. Photosynthetic Pigments

Following sample collection, leaf tissues were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Chlorophylls and carotenoids were quantified in approximately 200 mg (fresh weight) leaf tissue. Leaf tissue was ground in liquid nitrogen with sodium ascorbate ($C_6H_7NaO_6$) and immediately extracted in 5 mL of 100% acetone neutralized with calcium carbonate ($CaCO_3$) [70]; the extract (5 mL) was then sequentially filtered through 0.45 µm and 0.22 µm PTFE filters (Millipore, Merck KGaA, Darmstadt, Germany). Chlorophylls *a* and *b* were quantified spectrophotometrically after reading the extracts at 644.8 nm ($A_{644.8}$) and 661.6 nm ($A_{661.6}$) (Beckman-Coulter DU 650 spectrophotometer, Brea, CA, USA).

Quantification was performed using the equations of Lichtenthaler and Buschmann (2001) [71]:

$$\text{Chlorophyll } a\text{: Chl } a \text{ (µg mL}^{-1}\text{)} = 11.24\ A_{661.6} - 2.04\ A_{644.8} \quad (2)$$

$$\text{Chlorophyll } b\text{: Chl } b \text{ (µg mL}^{-1}\text{)} = 20.13\ A_{644.8} - 4.19\ A_{661.6} \quad (3)$$

Carotenoids (antheraxanthin, β-carotene, lutein, lutein epoxide, neoxanthin, violaxanthin, and zeaxanthin) were separated and quantified by high-performance liquid chromatography (HPLC) [68,72,73]. HPLC analysis was performed in an Alliance Waters 2695 separation module (Waters Corporation, Milford, MA, USA), with a Waters 2996 photodiode array detector (Waters Corporation, Milford, MA, USA) and a Phenomenex Synergi 4 µm Hydro-RP 80 Å LC Column, 150 × 4.6 mm (Phenomenex Inc., Torrance, CA, USA), Ea. During the process, extracts were maintained at 5° C, while the column was kept at 25 °C. Separation was performed by combining eluents in Isocratic mode, sequentially eluted by eluent R1 (acetonitrile, methanol and triethylamine, TEA) and R2 (acetonitrile, methanol, Milli-Q water, acetate ethyl and TEA), previously filtered and sonicated. Injection volume was set to 20 µL, and peak areas were monitored at 450 nm. Pigment concentrations were calculated through calibration curves of standards at known concentrations (pure pigments obtained from CaroteneNature, Lupsingen, Switzerland). The xanthophyll cycle epoxidation state was calculated as described by Silva et al. (2013) [68].

2.8. Adenylate Compounds (ATP, ADP and AMP) and Energy Charge (AEC)

Adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) were extracted and quantified following a protocol adapted from Coolen et al. (2008) [74] and Liu et al. (2006) [75]. Approximately 500 mg (fresh weight) of leaf tissue was ground in liquid nitrogen. Adenosine phosphates were extracted in 10 mL of 0.6 M perchloric acid ($HClO_4$). The extract was homogenized and then placed in a water bath at 100 °C for

10 min. Subsequently, the extracts were cooled in ice for 1 min and centrifuged at 4600× *g* at 4 °C for 30 min (Heraeus Megafuge 16 Centrifuge, Thermo Fisher Scientific, Waltham, MA, USA). Extracts' pH was adjusted to 6.5–6.8 with potassium hydroxide (KOH) 1 M, and the final volume was quantified. The extracts were then allowed to stand for 30 min in an ice bath to enable potassium perchlorate precipitation. The supernatants were filtered through 0.45 µm and 0.22 µm nylon filters and transferred to micro vials. Different concentrations of AMP, ADP and ATP (Sigma-Aldrich, St. Louis, MO, USA) were used as standard to calibrate the HPLC. Adenylate compounds were quantified by isocratic HPLC analysis [74] in an Alliance Waters 2695 separation module (Waters Corporation, Milford, MA, USA), with a Waters 2996 photodiode array detector (Waters Corporation, Milford, MA, USA) and a Phenomenex Kinetex 2.6 µm HILIC 100 Å, LC Column 30 × 2.1 mm (Phenomenex Inc., Torrance, CA, USA), Ea. Extracted ATPs were eluted in potassium phosphate buffer, and peaks were detected at 254 nm.

Adenylate energy charge (*AEC*) was calculated according to Atkinson and Walton (1967) [76] using the following equation:

$$AEC\left(\text{nmol gDW}^{-1}\right) = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]} \quad (4)$$

2.9. Data Analysis

Statistical analyses were made using Sigmaplot (Sigmaplot for Windows Version 11.0, 2008, Systat Software Inc., San Jose, CA, USA) and R Studio (R Studio for Windows Version 4.3.3, [77]). Shapiro–Wilk and Brown–Forsythe tests were performed to confirm normality distribution and equal variance, respectively. One-way ANOVAs were conducted to test the significant effects of the different treatments. Student–Newman–Keuls post hoc tests were used to reveal significant differences among treatments. In the case of heteroscedasticity, a Kruskal–Wallis test (ANOVA on ranks) was performed, followed by a Dunn–Bonferroni test to reveal differences among treatments. The significance level was set at $p < 0.05$ for all statistical tests.

3. Results

3.1. Water Physical–Chemical Parameters

The temperature in the aquaria ranged between 13.38 and 15.36 °C (Figure 1a). During the acclimation period (days 1 to 6), pH was stable at 8.35 ± 0.003. Following glyphosate addition (day 6), the pH of water with the intermediate and the higher concentrations of glyphosate decreased to 8.01 ± 0.04 and 4.72 ± 0.02, respectively (Figure 1b). Salinity was 36.40 ± 0.08‰ during the first day of acclimation due to water preparation and adjustments and then stabilized at 35‰ during the rest of the experiment (Figure 1c).

3.2. Photosynthetic Performance

The maximum quantum yield (F_v/F_m) showed similar values in all aquaria during the acclimation period (0.80 ± 0.001; Figure 1d). After adding glyphosate, control plants and plants exposed to the lower herbicide concentration continued to show values around 0.8, while those exposed to the higher concentration (5100 mg L^{-1}) faced a rapid decrease and reached zero after one day. *Z. marina* plants treated with the intermediate concentration of glyphosate (51 mg L^{-1}) displayed a continuous decrease of F_v/F_m until reaching 0.20 ± 0.1 after 4 days (Figure 1d). The effective quantum yield ($\Delta F/F_m'$) was also stable throughout the acclimation period, averaging 0.74 ± 0.01 (Figure 1e) and, as for F_v/F_m, the control plants and the plants exposed to the lower concentration of herbicide showed stable values during the experimental period. In plants exposed to the intermediate and higher

concentrations of glyphosate (51 and 5100 mg L^{-1}), $\Delta F/F_m'$ decreased to 0.35 ± 0.09 and 0 one day after adding glyphosate, respectively.

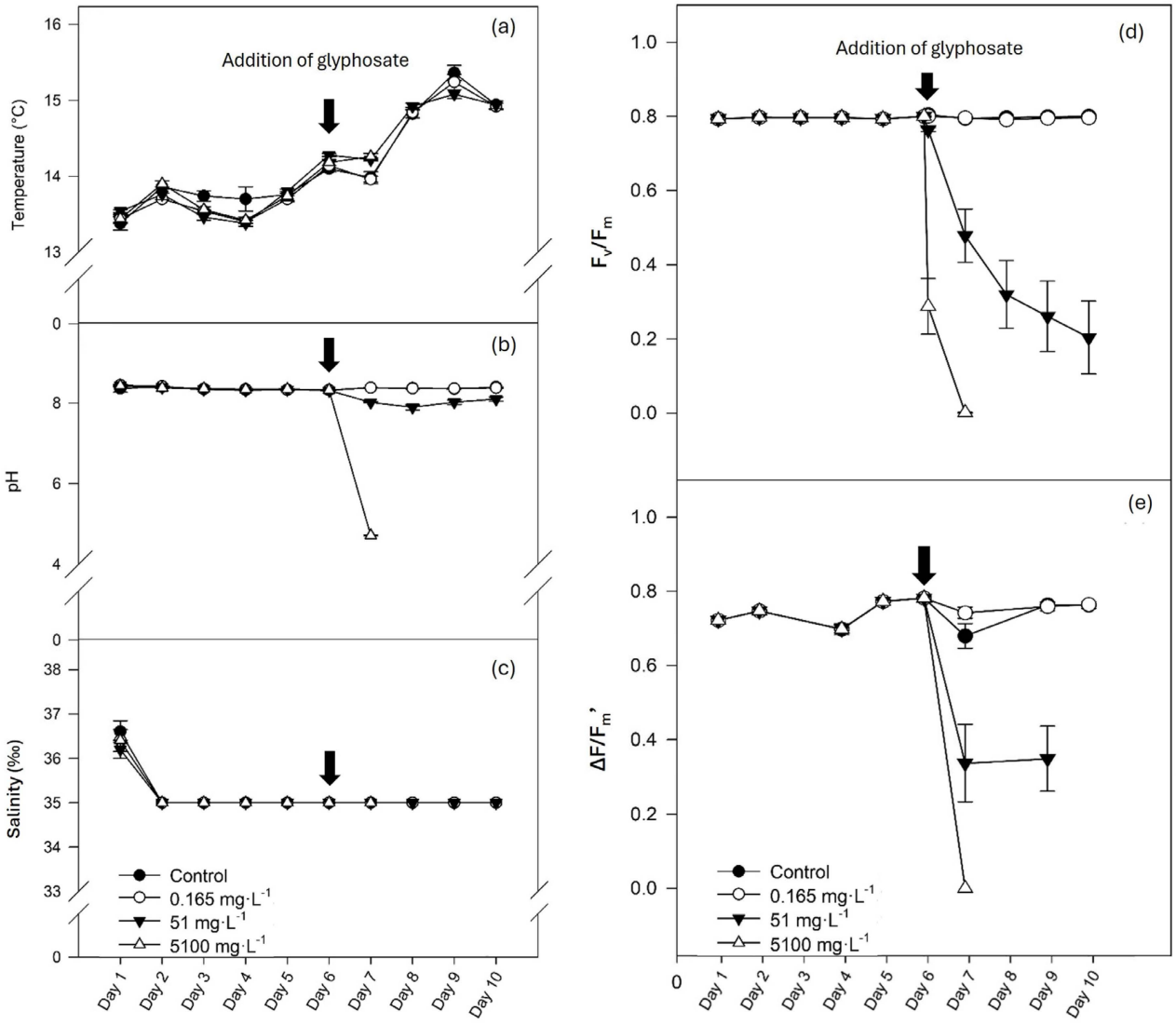

Figure 1. Water temperature (**a**), pH (**b**), salinity (**c**) (mean ± SE, *n* = 5) and maximum (F_v/F_m; (**d**)) and effective ($\Delta F/F_m'$; (**e**)) quantum yield of *Z. marina*'s photosystem II (mean ± SE, *n* = 10) in control conditions (no herbicide) and with addition of 0.165, 51 and 5100 mg L^{-1} of glyphosate equivalent.

3.3. Growth Rate

There was no statistical difference between the foliar growth rate of *Z. marina* exposed to 0.165 mg L^{-1} of glyphosate and the control. The foliar growth rate of *Z. marina* exposed to 51 mg L^{-1} of glyphosate was significantly lower than that of control plants and plants exposed to the lower concentration of glyphosate (Figure 2).

3.4. Photosynthetic Activity

There was a tendency for dark respiration to increase with glyphosate concentration (Table 1). The maximum photosynthetic rate (P_{max}) and minimum-saturation irradiance (I_k) were significantly lower in plants exposed to 51 mg L^{-1} of glyphosate compared to control and plants exposed to the lower glyphosate concentration. In contrast, the photosynthetic quantum efficiency (α) showed the opposite trend (Figure 3 and Table 1). It was not possible to obtain a photosynthesis-irradiance (*P-I*) curve for the plants exposed to the

higher concentration of glyphosate (5100 mg L^{-1}) as all individuals died in less than 24 h after exposure.

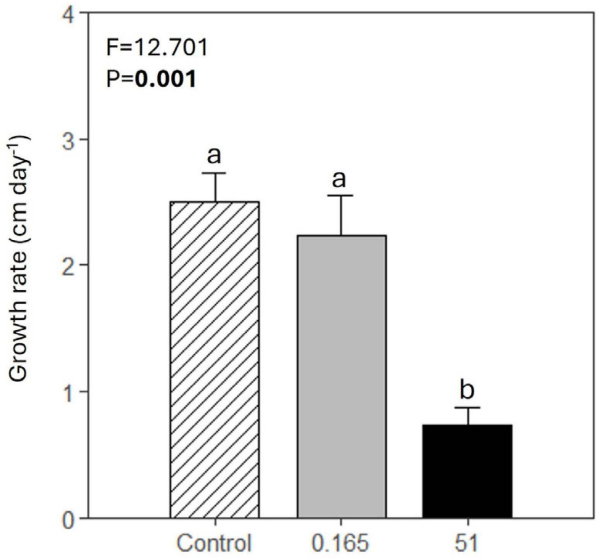

Figure 2. Foliar growth rate of *Z. marina* (mean ± SE, $n = 5$) in control conditions (no herbicide) and in plants exposed to 0.165 and 51 mg L^{-1} of glyphosate equivalent. Results from the one-way ANOVA testing for differences between treatments (F = test-statistic; P = *p*-value) are shown. The *p*-value in bold accounts for significant differences among treatments. Different letters indicate significant differences among treatments ($p < 0.05$).

Table 1. Dark respiration and photosynthetic parameters of *Z. marina*'s leaves (mean ± SE, $n \geq 4$) in control conditions (no herbicide) and after a four-day exposure to 0.165 and 51 mg L^{-1} of glyphosate equivalent. Photosynthetic parameters were obtained after Jassby and Platt (1976) [69] adjustment to the observed photosynthesis-irradiance data of *Z. marina*'s leaves. Dark respiration (µmol O_2 gDW^{-1} h^{-1}); P_{max}, maximum photosynthetic rate (µmol O_2 gDW^{-1} h^{-1}); α, photosynthetic quantum efficiency; and I_k, minimum-saturation irradiance (µmol photons m^{-2} s^{-1}). Results from one-way ANOVA testing for differences between treatments (F = test-statistic; *P* = *p*-value) are shown. The *p*-values in bold account for significant differences among treatments. Different letters indicate significant differences among treatments ($p < 0.05$).

Glyphosate Equivalent (mg L^{-1})	Dark Respiration (µmol O_2 gDW^{-1} h^{-1})	P_{max} (µmol O_2 gDW^{-1} h^{-1})	α	I_k (µmol m^{-2} s^{-1})
0 (Control)	−3.448 ± 0.807	832.704 ± 32.927 [a]	4.409 ± 0.450 [b]	188.869 ± 20.661 [a]
0.165	−17.336 ± 6.752	945.414 ± 89.636 [a]	5.149 ± 1.168 [b]	183.615 ± 45.154 [a]
51	−22.893 ± 4.750	108.436 ± 3.512 [b]	17.361 ± 4.237 [a]	6.246 ± 1.538 [b]
F	3.493	53.421	10.111	51.621
P	0.067	**<0.001**	**0.003**	**<0.001**

3.5. Photosynthetic Pigments

Total chlorophylls and total carotenoids did not show significant variations among treatments (Figure 4a,b). The chlorophyll *a*/*b* ratio decreased significantly with herbicide concentration (Figure 4c). The total chlorophylls to total carotenoids ratio was significantly higher in *Z. marina* exposed to the higher glyphosate concentration than control plants and plants exposed to the lower glyphosate concentration (Figure 4d).

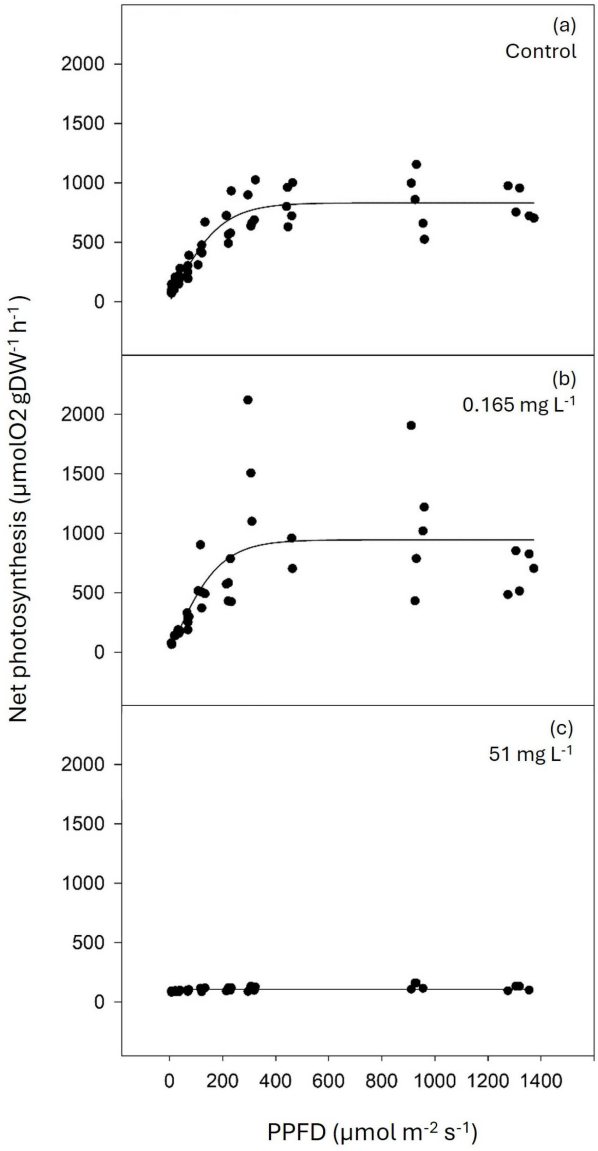

Figure 3. Photosynthesis-irradiance (*P-I*) curves of *Z. marina*'s leaves in control conditions (no herbicide) (**a**) and after a four-day exposure to 0.165 mg L^{-1} (**b**) and 51 mg L^{-1} (**c**) of glyphosate equivalent. Dots represent each measurement and curves represent the adjustment with the equation model of Jassby and Platt (1976) [69].

Most pigment concentrations did not vary among treatments. β-carotene and violaxanthin showed a significant reduction in plants exposed to the higher concentration of glyphosate compared to all the other treatments (Table 2). The sum of the xanthophylls violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z) was significantly lower in plants exposed to the higher concentration of glyphosate compared to all the other treatments. The de-epoxidation state (DES) index was not affected by the two lower glyphosate concentrations, but it was significantly higher in plants exposed to the higher glyphosate concentration. (V + A + Z)/Chl *a* + *b* decreased significantly in plants exposed to a glyphosate concentration of at least 51 mg L^{-1}.

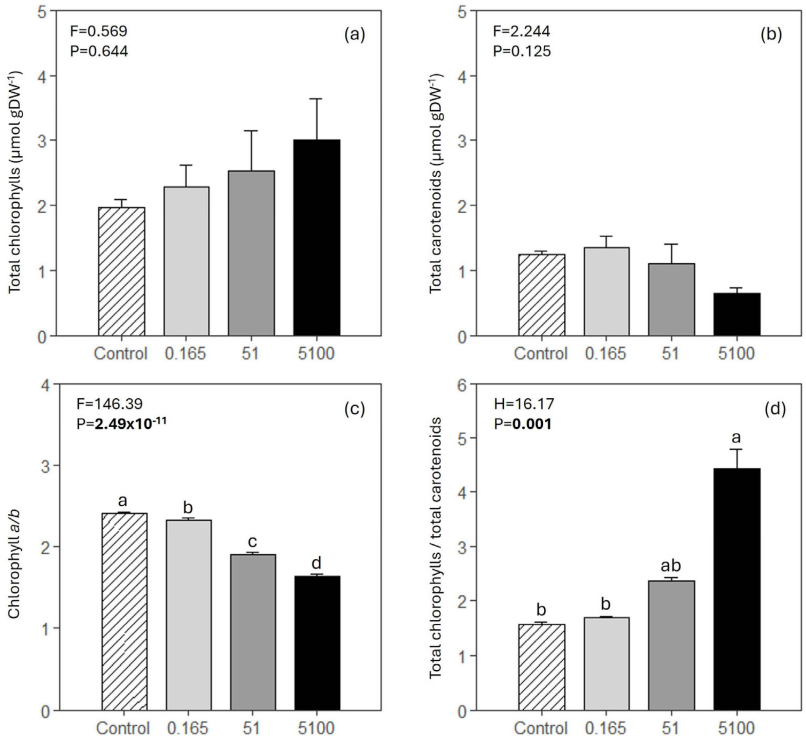

Figure 4. *Z. marina*'s foliar total chlorophylls (**a**), total carotenoids (**b**), chlorophyll *a*/*b* ratio (**c**) and total chlorophylls to total carotenoids ratio (**d**) (mean ± SE, $4 \leq n \leq 5$) in control conditions (no herbicide) and in plants exposed to 0.165, 51 and 5100 mg L^{-1} of glyphosate equivalent. Results from one-way ANOVA testing for differences between treatments (F and H = test-statistic; P = *p*-value) are shown. The *p*-values in bold account for significant differences among treatments ($p < 0.05$). In (**d**), a Kruskal–Wallis test was performed instead of ANOVA. Different letters indicate significant differences among treatments.

Table 2. Photosynthetic pigments content in *Z. marina* leaves (mean ± SE) in control conditions (no herbicide) and after exposure to 0.165, 51 and 5100 mg L^{-1} of glyphosate equivalent. Values are expressed in µmol gDW^{-1} ($4 \leq n \leq 5$). Results from one-way ANOVA testing for differences between treatments (F = test-statistic; P = *p*-value) are shown. The *p*-values in bold account for significant differences among treatments ($p < 0.05$). Different letters indicate significant differences among treatments.

Pigments (µmol gDW^{-1})	**Glyphosate Equivalent (mg L^{-1})**				**F**	**P**
	0 (Control)	**0.165**	**51**	**5100**		
Chl *a*	1.396 ± 0.081	1.604 ± 0.230	1.651 ± 0.396	1.850 ± 0.386	0.257	0.855
Chl *b*	0.580 ± 0.036	0.690 ± 0.095	0.879 ± 0.216	1.152 ± 0.256	1.434	0.272
β-Carotene	0.392 ± 0.015 ^{a}	0.426 ± 0.053 ^{a}	0.288 ± 0.077 ^{a}	0.105 ± 0.014 ^{b}	6.684	**0.004**
Lutein	0.362 ± 0.013	0.393 ± 0.057	0.467 ± 0.119	0.419 ± 0.082	0.226	0.877
Lutein epoxide	0.008 ± 0.001	0.006 ± 0.001	0.008 ± 0.002	0.005 ± 0.001	0.630	0.607
Neoxanthin	0.148 ± 0.005	0.167 ± 0.026	0.111 ± 0.034	0.087 ± 0.025	1.427	0.277
Violaxanthin (V)	0.332 ± 0.012 ^{a}	0.346 ± 0.039 ^{a}	0.227 ± 0.059 ^{a}	0.053 ± 0.09 ^{b}	8.256	**0.002**
Antheraxanthin (A)	0.006 ± 0.002	0.004 ± 0.001	0.003 ± 0.001	0.004 ± 0.001	0.949	0.442
Zeaxanthin (Z)	0.010 ± 0.001	0.007 ± 0.001	0.010 ± 0.002	0.007 ± 0.002	0.998	0.421
V + A + Z	0.346 ± 0.012 ^{a}	0.357 ± 0.041 ^{a}	0.240 ± 0.062 ^{a}	0.064 ± 0.011 ^{b}	7.550	**0.003**
DES = (A + Z)/(V + A + Z)	0.040 ± 0.008 ^{bc}	0.030 ± 0.004 ^{c}	0.056 ± 0.003 ^{b}	0.160 ± 0.009 ^{a}	73.875	**7.998×10^{-9}**
(V + A + Z)/Chl *a* + *b*	0.176 ± 0.006 ^{a}	0.160 ± 0.008 ^{a}	0.092 ± 0.003 ^{b}	0.027 ± 0.002 ^{c}	113.44	**4.643×10^{-10}**

3.6. Adenylate Compounds (ATP, ADP and AMP) and Energy Charge (AEC)

Adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), and, consequently, the total adenylates (AT) contents were lower in *Z. marina* exposed to all glyphosate concentrations, especially 51 and 5100 mg L^{-1} (Figure 5). Although some differences in the adenylate energy charge (AEC) were observed between treatments, no concentration-dependent tendency was observed (Figure 5).

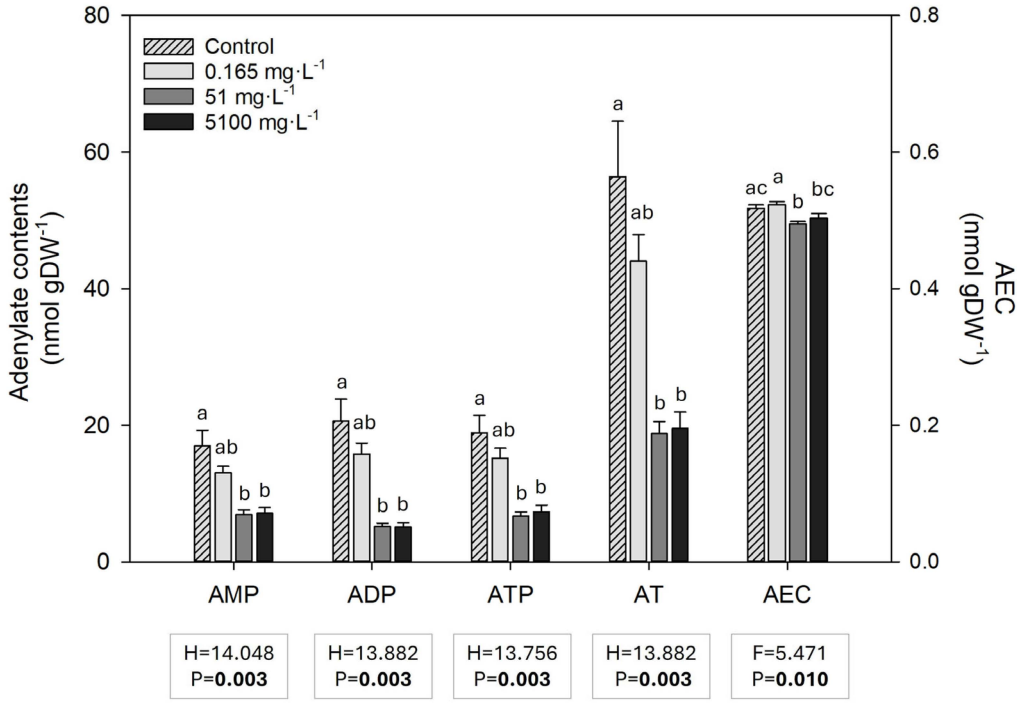

Figure 5. AMP, ADP, ATP, total adenylates content (AT) and adenylate energy charge (AEC) (mean ± SE) in *Z. marina* leaves in control conditions (no herbicide) and after exposure to 0.165, 51 and 5100 mg L^{-1} of glyphosate equivalent ($4 \leq n \leq 5$). Results from Kruskal–Wallis and one-way ANOVA testing for differences between treatments are shown (F and H = test-statistic; P = *p*-value). The *p*-values in bold account for significant differences among treatments ($p < 0.05$). Different letters indicate statistical differences among treatments ($p < 0.05$).

4. Discussion

This study demonstrated that *Z. marina* shows a certain short-term tolerance to an ecologically relevant low dose treatment of glyphosate-based herbicide (GBH). Short-term sublethal effects were observed at intermediate concentrations and exposure to high concentrations of GBH is lethal for *Z. marina*.

Exposure to the lower concentration of glyphosate (0.165 mg L^{-1}), a realistic concentration to be found in the environment, did not significantly impact the plant's physiology for the duration of the experiment. Exposure to the intermediate concentration of glyphosate (51 mg L^{-1}) caused a pH drop (of about 0.3 in 1 day, stable for the duration of the experiment) and had severe consequences on the plant's physiology. Even though *Z. marina* survived during the whole experiment in this treatment, the intermediate concentration tested severely hampered photosynthetic activity, leaf growth and adenylate compounds content. The application of the highest glyphosate concentration (5100 mg L^{-1}) caused pH to drop rapidly in the water (8.35 to 4.72 in 1 day) and lead to 100% mortality within 24 h. Although the present study does not demonstrate the effect of pH alone, the pH falling below 5 is likely one mechanism driving rapid death of *Z. marina* at the highest glyphosate concentration. Sudden seawater acidification, caused by the addition of the herbicide,

could have impacted seagrass physiology through various mechanisms, namely increasing susceptibility to stress [78], disrupting ionic homeostasis within the plants [79], interfering with symbiotic microbial communities [80] and altering nutrient uptake coupled with root and rhizome damage due to sediment chemistry changes [81]. Although the adenylate energy charge (AEC) did not change according to a clear pattern, the foliar concentration of adenylate compounds decreased in plants exposed to all glyphosate concentrations tested, reflecting a decrease in the availability of metabolic energy [82].

The GBH tested in this study interfered with the photosynthetic apparatus of *Z. marina* at the photosystem II (PSII)'s level, as previously observed in other seagrasses and in macroalgae [52,54,55,58,59]. F_v/F_m and $\Delta F/F_m'$ were negatively affected by a glyphosate concentration of 51 mg L^{-1}, evidencing a decrease in the photosynthetic performance. Plants exposed to the lethal concentration of 5100 mg L^{-1} had both F_v/F_m and $\Delta F/F_m'$ rapidly inhibited and dropping to 0 in less than 1 day. Such observations can be attributed to the glyphosate-induced alterations of PSII, inactivation of its reaction centers and oxidative damage [51,83,84]. Glyphosate is known to induce the production of reactive oxygen species (ROS) [85], whose accumulation leads to oxidative modification of PSII proteins [86] and lipid peroxidation of the thylakoid membranes [87]. Damaged thylakoid membranes can compromise the energy transfer towards the reaction centers and the electron transport chain. Previous studies showed that the PSII's integrity in marine macrophytes is negatively affected by glyphosate concentrations as low as 0.176 mg L^{-1} [55] and after a few minutes of exposure at higher concentrations (a few g L^{-1} [59]). The observed decreases in $\Delta F/F_m'$, P_{max}, and I_k in plants exposed to 51 mg L^{-1} of glyphosate reveal the decreased ability of PSII to deliver electrons to the electron transport chain (ETC), resulting in lower electron transport rate and diminished photosynthetic performance. The lower availability of ATP and NADPH, related to the lower efficiency of the ETC, has consequences for the Calvin cycle and the overall result of photosynthesis, i.e., triose phosphate synthesis and RuBP (ribulose-1,5-biphosphate) regeneration. The sugars produced in the Calvin cycle are the building blocks for the synthesis of molecules such as adenosine, the nucleoside constituting ATP, AMP and ADP (adenosine tri-, mono-, or diphosphate). Therefore, a decrease in sugar synthesis is likely to reduce the availability of the three adenylate compounds (ATP, AMP and ADP). Furthermore, part of the ATP generated in the chloroplast is needed for N assimilation [88]; this may again link to the availability of the adenylate nitrogenous compounds.

The photosynthesis-irradiance curve (*P-I*) shows three notable outliers for net photosynthesis at the low dose of glyphosate (Figure 3b). Retaining the original scatter points communicates the true extent of individual variability, dynamic responses, and the limits of the fitted *P-I* model. Removing the points would create a potentially misleading graph that would underrepresent the natural variability that is commonly observed in the physiological responses of plants submitted to a stressful situation such as herbicide exposure.

Some studies suggested the existence of a possible low-dose stimulatory growth response in the first few days of exposure to glyphosate in *R. maritima* (0.05 mg L^{-1} Roundup [62]) and *Z. marina* (10 µM glyphosate equivalent 17 mg L^{-1} [89]). The observed increase in P_{max} and β-carotene at the lowest dose tested in this study, although not supported by the statistical test, could account for a potential low-dose stimulatory response to the GBH ("hormesis" [65]). Conversely, the foliar growth rate was severely hampered under the intermediate concentration of GBH, meaning that the plant's carbon metabolism was impaired, which has been reported to be an effect of the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) [90,91]. Thus, the general reduction in energy availability in plants exposed to the intermediate concentration of GBH resulted in

the decrease in the syntheses essential to plant growth, nutrient absorption, and hormonal signals disruption, slowing down all cellular energy functions and provoking a drastic reduction in foliar growth rate [92].

There was a tendency for respiration to increase with glyphosate concentration, indicating the rise in metabolic activity in response to stress caused by the inhibition of the shikimate pathway [93]. The respiration increase could also possibly be attributed to increased release of plant exudates which in turn causes an increase in microbial respiration fueled by the highly labile exudates. Despite increased respiration being a stress response to maintaining energy balance, all ATP, ADP and AMP levels decreased with glyphosate concentration, especially in plants exposed to the two higher concentrations tested (51 and 5100 mg L^{-1} of glyphosate). Although the decrease in adenylate compounds was not statistically significant at 0.165 mg L^{-1} of glyphosate, given the short duration of the experiment (4 days of exposure to the GBH), we can consider that there was a strong tendency for adenylates to decrease. Several causes could explain this: disruption of the Krebs cycle due to the lack of aromatic amino acids [94], mobilization of energy resources to activate defense and repair pathways [95], or mitochondrial dysfunction [85,96], all causing an energetic disruption that contributes to the weakening and death of the plant. Alteration of the plant's carbon reserves could also impact its ability to uptake and assimilate nitrogen. Changes in energy balance as a stress response to GBH exposure have also been observed in other organisms [97]. We suggest that exposure to lower concentrations of glyphosate for an extended time, as it is likely to happen in nature, may be harmful to the plants, and this hypothesis should be evaluated in the future.

The chlorophyll content tended to increase with glyphosate concentration. In contrast, some studies showed that concentrations of GBH lower than those tested in our research provoked a reduction in chlorophyll synthesis and content in many terrestrial and aquatic plants, including seagrasses [58,62,63,98–100]. A higher chlorophyll content might reflect the onset of a compensation mechanism in response to the lower efficiency of the reaction centers and diminished photosynthetic performance. As the herbicide concentration increased, the chlorophyll *a/b* ratio decreased, meaning that the proportion of chlorophyll *b* increased relatively to that of chlorophyll *a*. This is commonly related to a higher investment in light-harvesting in comparison to the capacity to input electrons in the ETC [101]. A decreased chlorophyll *a/b* ratio can suggest changes in photosystem composition [102], a stress response to low light [101], nutrient deficiency (e.g., nitrogen deficiency) [103,104] and/or oxidative stress [105], as chlorophyll *a* may degrade faster than chlorophyll *b* under certain stress conditions, especially at low pH [106]. On the other hand, the total chlorophylls/total carotenoids ratio increased with glyphosate concentration, indicating that the carotenoid content decreased relative to chlorophylls. This can be a consequence of the inhibition of the shikimate pathway by glyphosate, leading to the reduction in plastoquinone synthesis, which directly affects carotenoid production [83] since plastoquinone is a co-factor of the enzymes involved in the carotenoid biosynthesis pathway [107]. The possible decrease in plastoquinone (the PSII to cytochrome *b6/f* electron carrier) synthesis could have also impaired the photosynthetic ETC [108]. Carotenoids act as antioxidants [109], and hence the increase in total chlorophylls/total carotenoids ratio in plants exposed to 5100 mg L^{-1} could indicate a lower protection against stress-induced ROS. In our experiment, plants were exposed to very low light intensity; thus, the de-epoxidation state (DES) index was always very low. Nonetheless, the decrease in violaxanthin content in *Z. marina*'s leaves exposed to glyphosate concentrations of 51 and 5100 mg L^{-1}, with high significance at 5100 mg L^{-1}, suggests the enhanced de-epoxidation of violaxanthin, supported by the increase in DES. This can be interpreted as a response

mechanism to protect the photosynthetic apparatus from chemical toxicity or other factors inducing oxidative stress [110–112].

Whereas β-carotene tends to increase in the low dose treatment, plants exposed to the higher concentration of glyphosate showed a significant decrease in β-carotene. β-carotene is the precursor of zeaxanthin, a key component of the xanthophyll cycle [113]. Lower β-carotene availability may lead to a reduced photoprotection capacity. Moreover, the impairment of the electron transport due to PSII's malfunction decreases the acidification of the thylakoid lumen, therefore impeding the induction of the xanthophyll cycle [114], decreasing non-photochemical quenching (NPQ) and increasing the damages caused to the PSII, starting a vicious circle of cumulative negative effects [115].

In the literature, a large discrepancy in the responses of seagrasses to GBHs can be found. Castro et al. (2015) [62] observed a high mortality of *R. maritima* exposed to 50 mg L^{-1} of GBH after 7 days. No significant adverse impacts were observed on *H. wrightii* and *R. maritima* at a glyphosate concentration of 1 mg L^{-1} [63], but mortality was observed at 100 and 1000 mg L^{-1}. Van Wyk et al. (2022) [66] observed that *Z. capensis* responded to the exposure to a 0.25–2.20 mg L^{-1} glyphosate formulation by decreasing leaf area and above-ground biomass after 3 weeks, whereas no variation in photosynthetic pigment concentration was observed. In a study targeting *Z. marina*, Nielsen and Dahllöf (2007) [89] observed that the exposure to glyphosate below 100 µM (equivalent to 16.9 mg L^{-1}) alone did not affect the relative growth rate neither the chlorophyll *a*/*b* ratio after 3 days; only at 1.69 mg L^{-1} a stimulation of the relative growth rate in weight was observed. Ralph (2000) [64] observed that the exposure of *H. ovalis* to glyphosate (1, 10 and 100 mg L^{-1}) did not affect fluorescence signals. Additionally, *H. ovalis* exposed to lower glyphosate concentrations showed lower chlorophyll content, lower chlorophyll/carotenoid ratio and higher chlorophyll *a*/*b* ratio with increasing glyphosate concentration, which is in opposition with our observations on *Z. marina*. Silvera et al. (2024) [65], who is, to our knowledge, the only study reporting long-term effects, tested the effect of a GBH on *H. ovalis* and *H. wrightii* and found that 3.74 mg L^{-1} of sprayed GBH did not affect shoot density over a 53-day experiment. However, when exposed to a concentration of 125 mg L^{-1}, plants' density started to decline after 5 and 15 days, respectively. This suggests a high variability in the response of seagrasses to GBHs, which could be attributed to differences in species and/or experimental set-up (i.e., glyphosate concentrations tested, duration of the experiment).

In some of those studies, the herbicide's origin and mixture composition are not disclosed, making comparisons difficult. The type of response could depend on the form of herbicide used (glyphosate as a pure salt vs. as formulations with surfactants and adjuvants). For example, Ralph (2000) [64] used a liquid glyphosate salt solution, which does not contain the surfactants present in many commercial formulations (1 to 10% of surfactants). Therefore, care must be taken when comparing these studies.

Numerous studies previously showed that the synergistic effect of glyphosate with its surfactant in commercial formulations (etheralkilamine ethoxylate, also known as polyethoxylated tallow amine, POEA) or its metabolite (aminomethylphosphonic acid, AMPA) increases toxicity to aquatic organisms (e.g., [60,116–124]). In the commercial formulation we used in our experiment, we cannot assess whether glyphosate, POEA or the combination of both are responsible for the observed deleterious effects on *Z. marina*. According to previous studies, however, we can suggest that POEA might be responsible for enhancing the toxicity of glyphosate-based herbicides in seagrasses. Nielsen and Dahllöf (2007) [89] showed the negative synergistic effect of low-concentration herbicide mixtures (glyphosate, bentazone and 4-chloro-2-methylphenoxyacetic acid, MCPA) relative

to exposure to glyphosate only, suggesting that the damages on seagrass physiology and fitness may be enhanced if several herbicides are present in the environment.

Other indirect effects of GBHs and associated water pH change on seagrass fitness could include impacts on the microorganisms of the sediment (microbiome), such as shifts in species composition [125]. In the medium-long term, these changes can lead to negative consequences at the ecosystem level [126,127]. Given the fundamental ecological importance of seagrass ecosystems as coastal engineers and biodiversity hotspots [38,128], any adverse effects can have far-reaching consequences. The potential harm to seagrasses may disrupt the ecological equilibrium of these habitats, with repercussions that extend throughout the associated biota. Such disturbances can impact the abundance and distribution of species within these habitats.

Although no data concerning herbicide concentration in Ria Formosa coastal lagoon is currently available, it is more likely that the actual presence of glyphosate approximates the magnitude of the lowest concentration tested in this study (0.165 mg L^{-1}), according to the concentrations found in other aquatic environments. If such is the case, *Z. marina* might not be at immediate risk in a 4-day experiment, but prolonged exposure may have harmful consequences. Further investigation is required to better understand the effects of long-term exposure and mixture toxicity and make ecologically realistic risk assessments [129,130]. Investigating a wider range of lower glyphosate concentrations is also required to assess this species' minimum adverse and lethal concentrations. Care must be taken when comparing different studies, as some mention the concentration of GBH used (including water and surfactants), whereas others mention the actual glyphosate concentration. Lastly, monitoring herbicide contamination in coastal waters is crucial to understand and prevent the deterioration of water quality and potential consequences on both seagrass beds and related ecosystems.

Author Contributions: Conceptualization, J.S. and I.B.; Formal analysis, A.D., K.P., B.V. and M.C.; Funding acquisition, J.S.; Investigation, K.P., B.V. and M.C.; Project administration, J.S.; Supervision, J.S. and I.B.; Visualization, A.D. and K.P.; Writing—original draft, A.D. and K.P.; Writing—review and editing, B.V., M.C., J.S. and I.B. A.D. and K.P. contributed equally to this work. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Portuguese national funds from FCT—Portuguese Foundation for Science and Technology through projects UIDB/04326/2020 (DOI:10.54499/UIDB/04326/2020) and LA/P/0101/2020 (DOI:10.54499/LA/P/0101/2020).

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request. Correspondence and requests for materials should be addressed to A.D.

Conflicts of Interest: The authors declare no conflicts of interest.

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