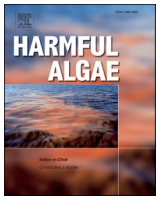
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Response of the toxic dinoflagellate *Alexandrium minutum* to exudates of the eelgrass *Zostera marina*

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ABSTRACT

Biotic interactions are a key factor in the development of harmful algal blooms. Recently, a lower abundance of planktonic dinoflagellates has been reported in areas dominated by seagrass beds, suggesting a negative interaction between both groups of organisms. The interaction between planktonic dinoflagellates and marine phanerogams, as well as the way in which bacteria can affect this interaction, was studied in two experiments using a non-axenic culture of the toxic dinoflagellate *Alexandrium minutum* exposed to increasing additions of eelgrass (*Zostera marina*) exudates from old and young leaves and to the presence or absence of antibiotics. In these experiments, *A. minutum* abundance, growth rate and photosynthetic efficiency (Fv/Fm), as well as bacterial abundance, were measured every 48 h. Toxin concentration per cell was determined at the end of both experiments. Our results demonstrated that *Z. marina* exudates reduced *A. minutum* growth rate and, in one of the experiments, also the photosynthetic efficiency. These results are not an indirect effect mediated by the bacteria in the culture, although their growth modify the magnitude of the negative impact on the dinoflagellate growth rate. No clear pattern was observed in the variation of toxin production with the treatments.

1. Introduction

Harmful Algal Blooms (HABs) widely occur in marine ecosystems around the world and show an apparent increase in their frequency and geographic distribution in the last decades (Anderson et al., 2015; Hallegraeff et al., 2004; Nwankwegu et al., 2019). Several causes have been suggested behind these observations as a greater monitoring effort of these events worldwide, the increasing use of coastal areas for aquaculture, the anthropogenic input of nutrients to coastal environments, unusual weather conditions or the transport of cysts of toxic species in the ballast water of ships or in shellfish stocks, among others (e.g. Hallegraeff et al., 2021). Model projections indicate that HAB events are expected to expand their impact in different coastal areas (Anderson et al., 2015; Xu et al., 2019).

The deleterious effects of HABs on aquatic organisms and ecosystems are different depending on the causative species and their properties (i.e. shellfish toxins and ictyotoxins production, reduced dissolved oxygen in the declining phase of high biomass blooms, mechanical irritation and bacterial infection of fish gills, etc. (FAO et al., 2023; Hallegraeff et al., 2021)). In the particular case of HABs formed by toxic species, these are often associated with substantial economic losses in the fisheries and mariculture sectors, and can also affect tourism services (Sanseverino et al., 2016).

In Galicia, North-West (NW) Spain, the main resource from mariculture derives from shellfish production, mainly blue mussel (*Mytilus galloprovincialis*) in rafts, together with seabed cultivation (clams, cockles and scallops) and raised intertidal beds for oysters growth (*Ostrea edulis* and *Crassostrea gigas*). Overall, Galician mussel's production accounts for most of the national and around 50 % of the European blue mussel production (APROMAR, 2022; Avdelas et al., 2021; Labarta and Fernández-Reiriz, 2019; Rodríguez-Rodríguez et al., 2011).

Mariculture activities in NW Spain are negatively affected by the seasonal development of toxin-producing HABs. These outbreaks force harvesting closures in production areas and represent a critical factor for the shellfish industry (Álvarez-Salgado et al., 2008; Rodríguez-Rodríguez et al., 2011). In this region, the shellfish toxins associated with

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HABs do not cause mortalities in aquatic organisms, but pose a public health risk due to their bioamplification in the food web including commercial resources (James et al., 2010; Vilariño et al., 2018).

The seasonal succession of potentially toxin-producers dinoflagellates (such as *Alexandrium minutum, Dinophysis* spp. and *Gymnodinium catenatum*) and diatoms (*Pseudo-nitzschia* spp.) and their associated poisoning syndromes in shellfish is well known in the area (Crespo et al., 2008; Díaz et al., 2016; Fermin et al., 1996; Nogueira et al., 2022; Reguera et al., 2008). Nonetheless, the causes explaining the triggering of these proliferations are complex and not fully understood, thus preventing the prediction of their occurrence (FAO et al., 2023; Ruiz-Villarreal et al., 2022).

The growth of toxic dinoflagellates is affected by diverse physical, chemical and biological factors (Anderson et al., 2012; Chambouvet et al., 2011; Glibert et al., 2005; Laabir et al., 2011). There is some consensus on the positive effect of some abiotic factors, such as high nutrient concentrations, high N:P ratios, continental inputs or thermohaline stratification (Glibert et al., 2005; Telesh et al., 2021). By contrast, knowledge on the role of biotic interactions on the development and termination of these blooms is relatively scarce. Microalgae cells closely interact with other organisms sharing the same habitat, these relationships being of different nature (symbiosis, predation, competition, allelopathy, etc.) and involving both micro- and macroorganisms. Early observations that some dinoflagellates are unable to be axenically cultivated illustrates the importance of biotic relationships for their survival (e.g. Guillard and Keller, 1984). Bacteria are also known to affect gene expression in some dinoflagellates (Moustafa et al., 2010). Recent studies based on DNA sequencing showed the existence of different and specific microbiomes associated with toxic dinoflagellates (Cui et al., 2020; Hattenrath-Lehmann and Gobler, 2017; Kang et al., 2021; Sörenson et al., 2019; Velo-Suárez et al., 2013), which may play an important role in bloom dynamics. It has been also reported that the dynamics of HABs and toxin production can be affected by the presence not only of bacteria, but also of predators or parasites (Albinsson et al., 2014; Doucette, 1995; Gallacher and Smith, 1999; Ho et al., 2006; Lu et al., 2016; Selander et al., 2019; Uribe and Espejo, 2003).

Recent investigations carried out in seagrass meadows suggest the existence of interactions between planktonic dinoflagellates and marine angiosperms. An increasing trend in the abundance of dinoflagellates from areas dominated by *Zostera marina* beds to salt marsh zones distant from the presence of *Z. marina* was observed from environmental DNA data (Jacobs-Palmer et al., 2020), suggesting that seagrass beds may display some negative allelopathic effect on dinoflagellates related to the presence of metabolites produced by seagrasses. For instance, *Zostera* species are known to contain phenolic compounds (Achamlale et al., 2009; Guan et al., 2017; Laabir et al., 2013; Todd et al., 1993), which may inhibit epiphytic organisms. In addition, several studies show that seagrass meadows harbor algicidal bacteria as part of their microbiomes, both forming biofilms on the leaves and suspended in the water column (Inaba et al., 2019, 2017; Onishi et al., 2020, 2014), which may affect dinoflagellate growth rates.

Few studies focused on the effect of marine seagrasses on toxic planktonic dinoflagellates. Harrison and Chan (1980) and Laabir et al. (2013) tested the effect of phanerogam extracts on cultures of planktonic dinoflagellates, reporting negative effects on their growth and morphology. However, in both studies the phanerogams were harshly treated by drying, boiling and using dissolvents to obtain the extracts, which could give rise to differences between the extract composition and the compounds naturally released to the environment. Other studies with non-axenic cultures yielded similar results and thus, it cannot be discarded that the negative effect could derive from bacterial overgrowth in the presence of the macrophyte exudates (Ben Gharbia et al., 2017).

In our study, naturally obtained macrophyte exudates were added to dinoflagellate cultures in the presence or absence of antibiotics aiming at preventing any potential effect associated with the dinoflagellate bacterial microbiome. To our knowledge, this is the first study focused on the effect of a marine phanerogam on the growth of planktonic dinoflagellates that takes into account the possible effect of the dinoflagellate-associated microbiome.

We hypothesized that exudates derived from the seagrass species *Zostera marina* have inhibitory effects on the growth and toxicity of the toxic dinoflagellate *Alexandrium minutum*, a common paralytic shellfish toxin producer in NW Spain. We aimed at 1) testing the inhibitory effect of *Zostera marina* exudates and 2) to determine the role of the *A. minutum* microbiome on the above mentioned effect.

2. Methods

2.1. Preparation of Z. marina exudates

Two different *Z. marina* exudates were prepared, one for each experiment, using leaves from the same plant but in different growth phase using old leaves for Exp_1 (defined as the fourth leaf from the inner part of each shoot) and young leaves for Exp_2 (defined as the third leaf from the inner part of each shoot). Several specimens of this species were collected at the same tidal level from Cesantes beach, in the Ría de Vigo (42.31 N, −8.62 W). Intact leaves were selected from the plants and 30 cm of them were taken, washed with Milli-Q water and dried with filter paper to prepare each exudate. Fragments were incubated for 24 h in transparent Teflon bottles with 0.5 L of a 35 g L^{-1} NaCl solution (previously calcined at 600 °C for 1.5 h) in Milli-Q water, in a chamber thermostated at 20 °C programmed with a light:dark cycle (L:D) of 12:12 h and continuous air bubbling. Three replicates of each exudate were incubated. The purpose of using exudates made from leaves of different ages was to study whether the growth state of the leaves can alter the negative effect of exudates on dinoflagellates.

After the incubation period, the content of the bottles with the leaves was filtered through a 0.2 μm polyetersulfone membrane (Pall, 60,301) (previously washed with diluted HCl and thoroughly rinsed with Milli-Q water) after filtering it through GF/F (Whatman, 1825-047) (previously calcined at 450 °C for 4 h). Dissolved organic carbon (DOC) and total nitrogen (TN) samples were taken at the end of incubation and measured in a Shimadzu TOC-V total organic C analyzer, in line with a Shimadzu TNM-1 total N measurement unit (Álvarez-Salgado and Miller, 1998), after which the three replicates of each type were combined, getting final concentrations of 2521 μM DOC and 455 μM TN for Exp_1 and 3944 μM DOC and 418 μM TN for Exp_2. Finally, each of the exudates was frozen at −20 °C until the experiments were carried out.

2.2. Experimental design

Two experiments (Exp_1 and Exp_2) were carried out at different times to test the effect of *Z. marina* exudates on the growth of a toxic strain (VGO1436) of the dinoflagellate *A. minutum* isolated from the Ría de Vigo (42°12′38.0″ N, −8°46′34.3″ W) during a red tide episode (strain S1 in Nogueira et al., 2022)). *A. minutum* was grown in L1 medium without silicates, at an initial density of 200–300 cell mL^{-1}. Different volumes of two different *Z. marina* exudates (one per experiment, see Table S1 for details on their chemical composition) were added to the cultures, reaching final concentrations of dissolved organic carbon (DOC) derived from the *Z. marina* of ca. 1200, 600, 300 and 0 μM (high, medium, low additions and control, respectively). The difference in volume between the treatments was corrected by adding a solution of 35 g L^{-1} of NaCl in Milli-Q water. The different DOC concentrations were assayed in parallel both on cultures treated or untreated with antibiotics to study the potential effect of the microbiome associated with *A. minutum* on the dinoflagellate response to the exudate addition. The antibiotic solution consisted in a commercial mixture of penicillin (ca. 5000 units mL^{-1}), streptomycin (ca. 3600 units mL^{-1}) and neomycin (ca. 6000 units mL^{-1}) (Sigma Aldrich, P4083). The reliability of this mixture of antibiotics to block bacterial growth without affecting

phytoplankton in natural samples was previously tested in our laboratory (Prieto et al., 2016). However, as bacterial abundances in cultures are higher than those found in the field, we employed higher concentrations of antibiotics than in that study (74 units mL^{-1}, repeated at the midpoint of each experiment), to effectively reduce the bacterial abundance of the *A. minutum* culture without compromising dinoflagellate growth. Each treatment was performed in triplicate (Table 1).

Cultures were incubated at 19 °C and 150 μE $m^{-2}s^{-1}$ light intensity with a L:D cycle of 12:12 h for two weeks. Samples to measure photosynthetic efficiency, bacterial abundance and *A. minutum* abundance were taken every 48 hrs. Samples for determining the toxin profile and levels of paralytic shellfish toxins (PST) were only collected at the end of the experiment in Exp_1 and one week later in Exp_2.

2.3. Bacterial abundance and culture density

Samples for the determination of bacterial and *A. minutum* abundances were preserved with 1 % paraformaldehyde + 0.05 % glutaraldehyde and frozen at −80 °C after liquid nitrogen immersion. Analysis of the samples was performed using a Beckman Coulter CytoFLEX S flow cytometer equipped with a laser emitting at 488 nm. Prior to analysis, bacteria were stained with SybrGreen DNA fluorochrome. Total bacterial abundance was determined by their green fluorescence after SybrGreen staining (using the FITC detector) and side scatter signals, while *A. minutum* abundance was determined by autofluorescence (using the PerCP and PE detectors).

2.4. Photosynthetic efficiency

Photosynthetic efficiency was estimated from fluorescence measurements (variable fluorescence/maximal fluorescence (Fv/Fm)). Samples (4 mL) were incubated in the dark for no less than 30 min before measurements, which were carried out using a WATER-PAM I Chlorophyll Fluorometer (Heinz Walz GmbH).

2.5. PSTs measurements

Samples from *A. minutum* cultures of each treatment were filtered through 25 mm diameter glass fiber filters (Whatman, 1825-025) at the end of the incubation. Each filter, containing the *A. minutum* cells, was placed in an Eppendorf tube. Then, 750 μL 0.05 M acetic acid was added and the tubes were frozen at −20 C until further use. Before analysis, the contents of each tube were sonicated for 1 min at 50 W and centrifuged at 5200 × *g* and 10 °C for 10 min. The supernatant was collected in a clean Eppendorf tube. The extraction was repeated with another 750 μL 0.05 M acetic acid. Both supernatants were combined (final volume 1500 μL) and then filtered through 0.22 μm PTFE syringe filters prior to HPLC analyses. The characterization of PSTs was carried out by liquid chromatography with fluorescence detection and post-column oxidation (LC_FLD-PCOX) following the method detailed by (Rodríguez et al., 2016) with slight modifications. Mobile phase A was composed of 11 mM heptane sulfonic acid sodium salt in a 5.5 mM phosphoric acid aqueous solution adjusted to pH 7.1 with 0.5 M ammonium hydroxide. Mobile phase B consisted of 88.5 % 11 mM heptane sulfonic acid sodium salt in a 16.5 mM phosphoric acid aqueous solution adjusted to pH 7.1 with 0.5 M ammonium hydroxide and 11.5 % acetonitrile. An isocratic elution of a mobile phases A and B mixture containing 95 % A and 5 % B was run at a flow rate 1 mL min^{-1}. The total run time was 20 min. Separation of toxins was performed onto a Kinetex, 5 μm, Biphenyl, 150 × 4.6 mm LC column maintained at 35 C. The Certified Reference standards of gonyautoxins GTX4, GTX1, GTX3 and GTX2 used were acquired from Cifga Laboratories S.A. (Lugo, Spain). Limits of detection (LOD, ng on column) of the LC_FLD-PCOX system were: for GTX1 (3.8 ng), GTX2 (0.1 ng), GTX3 (0.2 ng) and GTX4 (1.8 ng). In order to determine the PSTs concentration in the samples, the external standard calibration procedure was used. The EFSA (EFSA, 2009) STXs toxicity equivalency factors (TEFs) were used to calculate the toxin cell quota of the cultures as concentration of saxitoxin equivalents (STXeq) per cell.

2.6. Data analysis

Response ratios (RR) were calculated as T/C, where T and C are the value of the variable at the end of the experiment in each Treatment (low, medium and high) and in Control, respectively, to compare the effect of different exudate concentrations on *A. minutum* abundance and toxin cell quota.

A. minutum growth rates were estimated from the slope of the linear regression of the natural logarithm of the temporal evolution of cell abundances to study the effect of *Z. marina* exudates on the dinoflagellate growth.

A generalized linear model (GLM) with Poisson distribution of errors for count data was constructed to test for differences in bacterial abundance and the density of *A. minutum* cultures among the different treatments. The two experiments were analysed separately. The models included two fixed factors: Treatment (4 levels: control, low addition, medium addition, and high addition) and Antibiotic (2 levels: without antibiotics and with antibiotics) and their interactions. When over dispersion was detected (dispersion parameter, $\varphi \geq 2$), the model was corrected using a quasi-GLM model (Zuur et al., 2009). A generalized linear model (GLM) with Gamma distribution of errors for data of continuous variables with positive values was constructed to investigate differences in the growth rate (Exp_2) and photosynthetic efficiency (Zuur et al., 2009). Differences in growth rate (Exp_1) and STXeq concentrations at the end of the experiments were tested by two-way analysis of variance (two-way ANOVA) with the same factors and levels described above. Tukey's post-hoc tests were conducted to assess the main effects included in the analysis.

Table 1
Summary table of the experimental design, showing the volume of exudate added to each flask (EV), the total volume inside each flask (TV) and the concentration of dissolved organic carbon added to each flask (DOC) for each treatment (in triplicate) with and without antibiotics in each of the experiments.

	Experiment							
	Exp_1 (x3)				Exp_2 (x3)			
	Treatment	EV (mL)	TV (mL)	DOC (μM)	Treatment	EV (mL)	TV(mL)	DOC (μM)
No antibiotic added	Control	0	90	0	Control	0	190	0
	Low	11	90	308	Low	14.5	190	301
	Medium	21.5	90	602	Medium	29	190	602
	High	43	90	1204	High	58	190	1204
Antibiotics added	Control	0	90	0	Control	0	190	0
	Low	11	90	308	Low	14.5	190	301
	Medium	21.5	90	602	Medium	29	190	602
	High	43	90	1204	High	58	190	1204

3. Results

3.1. Exudates composition

Both exudates contained high concentrations of dissolved organic matter, mainly DOC, although the relative concentrations of dissolved carbon and nitrogen differed between exudates. The highest concentration of DOC was observed in the exudate used in Exp_2 (3944 μM vs. 2521 μM in Exp_1), while the highest concentration of TN was in the exudate used in Exp_1 (455 μM vs. 418 μM in Exp_2). As a result, when using DOC concentration as a reference to set-up the exudate additions the added TN concentrations differed between experiments (Table S1).

3.2. Bacterial abundance

The addition of exudates derived from *Z. marina* significantly affected bacterial abundance, as did the addition of antibiotics and the interaction between both factors in the two experiments (Table 2).

The addition of *Z. marina* exudates stimulated the growth of bacteria associated with *A. minutum* in both experiments, with the greatest increase in bacterial abundance of the treated cultures occurring during the first 48 h of the experiments (Fig. 1). In these cases, maximum bacterial abundance occurred in the high addition treatments, while lowest ones were observed in the controls. The increase in bacterial abundance observed in Exp_2 outweighed that observed in Exp_1. Overall, bacterial abundance in all treatments was significantly higher than in the controls at the end of both experiments (Tukey post hoc test, $p < 0.05$).

The addition of antibiotics blocked bacterial growth significantly (Tables S2 and S3). Bacterial abundance decreased to mean values per treatment below 500 cell μL^{-1} in Exp_1 and 250 cells μL^{-1} in Exp_2 during the first 10 days of incubation. Some of the treatments showed small rebounds from that time onwards. Bacterial abundance in the antibiotic treatments never exceeded 3000 cells μL^{-1}.

3.3. Alexandrium minutum abundance and growth rate

At Exp_1, *A. minutum* abundance was significantly affected by the addition of *Z. marina* exudates, but not by the addition of antibiotics or by the interaction of both experimental factors (Table 3). *A. minutum* growth was impaired even at the low addition treatment; only a slight increase in cell abundance was observed at the end of the experiment (Figs. 2 and 3). Cell abundance in the controls raised from an initial mean value of 289 ± 13 cell mL^{-1} to 22,141 ± 4210 cell mL^{-1} and 14,480 ± 3867 cell mL^{-1} (without and with antibiotic treatments (-AB and +AB), respectively) at day 14. In the high addition treatment, *A. minutum* abundance was extremely low. Culture growth was nearly arrested and cell abundance even decreased by the end of the experiment (395 ± 25 cell mL^{-1} and 151 ± 53 cell mL^{-1}, -AB and +AB, respectively). Post-hoc tests showed that the abundance of *A. minutum* differed significantly between the control and the different additions of *Z. marina* exudate at the end of the experiment. This was also the case among the different addition treatments (Tukey post hoc test, $p < 0.001$), except between the 600 μM and 1200 μM additions (Tukey post hoc test, $p > 0.1$).

The results from Exp_2 showed a similar trend but also some differences (Figs. 2 and 3). As in Exp_1, *A. minutum* abundance was also significantly affected by the addition of *Z. marina* exudates, but, in this case, it was also affected by the addition of antibiotics and by the interaction of both factors (Table 3). The higher abundances of *A. minutum* were measured in the control treatments both -AB and +AB. However, in this case, *A. minutum* growth was lower in the control treatment -AB than +AB, reaching final mean abundances of 15,613 ± 699 cells mL^{-1} and 21,472 ± 2425 cells mL^{-1}, respectively. Post-hoc tests showed that the abundance of *A. minutum* differed significantly between the high addition of *Z. marina* exudate and the other addition treatments (Tukey post hoc test, $p < 0.001$), as well as between the control and the low addition treatment (Tukey post hoc test, $p < 0.05$), after 14 days. The abundance of *A. minutum* in cultures not exposed to antibiotics decreased along increasing levels of *Z. marina* exudate, but no significant differences were found between treatments (Tukey post-hoc test, $p > 0.05$). Nonetheless, when exposed to antibiotics, *A. minutum* cultures reached higher abundances in the medium vs the low addition treatment. The high addition treatment showed significantly lower abundances than any other condition. The control and low addition treatments +AB were significantly different by the end of the experiment (Tukey post hoc test, $p < 0.05$).

Fig. 3 showed the response ratio of each of the treatments -AB and +AB compared to their respective controls in each experiment. In Exp_1 the addition of antibiotics did not modify the effect on the abundance of *A. minutum* by the different exudate additions. In Exp_2 the abundance response of *A. minutum* differed between cultures coexisting or not with their active microbiome.

At Exp_1, *A. minutum* growth rate was significantly affected by the addition of *Z. marina* exudates, but not by the addition of antibiotics or by the interaction of both factors (Table 4, Fig. 4). It should be noted, however, that in the case of the antibiotics factor, the p-value (0.061) is very close to the 0.05 significance level. Controls achieved average growth rates of 0.291 ± 0.016 and 0.265 ± 0.020 day^{-1} (without and with antibiotic treatments (-AB and +AB), respectively) throughout the experiment. In the high addition treatment, *A. minutum* growth rates were extremely low, even becoming negative when antibiotics were added (0.008 ± 0.006 day^{-1} and −0.033 ± 0.017 day^{-1}, -AB and +AB, respectively). Post-hoc tests showed that *A. minutum* growth rate differed significantly between the control and the different *Z. marina* exudate additions, both without and with antibiotics. This was also the case among the different addition treatments (Tukey post hoc test, $p < 0.001$).

In Exp_2, *A. minutum* growth rate was not as rigorously affected by the addition of *Z. marina* exudates as in Exp_1 (Fig. 4), although the p-value (0,069) was close to the 0.05 significance level (Table 4). In this case the interaction of exudates and antibiotics addition on *A. minutum* growth rate was statistically significant, (Table 4). The highest growth rates were measured in the control treatments both -AB and +AB, being slightly lower in the -AB one. When no antibiotics were added, the greater the addition of exudate, the lower the *A. minutum* growth rate, showing a maximum mean growth rate of 0.302 ± 0.001 day^{-1} in the control and a minimum of 0.236 ± 0.025 day^{-1} in the high treatment. Post-hoc tests showed no significant differences between -AB treatments (Tukey post hoc test, $p > 0.05$). When exposed to antibiotics, the trend continues negative with the exception of the cultures with medium addition treatment, which presented a higher growth rate than that observed in the low concentration treatment. In this case, post-hoc tests showed significant differences between the high addition treatment and any other condition (Tukey post hoc test, $p < 0.001$).

Table 2
Summary of results of GLMs testing the effects of the factors Treatment (4 levels: high vs medium vs low vs control) and Antibiotic (2 levels: without antibiotic vs with antibiotic) on bacterial abundance at time 14 in Exp_1 and Exp_2. Significant effects are indicated in bold.

	Experiment					
	Exp_1			Exp_2		
	LR χ^2	df	p	LR χ^2	df	p
Treatment	60.293	3	**< 0.001**	500.41	3	**< 0.001**
Antibiotic	184.839	1	**< 0.001**	901.08	1	**< 0.001**
Treatment: Antibiotic	10.739	3	**0.013**	46.13	3	**< 0.001**

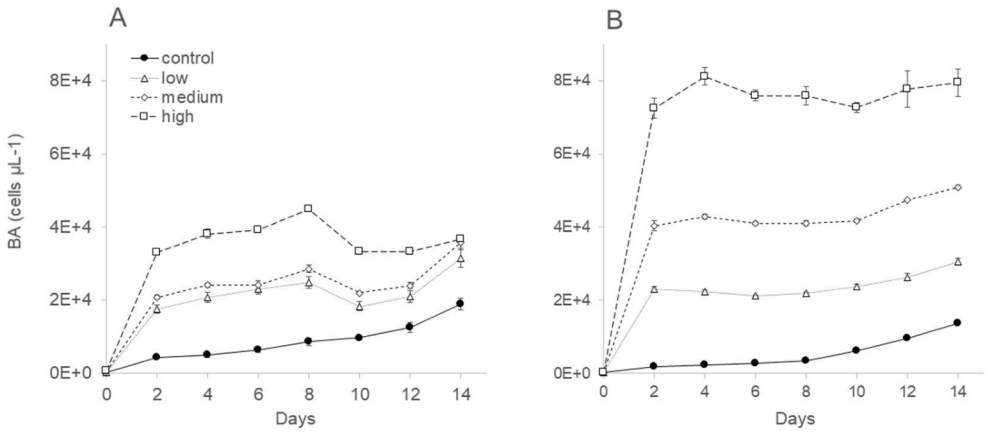


Fig. 1. Temporal evolution of the mean bacterial abundance (BA) by treatment without antibiotics throughout Exp_1 (A) and Exp_2 (B) in cells μL^{-1}. The bars represent the standard error of each of the treatments.

Table 3
Summary of results of GLMs testing the effects of factors Treatment (4 levels: high vs medium vs low vs control) and Antibiotic (2 levels: without antibiotic vs with antibiotic) on *A. minutum* abundance at time 14 in Exp_1 and Exp_2. Significant effects are indicated in bold.

	Experiment					
	Exp_1			Exp_2		
	LR χ^2	df	p	LR χ^2	df	p
Treatment	130.312	3	**< 0.001**	8.5657	3	**0.036**
Antibiotic	0.41	1	0.522	8.54	1	**0.003**
Treatment:Antibiotic	0.329	3	0.955	14.305	3	**0.003**

3.4. Photosynthetic efficiency

Fig. 5 showed the temporal evolution of the photosynthetic efficiency (Fv/Fm) throughout the experiments. The effect of the addition of *Z. marina* exudates, the addition of antibiotics as well as the interaction between the *Z. marina* exudate additions and the antibiotic on *A. minutum* photosynthetic efficiency were statistically significant in the first sampling after the addition of *Z. marina* exudate ($t = 48$ h), while only the effect of the addition of antibiotics and the interaction between the addition of exudates and antibiotics were statistically significant by the end of Exp_1 (Table 5). Both the control and the low addition treatments showed Fv/Fm values ranging from 0.6 to 0.7 throughout the entire experiment, either in -AB or +AB treatments. A subtle decrease in photosynthetic efficiency was observed in the first sampling after the addition of *Z. marina* exudate in the medium addition treatments, with mean Fv/Fm values slightly over 0.5. These values remained rather constant until the sixth day of incubation, when Fv/Fm steadily increased reaching similar or higher values than in the control treatments.

The largest effect was found in the high addition treatments. In the high addition treatment exposed to antibiotics, Fv/Fm decreased to 0.4–0.5 immediately after the addition of the exudate and remained rather constant afterwards. The differences in photosynthetic efficiency in this treatment and the rest of -AB treatments were statistically significant 48 h after the addition of exudate (Tukey post-hoc test, $p < 0.01$), but not by the end of the experiment (Tukey post-hoc test, $p > 0.05$). In the high addition treatment +AB, Fv/Fm decreased after the addition of the exudate to 0.36 ± 0.02, then finally dropped to 0.14 ± 0.08. Post-hoc tests showed significant differences between the high addition treatment +AB and the low and medium ones at final time (Tukey post-hoc test, $p < 0.05$), but not between the high addition treatment and the control +AB (Tukey post-hoc test, $p = 0.053$). Fv/Fm values at 48 h were significantly lower in the high addition treatment +AB compared with the rest of the +AB treatments (Tukey post-hoc test, $p < 0.001$).

In Exp_2, mean Fv/Fm values were always higher than 0.55. The lowest mean values were measured after 48 h for the high addition treatments (with and without antibiotics), with mean values of 0.579 ± 0.003 and 0.568 ± 0.017, respectively. At this time, differences in Fv/Fm values between the high addition treatments and the rest were significant (Tukey post-hoc test, $p < 0.001$) but the measured Fv/Fm values were typical of healthy cells. Thus, it does not seem that the *Z. marina* exudate in Exp_2 exerted a negative effect on the photosynthetic efficiency of *A. minutum*. From 48 h onwards, both high-addition treatments increased their photosynthetic efficiency and all treatments showed similar Fv/Fm. By the end of the experiment the highest Fv/Fm were measured in the -AB control (0.676 ± 0.012) and the lowest in the +AB control (0.600 ± 0.024).

3.5. Alexandrium minutum PSTs

Alexandrium minutum toxin cell quota was measured as the concentration of saxitoxin equivalents per cell (STXeq $cell^{-1}$). Toxin concentrations measured in the two experiments were different (Fig. 6), including the controls, with a mean value of more than 3 pg STXeq $cell^{-1}$ in the -AB control of Exp_1, while in Exp_2 that control yielded a mean value <0.5 pg STXeq $cell^{-1}$. The addition of *Z. marina* exudates decreased intracellular toxin concentration in Exp_1, though the converse occurred in Exp_2, being these differences statistically significant (Table 6).

In Exp_1, toxin concentration per cell decreased with the addition of *Z. marina* exudate in the presence of an active bacterial community from a maximum of 3.20 ± 0.48 pg STXeq $cell^{-1}$ measured in the control treatment and a minimum of 1.40 ± 0.16 pg STXeq $cell^{-1}$ in the medium addition treatment (Figs. 6 and 7). However, the addition of antibiotics increased the toxin concentration per cell in the exudate treatments compared to the control. The very low cellular density measured in the high addition treatment (see Fig. 2) precluded the quantitation of any PSTs, as they were below the LOD of the analytical method.

In Exp_2, the addition of *Z. marina* exudate and the interaction between exudate and antibiotic additions had significant effects on PSTs cellular quota (Table 6). The addition of eelgrass exudates increased PST levels either in -AB or +AB treatments, showing significant differences between the values measured in the high addition treatments and those in the low addition or the control (Tuckey post-hoc tests, $p < 0.001$). In cultures not exposed to antibiotics, the minimum toxin concentration per cell was found in the control and the maximum value in the medium addition treatment. However, the differences in cellular toxin concentration between -AB treatments and controls were not significant

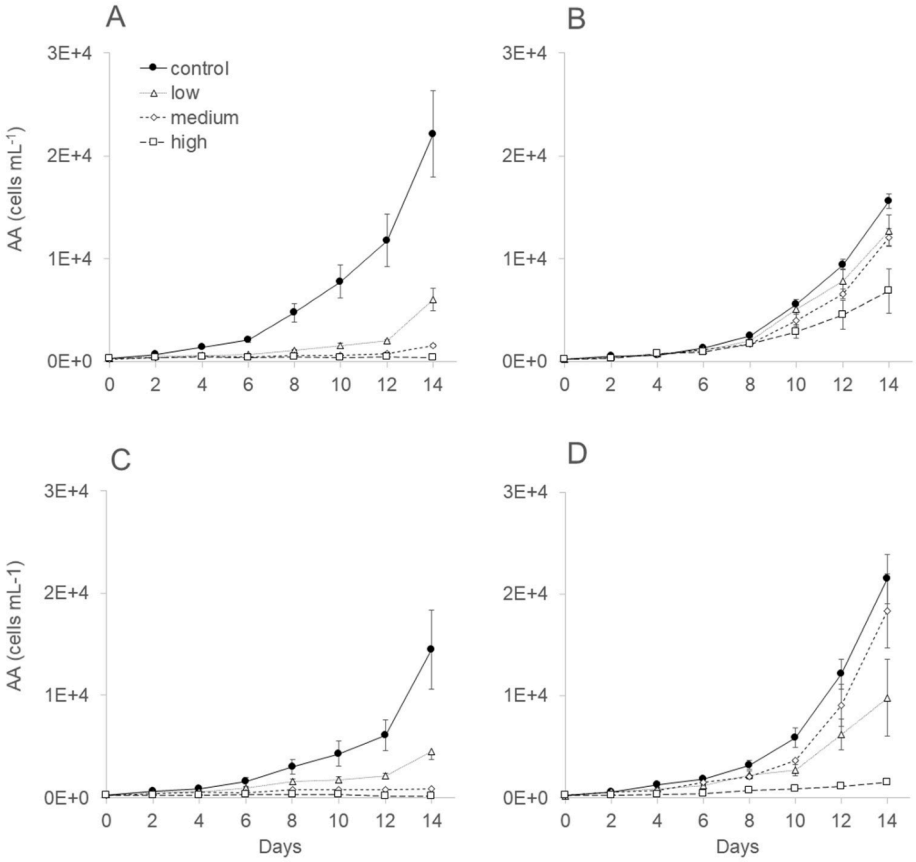


Fig. 2. Temporal evolution of the mean *A. minutum* abundance (AA) per treatment throughout Exp_1 without (A) and with (C) antibiotics and Exp_2 without (B) and with (D) antibiotics, measured in cells mL^{-1}. The bars represent the standard error of each of the treatments.

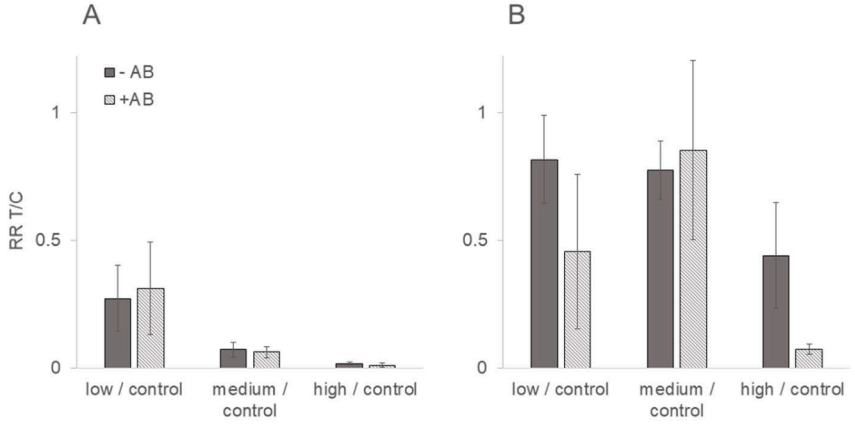


Fig. 3. Abundance response ratio of the culture at the final time in each of the treatment additions versus the control without (grey) and with (striped) antibiotics in Exp_1 (A) and Exp_2 (B). The bars represent the absolute error propagated.

Table 4
Summary of results of the two-way ANOVA (Exp_1) and the GLM (Exp_2) testing the effects of factors Treatment (4 levels: high vs medium vs low vs control) and Antibiotic (2 levels: without antibiotic vs with antibiotic) on *A. minutum* growth rate. Significant effects are indicated in bold.

	Experiment						
	Exp_1				Exp_2		
	df	SS	F	p	LR χ^2	df	p
Treatment	3	0.27812	100.437	**< 0.001**	7.1027	3	0.069
Antibiotic	1	0.00375	4.057	0.061	0.1713	1	0.679
Treatment:Antibiotic	3	0.00072	0.261	0.853	25.7237	3	**< 0.001**

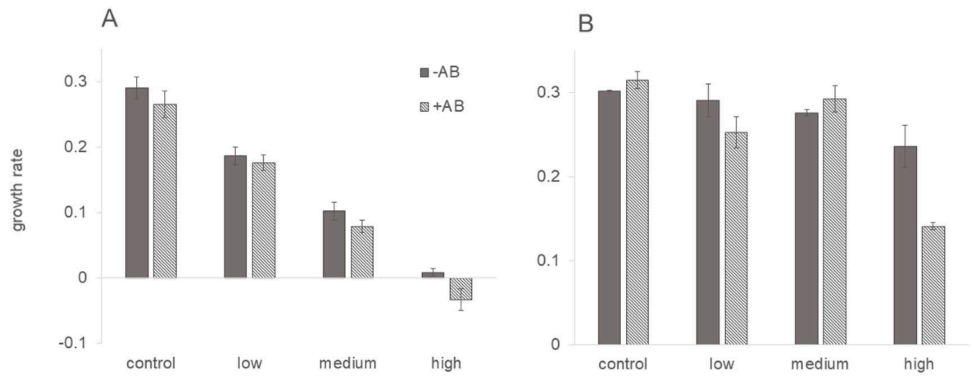


Fig. 4. Mean growth rate of the culture per treatment throughout each of the experiments without (-AB) and with (+AB) antibiotics in Exp_1 (A) and Exp_2 (B). The bars represent the standard error of each of the treatments.

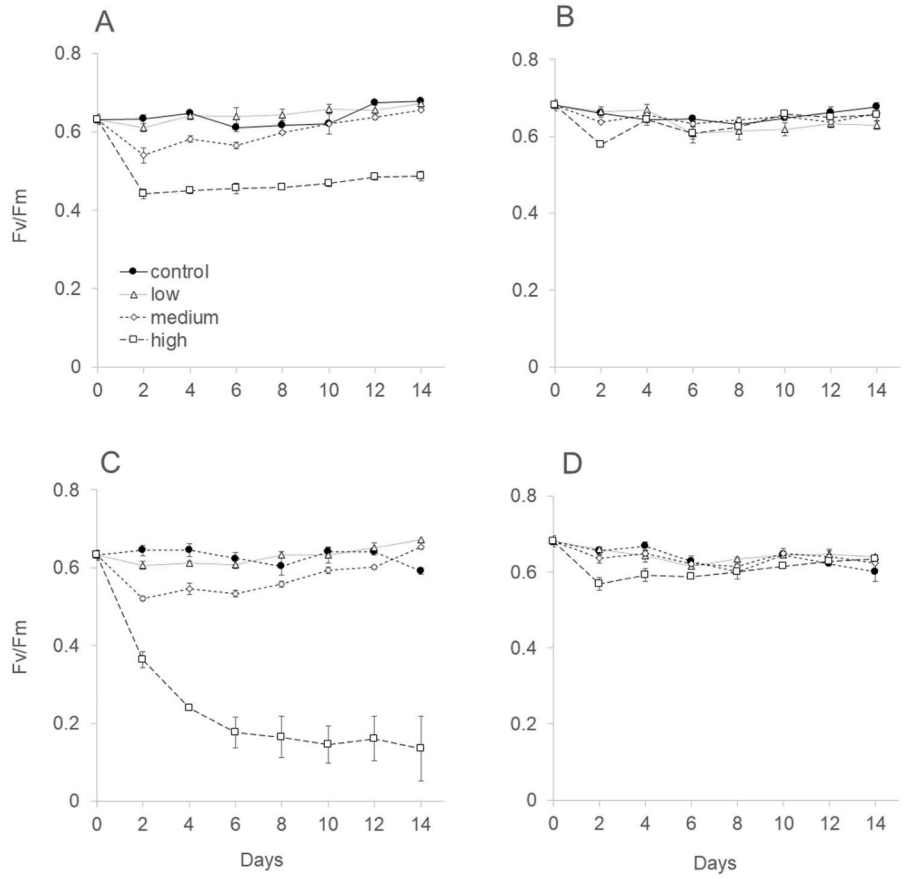


Fig. 5. Temporal evolution of the mean Fv/Fm by treatment without (-AB) and with (+AB) antibiotics throughout Exp_1 without (A) and with (C) antibiotics and Exp_2 without (B) and with (D) antibiotics. The bars represent the standard error of each of the treatments.

(Tuckey post-hoc test, $p > 0.05$). On the contrary, in the +AB treatments, statistically significant increases in toxin concentration per cell were observed in the high addition exudate compared to the other additions (Tuckey post-hoc test, $p < 0.05$).

4. Discussion

4.1. Macrophyte effects on dinoflagellates growth

The exudates used here were obtained after incubating *Z. marina* leaves in a solution of NaCl without any external manipulation relative to those occurring in the natural environment. The aim was to obtain exudates that were representative of field conditions. Moreover, the concentrations at which these exudates were added in the different treatments fell within the range of DOC concentrations in areas dominated by seagrasses, where values up to 2500 μM have been measured (Velimirov, 1986).

Marine angiosperms are known to release dissolved organic matter, including DOC, through their leaves into the water column (Duarte et al., 2010; Penhale and Smith, 1977), which can be taken up by bacteria (Moriarty and Pollard, 1982; Ziegler and Benner, 1999). In this regard, enhanced bacterial production associated with the exudation of organic matter by seagrasses linked to its photosynthetic activity has been reported (Moriarty and Pollard, 1982). In contrast, the production

Table 5
Summary of results of GLMs testing the effects of factors Treatment (4 levels: high vs medium vs low vs control) and Antibiotic (2 levels: without antibiotic vs with antibiotic) on photosynthetic efficiency at time 2 and time 14 in Exp_1 and Exp_2. Significant effects are indicated in bold.

	Experiment					
	Exp_1			Exp_2		
	LR χ^2	df	p	LR χ^2	df	p
Time 2						
Treatment	91.567	3	**< 0.001**	42.479	3	**< 0.001**
Antibiotic	22.56	1	**< 0.001**	0.649	1	0.421
Treatment: Antibiotic	19.769	3	**< 0.001**	0.26	3	0.967
Time 14						
Treatment	1.4884	3	0.685	5.1061	3	0.164
Antibiotic	16.063	1	**< 0.001**	1.0149	1	0.314
Treatment: Antibiotic	14.7965	3	**0.002**	9.4243	3	**0.024**

of bactericidal compounds or inhibitors of bacterial growth by marine phanerogams, such as rosmarinric acid and other phenolic compounds, has been also described (Guan, 2017).

The negative effect of the exudates of marine angiosperms on toxic dinoflagellate growth had been already reported by Harrison and Chan (1980), who tested the effect of adding extracts of *Z. marina* on axenic cultures of the planktonic dinoflagellates *Gonyaulax polyedra* and *Protogonyaulax tamarensis* (=*Lingulodinium polyedra* and *A. tamarense*), observing a significant decrease in viable cells, accompanied by loss of motility, deformation and loss of cellular structure. In that case, extracts were obtained from dead stranded leaves, using methanol as solvent and boiling them to help the release of substances from the leaves. This method could facilitate the extraction of compounds which are not naturally released by *Z. marina* at sea. Moreover, the composition of the plants can vary depending on their physiological state and, therefore, on the amount and quality of the substances they release into the environment. Hence, it can be expected that the extracts used in Harrison and Chan (1980) would differ in composition from what is actually exudated by *Z. marina* meadows in the natural environment.

Similar experiments were carried out by Laabir et al. (2013) with the planktonic dinoflagellate *A. catenella* in which a significant decrease in growth rate when exposed to the marine angiosperms *Z. marina* and *Z. noltii* was shown. These authors used extracts from both live leaves and others recently detached from the plants and two different solvents for the extractions: deionized water and methanol. However, the exudates made with fresh leaves were extracted with methanol, so none of the exudates tested came close to a natural exudate. In addition, the extractions were carried out after the leaves dried out, a process that does not occur in *Z. marina* meadows.

Recently, Ben Gharbia et al. (2017) described several negative effects of fresh leaf fragments from *Cymodocea nodosa* on the planktonic dinoflagellate *A. pacificum*, including behavioral inhibition, malformations, loss of motor capacity, etc. In these experiments, plants were carefully washed and added to the cultures without subjecting them to any process which is not present in their natural environment. However, in that study the cultures used were not axenic and the possibility of an effect of bacteria on the interaction between the dinoflagellate and the plants could not be discarded.

4.2. Role of bacteria on the effect of macrophyte exudates

The inhibitory effect of *Z. marina* exudates on *A. minutum* growth rates observed in this study could be due to both a direct effect on the dinoflagellate, or to an indirect effect caused by the potential effect of the exudates on the dinoflagellate bacterial microbiome, since a close relationship between different species of planktonic dinoflagellates and their associated microbiome has been reported (Hattenrath-Lehmann and Gobler, 2017; Sörenson et al., 2019). A positive effect of *Z. marina* exudates on bacteria could promote competition for resources between bacteria and the dinoflagellates, while a potential bactericidal action of *Z. marina* (Natrah et al., 2015; Sathyanathan et al., 2016) would

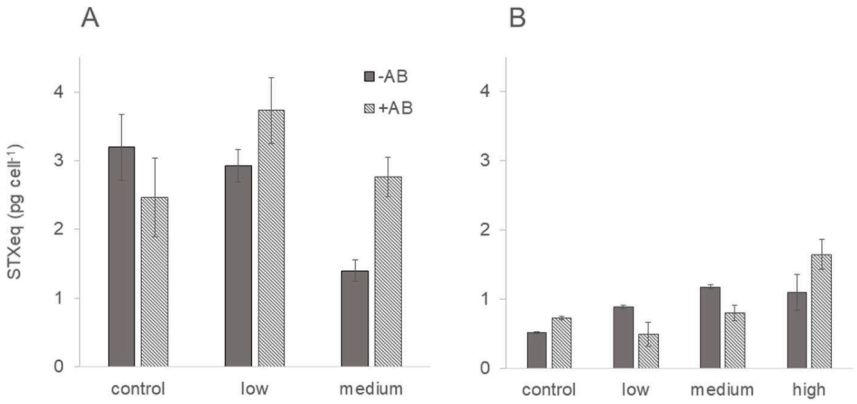


Fig. 6. Mean *A. minutum* PST toxin concentration, measured in pg of STXeq per cell, of *A. minutum* cultures subjected to each of the additions of *Z. marina* exudate without (-AB) and with (+AB) antibiotics in Exp_1 (A) and Exp_2 (B). The toxin cell quota of the high addition treatments in Exp_1 is not represented because the low abundance of *A. minutum* preculed toxins quantitation. The bars represent the standard error.

Table 6
Summary of results of two-way ANOVAs testing the effects of factors Treatment (4 levels: high vs medium vs low vs control) and Antibiotic (2 levels: without antibiotic vs with antibiotic) on *A. minutum* PST concentrations at time 14. Significant effects are indicated in bold.

	Experiment							
	Exp_1				Exp_2			
	df	SS	F	p	df	SS	F	p
Treatment	2	5.27	5.358	**0.024**	3	2.0897	11.71	**< 0.001**
Antibiotic	1	0.729	1.482	0.250	1	0	0.001	0.978
Treatment:Antibiotic	2	3.262	3.317	0.075	3	0.9748	5.462	**0.009**

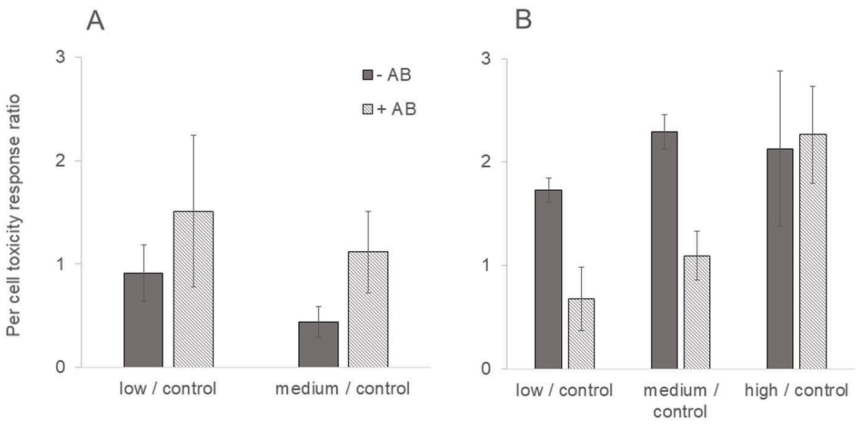


Fig. 7. Response ratio of *A. minutum* PST toxins, measured as pg of STXeq per cell, at the end of each experiment. Bars represent STXeq per cell of the *Z. marina* exudate additions with respect to control in cultures not exposed (-AB) or exposed (+AB) to antibiotics in Exp_1 (A) and Exp_2 (B). The toxin cell quota of the high addition treatments in Exp_1 is not represented because the low abundance of *A. minutum* preculed toxins quantitation. The bars represent the absolute propagated error.

neutralize the possible beneficial impacts of the bacterial microbiome on the studied dinoflagellate.

In the present study, the addition of *Z. marina* exudates to non-axenic cultures of *A. minutum* inhibited its growth, but stimulated the bacterial community. This negative effect on *A. minutum* also occurred when bacterial growth was blocked by the addition of antibiotics. These observations were consistent between experiments, despite differences in the magnitude of the measured parameters. In should be noted that the variable used in our study to establish the addition experiments, DOC concentration, is not expected to be the factor negatively affecting dinoflagellate growth. Rather, the cause of this effect is likely to be associated with other still unknown chemical substance, included in the DOM pool, released by *Z. marina*. Thus, although the added DOC concentrations were the same in both experiments, the additions of the probable compound with the negative effect on dinoflagellates could have been different, which helps explaining the variability observed in the response of *A. minutum* to the addition of different exudates. Different exudates, made from leaves in different growth stages (old leaves in Exp_1 and young leaves in Exp_2), would be expected to induce somewhat distinct responses on dinoflagellate growth, although the overall trends found in this investigation were similar in the two experiments.

The increase in bacterial abundance measured in treatments occurred mainly in the first 48 h of the experiments, this increase being much smaller or even non-existent thereafter. This indicates that the organic matter present in the exudates is pretty labile, so the high amount of organic nutrients present in the exudates was mostly consumed soon after the beginning of the experiments.

The dinoflagellate photosynthetic efficiency (Fv/Fm) values remained close to those of healthy cells in the controls but slightly decreased in some of the exudate treatments immediately after the start of both experiments. Only the high addition treatment in Exp_1 showed a significant and sustained decrease in Fv/Fm over time. The impact of *Z. marina* exudates on the PSII photosynthetic efficiency points out to a direct negative effect on the dinoflagellate photosynthetic metabolism, an observation consistent with previous studies where *A. pacificum* cultures were exposed to fresh fragments of *C. nodosa* (Ben Gharbia et al., 2017).

The comparison of the abundance, growth rate and photosynthetic efficiency of *A. minutum* cultures grown with the bacteria of its culture with respect to those where the bacterial growth was blocked demonstrates that inhibitory effect of *Z. marina* exudates on *A. minutum* growth should be mainly attributed to some substance directly released by the angiosperm and not to an indirect effect on the dinoflagellate microbiome. Notwithstanding, the associated bacterial community may also play some role since differences between cultures with and without bacteria were found in both experiments.

The toxin cell quotas measured in this study showed a large variability, even in the control treatments, with values more than 6-fold higher in Exp_1 versus Exp_2. In this regard, mean total toxin cell quota values in Exp_2 were of similar magnitude to those originally determined for *A. minutum* VGO1436 and other 44 strains obtained from close locations and dates (Ben-Gigirey et al., 2020).

Alexandrium is among the most studied dinoflagellate genera and the variations in toxin contents and composition, as well as the production of bioactive metabolites, have been detailed elsewhere (Anderson et al., 2012; Han et al., 2016; Lewis et al., 2018; Long et al., 2021). It is well known that range of PST contents in microalgae varies considerably within and between populations, and culture-induced variation is also evident (Hallegraeff et al., 2012; Negri et al., 2007). Nevertheless, the reasons for this high level of variation are poorly understood, and they include growth phase and multiple abiotic (e.g. salinity, temperature, nutrient availability), biotic (the direct or indirect effects of the microbial community, the exposure to grazers and its cues; Park et al., 2023) and genetic factors (Verma et al., 2019). In general, conditions that inhibit growth usually increase toxin concentration per cell in *Alexandrium* (Han et al., 2016; Navarro et al., 2006). However, there exist conflicting results about the outcome of some factors which make difficult to draw general statements. For instance, different studies reported maximum cell toxin concentration values during the exponential growth phase in *Alexandrium* spp, followed by a rapid decrease in the toxin concentration per cell as the cultures approached the stationary phase (Anderson et al., 1990; Boczar et al., 1988; Siu et al., 1997; Suikkanen et al., 2013), while others found the maximum cell toxin content in the post-stationary phases (Hwang and Lu, 2000). Similar experiments studying the effect of macrophyte exudates on toxin production (Ben Gharbia et al., 2017), showed no significant differences in the toxin production of *A. pacificum*, due to large within-treatments variability.

In our investigation, both experiments were carried out separately in time and although the same strain was used in the two experiments, the growth state of the inoculums and the toxin concentration per cell of each of them at the initial time differed. Additionally, taking into account that PST synthesis occurs only during the G1 phase of cell division (Taroncher-Oldenburg et al., 1997) differences in division synchrony among cultured treatments may have affected our results.

The effect of the addition of *Z. marina* exudates on toxin cell quota production clearly differed in the two experiments depending on the presence or absence of antibiotics although neither the decrease in toxin concentration per cell of Exp_1 nor the increase in Exp_2 in the

treatments without antibiotics compared to those with antibiotic were statistically confirmed.

4.3. Conclusions

In this study we assessed the effect of exudates from the eelgrass *Zostera marina* on the abundance, growth rate, photosynthetic efficiency and toxin cell quota of the PSP toxin-producing dinoflagellate *Alexandrium minutum*. We have shown that exudates of *Z. marina* collected in spring negatively affected the abundance of *A. minutum* in culture and that they also affect its photosynthetic efficiency. However, the negative effects are variable, obtaining different but consistent results even when leaves from the same plants are used but in different growth stages. Although a significant effect of the exudates on the *A. minutum* toxin cell quota was observed, we did not find a consistent pattern as contrasting results were obtained in the two experiments. Future studies should focus on the effect of the exudates of *Z. marina* collected at different seasons to test if the negative effect detected in this study remains throughout the seasonal cycle of *Z. marina* growth, as well as to verify if the meadows efficiently release exudates that negatively affect *A. minutum* in the natural environment.

CRediT authorship contribution statement

Alexandra Díaz-Alonso: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Francisco Rodríguez:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition, Investigation, Writing – original draft. **Pilar Riobó:** Methodology, Writing – review & editing, Data curation. **Xose Álvarez-Salgado:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Eva Teira:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Emilio Fernández:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2024.102605.

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