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How Does *Mytilus galloprovincialis* Respond When Exposed to the Gametophyte Phase of the Invasive Red Macroalga *Asparagopsis armata* Exudate?

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Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). **Abstract:** *Asparagopsis armata* is classified as an invasive species in Europe. Through the exudation of secondary metabolites, this macroalga holds a chemical defence against consumers, with potential toxic effects to native rocky shore communities. This study aims to evaluate the potential impact of *A. armata* (gametophyte) exudate in a native species, the mussel *Mytilus galloprovincialis*, in terms of biochemical and organismal effects. The 96 h-LC50 was 3.667% and based on it, exudate concentrations (0.25; 0.5; 1; 2%) were determined to further sublethal experiments. These sublethal concentrations caused no oxidative damage in the digestive gland since lipid peroxidation and protein carbonylation were not affected. Nevertheless, there was a significant rise in the electron transport system activity and total glutathione content in muscle, suggesting an increased non-enzymatic antioxidant capacity and consequent energy consumption to cope with potential prooxidant compounds. This might have contributed to the observed decline in cellular energy allocation of the exposed mussels. At the organismal level, clearance capacity declined along the concentrations and a significant reduction in their attachment strength was observed. These findings suggest that the presence of *A. armata* may compromise *M. galloprovincialis* integrity in the invaded coastal areas.

Keywords: invasive macroalgae; marine; biochemical markers; clearance rate; byssus production

1. Introduction

The continuous proliferation of invasive seaweeds has grown a great concern considering their impacts on native marine communities, mainly because they naturally threaten the availability of resources compromising the natural ecosystem functioning [1]. The red marine macroalgae Asparagopsis armata Harvey, 1855 (Bonnemaisoniales, Rhodophyta), native from southern Australia and New Zealand, is identified as an invasive species and widely distributed in the Mediterranean and Atlantic coasts of Europe including Portugal [2,3]. This temperate alga can overcome the ecological barriers and colonise diverse environmental conditions, mainly due to its fast growth rate, lack of predators and its effective spread, both through the tetrasporophyte floating balls and the gametophyte phase that hooks onto floating material [4,5]. A. armata sets mainly in the coastal low intertidal zone extending to the upper meters of the subtidal zone [3,6]. Therefore, it competes for resources (mainly space and nutrients) with specific native species, such as epifaunal assemblages. Furthermore, this macroalga is adapted to strong physicochemical changes [7,8], characteristic from this zone, imposed by wave action and the ebb and flow of the tide. These tidal cycles lead to the formation of microenvironments within intertidal tide pools, wherein the organisms face adverse conditions [9]. Like other seaweeds (particularly concerning in case

of invasive macroalgae [10,11]), *A. armata* is known to exude a wide array of secondary metabolites with potentially deleterious effects, including a large number of halogenated compounds (e.g., haloketones, haloacids and haloforms) [12,13]. This mixture is usually produced as a chemical defence against consumers, epiphytes such as bacteria and may act in space competition (allelopathy) [4,14]. Exudation of such compounds may therefore cause toxic effects to surrounding species which together with the competition for natural resources, especially in the intertidal tide pools, can enhance the impacts of *A. armata* on ecosystem integrity and functioning. Given this potential as an ecosystem engineer, very common to marine macroalgae [15], it is crucial to evaluate this non-indigenous alga' effects. Although few studies addressed this problem, little is known about it. For example, it was demonstrated that *A. armata* halogenated metabolites deterred feeding by two marine mesograzers (*Hyale nigra* and juvenile abalone *Haliotis rubra*) [16]. Exposure to its exudate caused physiological status impairment in exposed *Palaemon elegans* and *Gibbula umbilicalis*, as well as feeding inhibition in this gastropod [17].

There is limited knowledge on the impact of toxic secondary metabolites derived from macroalgae on bivalve species, contrary to the deleterious effects of bioactive compounds and toxins produced by cyanobacteria and dinoflagelates, e.g., [18–20]. Nonetheless, the physiological status of the mussel Pinna nobilis was found to be negatively affected when colonized by the invasive red macroalga Lophlocladia lallemandi, which is known to produce lophocladines with potential cytotoxic effects [21]. Thereby, the present study arises at assessing possible effects of A. armata exudates on the native mussel Mytilus galloproviancialis Lamarck, 1819. This species forms large and dense mono-and multi-layered beds in the low to mid intertidal rocky shores [22,23]. M. galloprovincialis has been employed as a sentinel species for monitoring coastal environments due to its widespread distribution, abundance and sedentary lifestyle [24–27]. They have an impressive filter-feeding behaviour, being capable to uptake several waterborne contaminants, thus reflecting the impacts of different environmental disturbances, e.g., [28–31]. Furthermore, M. galloprovincialis has great ecological relevance in coastal ecosystems by playing a critical role in the water quality through the filtration of particles and excess nitrogen from the aquatic environment [32,33]. They are also considered important links in coastal trophic chains between bottom-dwelling organisms and phytoplankton [32]. In addition, mussel beds contribute to increasing habitat complexity and therefore biodiversity [34,35], by providing shelter to a variety of organisms and nurseries of juvenile fish and crustaceans. Therefore, eventual ecological consequences may derive from M. galloprovincialis decline. Aside from its ecological importance, M. galloprovincialis has a high economic value, both in Portugal and worldwide. It is considered an important food resource for human consumption, thus being among the most harvested species and representing a significant part in the overall marine aquaculture [32,36–38].

In this sense, this work intends to understand how this mussel species respond in the presence of *A. armata* exudate, by evaluating its toxicity in terms of biochemical and organismal responses.

2. Materials and Methods

2.1. Asparagopsis armata Sampling and Exudate Production

A. armata (gametophyte phase) was collected by hand through free diving in the subtidal zone at the Terceira island Azores (Portugal) (38°38′59.2″ N, 27°13′16.4″ W) in January 2019. The macroalgae were kept in aerated seawater tanks until next day and packed in sealed containers to be transported to the laboratory in Aveiro (Portugal). Immediately upon arrival, *A. armata* was cleared from visibly associated fauna and allocated to a tank with artificial seawater (marine RedSea[®] Salt premium grade) in a 1:10 proportion (salinity 35 ± 1, pH 8.0 ± 0.1, temperature 20.0 ± 0.5 °C) in the dark and no aeration for 24 h to produce the exudate, adapted from [39–41]. These conditions were previously optimized to obtain exudate from the stressed alga without causing its death during the process. Afterwards, the

alga was removed from the tank and the resulting media (considered as the stock solution and representing 100% of exudate) was preserved at -20 °C, until further use.

2.2. Mytilus galloprovincialis Sampling and Acclimatization

M. galloprovincialis specimens (4 ± 0.5 mm length), were collected by hand, during low tide, from the intertidal rocky shores of the Barra de Aveiro (Portugal) (40°38′38.8″ N, 8°44′44.6″ W). Organisms were transported from the field to the laboratory and were immediately cleaned of superficial debris and encrusting organisms. Then, they were acclimated for seven days in an aquarium containing aerated artificial seawater (salinity 35 ± 0.5 , pH 8.0 ± 0.1, temperature 19.0 ± 0.5 °C, oxygen saturation > 80%) with a 14 h light: 10 h dark photoperiod. No food was provided during this period.

2.3. Lethal Toxicity Test

After acclimatising, mussels were used in a 96 h static acute toxicity test with the exudate from *A. armata*, which was slowly defrosted in the dark in a 4 °C chamber, before the preparation of the solutions. Eight different exudate treatments (0.5, 1, 2, 4, 8, 16, 32 and 64%) were tested, based on a range-finding experiment, plus an artificial seawater control. Exposure was performed in 5 L aquarium containing 2.5 L of the aerated solution with five animals each and four replicates per treatment and a 14 h light: 10 h dark photoperiod. Test parameters were salinity 35 ± 0.5 , pH 8.0 ± 0.3 , 19.0 ± 0.5 °C and oxygen saturation > 80%. Mussels' mortality was daily checked through the presence of open valves that were unresponsive to stimuli.

2.4. Sublethal Toxicity Tests

To assess the effects of the exposure of *M. galloprovincialis* to sublethal exudate concentrations, several endpoints were considered regarding biochemical (neurophysiological, oxidative stress, energy metabolism) and organismal (clearance rate and byssal thread production) responses. After the acclimatisation period (Section 2.2), mussels (4 ± 0.3 mm length) were exposed to four nominal exudate concentrations (0.25; 0.5; 1; 2%), based on the 96 h-LC50 estimated through the survival test, plus the control, during 96 h. Seven replicates were used per treatment with one mussel each, in 1 L glass flasks containing 500 mL of aerated solution and a 14 h light: 10 h dark photoperiod. Test parameters were salinity 35 ± 0.5 , pH 8.0 \pm 0.2, 19.0 \pm 0.3 °C, oxygen saturation > 80%.

2.4.1. Biochemical Responses

Sample Preparation for Biomarker Analysis

Upon exposure, *M. galloprovincialis* tissues (digestive gland, adductor muscle and gills) were dissected, and samples were individually homogenised on ice using a sonicator (pulsed mode of 10%, 250 Sonifier, Branson Ultrasonics, Danbury, CT, USA).

The digestive glands were homogenised in 2000 μ L of ultra-pure water. From each sample, one aliquot containing 4% butylated hydroxytoluene (BHT) in methanol was used for the determination of lipid peroxidation (LPO); another sample was stored for protein carbonylation (PC) quantification. The remaining homogenate was diluted in 0.2 M K-phosphate buffer, pH 7.4, and centrifuged for 15 min at $10,000 \times g$ (4 °C), the post-mitochondrial supernatant (PMS) was divided into microtubes and kept in -80 °C until further analyses of catalase (CAT) and glutathione S-transferase (GST) activities, total glutathione (tGSH).

The adductor muscles were homogenised in 2400 µL of ultra-pure water. From each individual, samples were stored for lactate dehydrogenase (LDH) activity, acetyl-cholinesterase (AChE) activity and tGSH determination, diluted in 0.2 M K-phosphate buffer, pH 7.4, and centrifuged for 15 min; 3 aliquots were taken for the analysis of lipid, sugar and protein contents and electron transport system (ETS) activity.

The homogenisation of gills was performed in 1600 μ L of ultra-pure water. From each sample, one aliquot for the determination LPO with 4% BHT in methanol was stored; other

aliquots were stored for ETS quantification; the remaining homogenate was diluted in 0.2 M K-phosphate buffer, pH 7.4, and centrifuged for 15 min at $10,000 \times g$ (4 °C) and the PMS divided into microtubes and kept in -80 °C until further analyses of CAT and GST activities and tGSH content.

All biomarkers determinations were done in micro-assays set up in 96 well flat-bottom plates and read spectrophotometrically with the Microplate reader MultiSkan Spectrum (Thermo Fisher Scientific, Waltham, MA, USA).

Neurophysiological and Oxidative Stress Biomarkers

The Bradford method [42] was applied to determine the protein concentration of PMS, using as standard the bovine γ -globulin. AChE activity was measured using acetylthiocholine as substrate and following the absorbance increase at 412 nm, as described by Ellman's method [43] adapted to microplate [44,45]. CAT activity was determined in the PMS by following the decomposition of the substrate H₂O₂ at 240 nm [46]. GST activity was measured in the PMS following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm [47]. tGSH content was determined in the PMS fraction using a recycling reaction of reduced glutathione (GSH) with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase (GR) excess at 412 nm [48,49]. Endogenous LPO was measured as thiobarbituric acid-reactive substances (TBARS) at 535 nm [50]. PC was quantified based on the reaction of 2,4-dinitrophenylhydrazine (DNPH) with carbonyl groups, according to the DNPH alkaline method described by Mesquita et al. [51] at 450 nm.

Samples for LDH determination were submitted to 3 freezing/melting cycles, centrifuged at $1000 \times g$ for 10 min at 4 °C. The supernatant was used to determine LDH activity by following the NADH oxidation caused by pyruvate consumption at 340 nm [52], adapted to microplate [53].

Cellular Energy Allocation (CEA)

CEA value is calculated as CEA = Ea/Ec [54], whereas Ea is the energy available (the sum of sugars, lipids and proteins) and Ec is the aerobic energy production (estimated as ETS activity) and were determined by the methods described by De Coen and Janssen [55] with slight modifications for microplate [56]. Total lipid content was determined by adding chloroform, methanol and ultra-pure water in a 2:2:1 proportion. After centrifugation, H₂SO₄ was added to the organic phase of each sample in clean glass tubes and were incubated at 200 °C, 15 min. The absorbance was measured at 375 nm using tripalmitin as a lipid standard. 15% TCA was added to 300 μ L of homogenate and incubated for 10 min at -20 °C. After centrifugation ($1000 \times g$ for 10 min at 4 °C), carbohydrates quantification was performed in the supernatant by adding 5% phenol and H_2SO_4 to the samples, with glucose as a standard, at 492 nm. The remaining pellet was resuspended with 1 M NaOH (incubated for 30 min at 60 °C) and then neutralised with 1.67 HCL. Total protein content was quantified in this fraction following the Bradford method [42], at 520 nm and using bovine serum albumin as a standard. These energy available fractions were converted into energetic equivalent values using the corresponding energy of combustion: 39,500 mJ/g lipids, 17,500 mJ/g glycogen, 24,000 mJ/g protein [57].

ETS activity was assessed using the INT (Iodonitrotetrazolium) reduction assay, measuring the rate of INT reduction in the presence of the nonionic detergent Triton X-100, at 490 nm. The stoichiometrical relationship in which for 2 µmol of formazan formed, 1 µmol of oxygen is consumed was applied to calculate the cellular oxygen consumption rate. The specific oxyenthalpic equivalent for an average lipid, protein and carbohydrate mixture of 480 kJ/mol O₂ was used to convert the final Ec value into energetic equivalents [57].

2.4.2. Organismal Responses

Clearance Rate

Clearance rate estimates the volume of water cleared of particles by the mussel per hour [58] and this way evaluates their filter-feeding behaviour. For this procedure,

the green microalga *Isochrysis galbana* (Haptophyta) was used as a food item for mussels since it is representative of the marine phytoplankton communities and is known to be well ingested by bivalves. *I. galbana* was cultivated using the GoldMedium (aqualAlgae[®]) prepared according to manufacturer instructions. Cultures were maintained under the same conditions as mentioned by Kaczerewska et al. [59]. To create the growth curve, a dilution series with a factor of two (with eleven points) was used. The cell density was then determined by direct counting in a Neubauer hemocytometer using an optical microscope and, by fluorescence ($\lambda_{excitation}$: 475 ± 20 nm; $\lambda_{emission}$: 645 ± 40 nm) in a microplate reader (Biotek[®] Synergy HT, VT, USA) [59]. The initial cell density was approximately 10⁶ cells/mL and the linear regression equation used to convert fluorescence (X) into microalgae cell density (cells/mL, as cell number per millilitre) (Y) was:

$$Y = 465.17 X - 500,951 (r^2 = 0.9938)$$

Mussels were transferred, after exposure, to glass flasks containing 300 mL of new artificial seawater (conditions aforementioned) for 30 min to allow the opening of the valves so that the feeding starts in the same conditions for all organisms. Then, about 4.5×10^6 cells/mL of the microalga *I. galbana* were added and the aeration was maintained to prevent the algae from settling. Three flasks were used to monitor *I. galbana* concentration over time, following the same procedure though without mussels. The number of algal particles per mL was quantified through fluorescence measurements of 2 mL subsamples of overlying seawater collected at 0, 10, 20, 40, 60, 120 and 180 min. Fluorescence was then converted into cell density (using the mentioned regression equation). The clearance rate was determined through the following formula [60]:

$$CR (Lh^{-1}) = ((ln C_0 - ln C_t)/t(h)) \times Vol (L)$$

where CR is the clearance rate, C_0 and C_t are concentrations of cells at t = 0 and at a given time (t = t), respectively; t is the time elapsed since t = 0 and Vol is the volume of water.

Byssal Thread Production

The byssal thread production is a good physiological biomarker considering mussels' general stress conditions [61]; therefore, the number of produced byssal threads and the attachment efficiency to the flask by each individual were assessed. After the 96 h of exposure to the different treatments (0; 0.25; 0.5; 1; 2%), the number of functional byssuses produced by each individual was counted and their byssal attachment strength (i.e., the force necessary for detachment) was manually evaluated while still immersed in the solutions to avoid modification of the mechanical properties of the byssus by air-dryness conditions. Four attachment strengths were considered: strong, medium, weak and detach.

2.5. Data Analysis

Data from the lethal toxicity test were analysed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA).

All variables were assessed for normality using the Kolmogorov–Smirnov test and Levene's test verified the homoscedasticity of data (p > 0.05). Biochemical and byssus production data were analysed through one-way analysis of variance (ANOVA) followed by multiple comparisons with Dunnett's post hoc test to discriminate statistically significant differences between *A. armata* exudate treatments and the non-exposed control (IBM SPSS Statistics 26). For data showing no normal distribution, the non-parametric test Kruskal–Wallis one-way analysis of variance on ranks was used, followed by Dunn's test.

A linear mixed model was used to investigate the influence of *A. armata* exudate concentration on *M. galloprovincialis* clearance rate over time. Exudate concentration and time were considered as fixed effects in the model, while animal ID as a random effect to consider the repeated measures. This analysis was performed in R software v4.0.0 [62] using Ime4 package v1.1-23 [63].

3. Results

3.1. Lethal Toxicity Test

The LC₅₀ for *M. galloprovincialis* was 3.667% (95% CI: 1.820–7.387%) after 96 h of exposure to *A. armata* exudate, no mortality occurred in the control treatments. Based on this concentration, the sublethal concentrations were determined to the following endpoints.

3.2. Sublethal Toxicity Tests

3.2.1. Biochemical Responses

Neurophysiological and Oxidative Stress Biomarkers

Considering the digestive gland, there were no alterations in the levels of LPO (One Way ANOVA, $F_{4,30} = 0.774$, p = 0.551) and PC (Kruskal–Wallis, H = 2.276, d.f. = 4, p = 0.685) in mussels exposed to *A. armata* exudate (Figure 1). On their turn, the activity of CAT (One Way ANOVA, $F_{4,30} = 1.081$, p = 0.383), tGSH (One Way ANOVA, $F_{4,30} = 1.316$, p = 0.287) and GST (One Way ANOVA, $F_{4,30} = 0.664$, p = 0.622) were also not affected (Figure 1).



Figure 1. Oxidative stress biomarkers of *Mytilus galloprovincialis* digestive gland after 96 h of exposure to *Asparagopsis armata* exudate. (a) Lipid Peroxidation (LPO); (b) Protein Carbonylation (PC); (c) Catalase (CAT); (d) Glutathione-S-Transferase (GST); (e) Total Glutathione (tGSH). All values are presented as mean \pm SE.

In the muscle, the levels of tGSH increased in mussels exposed to the exudate, when compared to the control treatment (0%, Figure 2a), this increase was significant at 0.5% and 2% (51.0 \pm 13.5 μ M/mg prot vs. 91.8 \pm 40.5 and 84.9 \pm 27.0 μ M/mg protein, respectively; Kruskal–Wallis, H = 9.681, d.f. = 4; post-hoc analysis, p < 0.05). A similar pattern was observed in the activity of AChE (Figure 2b), which significantly increased in the concentration 0.5% (2.756 \pm 0.802 nmol/min/mg prot vs. 5.027 \pm 1.909 nmol/min/mg prot; One Way ANOVA, F_{4,28} = 3.065; post-hoc analysis, p = 0.009).



Figure 2. (a) Levels of total glutathione (tGSH) in *M. galloprovincialis* muscle after 96 h of exposure; (b) Acetylcholinesterase (AChE) activity in *M. galloprovincialis* muscle after 96 h of exposure. All values are presented as mean \pm SE. * denotes a statistically significant difference compared to control (0%).

Regarding the gills (Figure 3), no alterations were detected for LPO (One Way ANOVA, $F_{4,29} = 0.708$, p = 0.593), CAT (One Way ANOVA, $F_{4,30} = 3.855$, post-hoc analysis, p > 0.05) and GST (Kruskal–Wallis, H = 6.211, d.f. = 4, p = 0.184), with the exception of tGSH, which levels decreased significantly in mussel exposed to 1% and 2% of *A. armata* exudate (39.0 ± 14.6 μ M/mg prot *vs.* 19.4 ± 7.8 and 16.7 ± 12.9 μ M/mg prot, respectively; Kruskal–Wallis, H = 11.049, d.f. = 4; post-hoc analysis, p < 0.05).



Figure 3. Oxidative stress biomarkers of *M. galloprovincialis* gills after 96 h of exposure to *A. armata* exudate. (a) Lipid Peroxidation (LPO); (b) Catalase (CAT); (c) Glutathione-S-Transferase (GST); (d) Total Glutathione (tGSH). All values are presented as mean \pm SE. * denotes a statistically significant difference compared to control (0%).

Energy Metabolism

The activity of LDH (One Way ANOVA, $F_{4,27} = 0.454$, p = 0.768) in muscles was not affected in *M. galloprovincialis* exposed to *A. armata* exudate (Figure 4a).

Although the lipid content in muscle slightly decreased in mussels exposed to 0.25% of exudate, it increased in the other concentrations, being this rise significant at2% (88.2 \pm 38.2 mJ lip/mg tissue vs. 150.6 \pm 50.0 mJ lip/mg tissue; One Way ANOVA, F_{4,29} = 6.277; post-hoc analysis, *p* = 0.012, Figure 4b). Sugars (One Way ANOVA, F_{4,30} = 0.169, *p* = 0.953) and proteins (One Way ANOVA, F_{4,30} = 2.289, *p* = 0.083) were not altered (Figure 4c,d).

In the muscle, the aerobic energy production, measured as ETS activity, increased significantly in individuals exposed to exudate 0.5%, 1% and 2% concentrations of compared to the control (Kruskal–Wallis, H = 12.544, d.f. = 4; post-hoc analysis, p < 0.05; Figure 4e). In line with the few changes previously observed for antioxidant defences, ETS activity was not altered in the gills (One Way ANOVA, $F_{4,30} = 1.588$, p = 0.203) (Figure 5).

In the final balance of CEA, all treatments exhibited a decrease on the energy budget of *M. galloprovincialis* compared to that of the control; this decrease was significant in the 0.25% and 0.5% treatments (Kruskal–Wallis, H = 10.844, d.f. = 4, post-hoc analysis p < 0.05) (Figure 4f).



Figure 4. Energy metabolism biomarkers of *M. galloprovincialis* muscle after 96 h of exposure to *A. armata* exudate. (a) Lactate dehydrogenase (LDH); (b) Lipids content (E_{lipids}); (c) Sugars content (E_{sugars}); (d) Proteins content ($E_{proteins}$); (e) Electron transport system (ETS); (f) Cellular energy allocation (CEA). All values are presented as mean \pm SE. * denotes a statistically significant difference compared to control (0%).



Figure 5. Electron transport system (ETS) in M. galloprovincialis gills after 96 h of exposure to A. armata exudate.

3.2.2. Organismal Responses Clearance Rate

To evaluate the filtration performance of *M. galloprovincialis* after a 96-h exposure to *A. armata* exudate, the CR of each mussel was estimated (Figure 6). The cell density of *I. galbana* remained consistent in the vessels (without mussel) used to monitor its growth over the 3 h of experiment.



Figure 6. *M. galloprovincialis* clearance rates (Lh⁻¹) exposed to different *A. armata* exudate concentrations over time.

In all evaluated times (in which the cell density of *I. galbana* was measured), the CR means were higher in the controls (from 0.41 to 0.21 Lh⁻¹, Figure 6). Specifically, mussels exposed to the highest exudate concentration (2%) generally exhibited the lowest CR means (between 0.12 and 0.21 Lh⁻¹) except for the 60 min, in which the CR mean was lower in mussels of the 1% treatment (Figure 6).

The *linear mixed model* showed that mussels' CR decreased significantly with increased concentrations of *A. armata* exudate (p < 0.05, Table 1), it also displayed a significant tendency of CR decreasing with time (time p < 0.001, Table 1). In addition, there is a significant interaction between time and exudate concentration, displaying that the CR differs significantly between distinct concentrations of exudate (exudate concentration*time interaction p < 0.05, Table 1).

Fixed Effects	Estimate	Std. Error	t Value	<i>p</i> -Value
intercept	$3.307 imes 10^{-1}$	$3.328 imes 10^{-2}$	9.939	< 0.001
exudate concentration	-8.422×10^{-2}	$3.404 imes 10^{-2}$	-2.474	< 0.05
time	$-8.444 imes10^{-4}$	$1.221 imes 10^{-4}$	-6.914	< 0.001
exudate concentration * time	2.929×10^{-4}	$1.293 imes 10^{-4}$	2.265	< 0.05

Table 1. Results of the linear mixed model on *M. galloprovincialis* clearance rates (Lh^{-1}) exposed to *A. armata* exudate over time. Significant at *p* < 0.05 and *p* < 0.001. "exudate concentration * time" term represents the interaction between these two fixed effects.

Byssal Thread Production

After the 96-h exposure, *M. galloprovincialis* secretion of byssal threads was affected, since the total number of newly secreted functional byssus tended to decrease along the gradient of *A. armata* exudate concentrations (Figure 7), this decrease was significant at 1 and 2% treatments (Kruskal–Wallis, H = 10.528, d.f. = 4, post-hoc analysis, p < 0.05).



Figure 7. Number of byssal threads produced by *M. galloprovincialis* during 96 h of exposure to different concentrations of *A. armata* exudate. * denotes a statistically significant difference compared to control (0%).

In addition, there was a significant reduction in their byssal attachment strength (Fisher exact test, p = 0.025), as represented in Figure 8).



Figure 8. Byssal attachment strength on threads produced by *M. galloprovincialis* during 96 h to different concentrations of *A. armata* exudate. Δ represents each mussel.

4. Discussion

The present study focused on the exposure of *M. galloprovincialis* to different concentrations of the exudate of *A. armata* and helped to evaluate the potential impact on the local ecosystems' integrity promoted by the presence of this invasive macroalga. Although there is evidence that the main effects may be a consequence of the biologically-active metabolites present in the *A. armata* exudate with strong antiherbivore, antifungal and antibiotic activity [16,64–66], especially volatile halocarbons such as bromoform, dibromomethane, 1,2-di-bromoethylene, dibromochloromethane and tribromoethylen [39], there is still a poor understanding on the impact this invasive seaweed may have in the surrounding biota. This is particularly important in the microenvironments within intertidal pools due to the encountered extreme conditions and where major algal blooms may form, especially in late spring/early summer, when the temperature starts to rise [2].

The lethal toxicity test demonstrated that the exudate produced by *A. armata* might be toxic to *M. galloprovincialis* compromising their survival even in short exposures. Mussels' survival decreased to 50% when exposed to only about 3.67% of exudate. Similar 96h-LC₅₀ values were reported by Silva et al. [17] for the gastropod *G. umbilicalis* (2.79%) and the prawn *P. elegans* (5.04%). Another study comparing the communities of peracarid species inhabiting *A. armata* and *Corallina elongata*, a red macroalga native from the Atlantic coast and abundant in the subtidal zone of Azores [67], showed that even though *A. armata* hosts a diversity of crustacean assemblages [6], its presence might lead to an impoverishment of epifaunal peracarid. Along the Atlantic coast and the Iberian Peninsula, the species richness was higher in *C. elongata* comparing to *A. armata*, especially for isopods [3].

Moreover, marine macroalgae are considered a possible source of reactive oxygen species (ROS), especially when under stress [68], compromising the organisms exposed to the exuded halogenated compounds. The short exposure of *M. galloprovincialis* to sublethal exudate concentrations caused no oxidative damage in the digestive gland of mussels since the levels of LPO and PC were not affected by the increase of exudate concentrations. In the same tissue, no alteration was detected in the activity of the enzymatic antioxidant CAT, the enzymatic and non—enzymatic antioxidants GST and tGSH that have the main role in phase II of the biotransformation of reactive oxygen species and detoxification [69].

Oxidative stress-related biomarkers were also assessed in mussels' gills since these organs can strongly reflect the adverse environmental impact on the animals as they are directly exposed to surrounding factors due to their large surface and role in gas exchange and feeding [70]. Although most biomarkers were not affected (LPO, CAT, GST and ETS), tGSH levels declined in exudate-exposed individuals. The increased consumption in tGSH allowed to counteract increased oxidative stress and avoid oxidative damage.

In its turn, the significant increase in the levels of tGSH and ETS activity in M. galloprovincialis muscles suggests an increment of non-enzymatic antioxidant capacity, and consequently increased aerobic energy production, to cope with potential pro-oxidant compounds produced at higher exudate concentrations. This energy consumption led to a significant decrease in the energy budget of exposed mussels. Interestingly, an increase in total lipid content was observed at the highest tested exudate concentrations. Lipids are essential constituents of biologically active molecules as well as important energy reserves in bivalves. Although we did not measure some peroxisomal enzymes (such as CAT) in the muscle tissue, a correlation between lipids metabolism and peroxisomal enzyme activities has been previously described [71]. On the other hand, and in agreement with our results, an increase in lipid content of G. umbilicalis exposed to A. armata exudate was observed and related with a concomitant decrease in feeding activity, leading to less energy expenditure [17]. On the other hand, the enzymatic activity of LDH, that plays an important role in the anaerobic pathway of energy production and is responsible for the reduction of pyruvate to lactate in glycolysis [72], was not altered. A. armata exudate caused an induction in the activity of the neurotoxicity biomarker AChE, mainly in an intermediate concentration. Hormesis response after exposure to several contaminants has been observed for AChE activity, mainly in the freshwater crustacean Daphnia magna,

e.g., [73], but also verified in *in vitro* testing with cell cultures [74]. This response may reveal the activation of a compensatory mechanism allowing for plasticity in response to moderate adverse environments [75]. Hormesis responses after exposure to organic compounds were also observed regarding other oxidative stress-related biomarkers in marine bivalves, such as CAT activity of *Crassostrea gigas* or GSH levels of *M. edulis* [76]. This enzyme plays a main role in regulating cholinergic neurotransmissions [77,78], therefore adverse effects on AChE may disrupt many essential nervous system functions. The final balance of the energy budget of exposed animals reflects the use of energy reserves for the antioxidant defence system and behaviour and byssus development, highlighting a higher consumption of lipids at lower concentrations of exudate.

In relation to the clearance rate, data revealed that the ability of *M. galloprovincialis* to filter was clearly affected by the individuals exposed to A. armata exudate. Immediately after 10 min, mussels from the control treatment cleared the microalgae cells more efficiently than the organisms exposed to the exudate, this difference remained during the test timeline, although the values ended up converging at 180 min. This clearance rate decline may be the result of behavioural impairment, which is in accordance with the observed changes in AChE activity. One possible explanation for these results may be related to a prolonged valve closure that bivalves usually activate as a defence mechanism resulting in a decreased feeding activity [58]. On the other hand, the bioactive compounds present in the exudate may also pose a direct effect on the gills' tissues also contributing to a filtration reduction. Similar responses on clearance rate were also reported in different bivalve species exposed to metals [58,79], pharmaceuticals [80,81], micro- and nanoplastics [82,83], but also to other natural toxins [18–20]. In fact, Comeau et al. [84] demonstrated behavioural alterations in terms of valve activity in M. galloprovincialis exposed to the dinoflagellate Alexandrinum minutum. Prasertiya et al. [85] also detected changes in Mytilus edulis valve opening in response to a pigment released by the diatom Haslea ostrearia. The present findings suggest that the presence of A. armata may impact the suspension-feeding process in M. galloprovincialis, compromising not only their individual performance, fitness and even survival, but also leading to magnified consequences on this important intertidal community. By decreasing their feeding activity, these individuals will grow less and possibly mature later, with potential consequences to the mussel population dynamics. Decreased feeding may also reflect into a lower nutritional value to their predators, including human consumers. In addition, their pivotal role as a water filterer may be compromised under exposure to A. armata exudate, as well as the critical ecosystem service they provide on water quality improvement and nutrient cycling.

Furthermore, the mechanism of byssus secretion in *M. galloprovincialis* is influenced by several endogenous and exogenous factors (e.g., related to the hydrodynamic character of the environment, available food resources and reproductive status) [86]. Byssus promotes mussels' ability to firmly anchor to a wide range of solid surfaces, including to each other [87]. Any interference with the byssogenesis can compromise mussels' attachment to the substratum, making them more susceptible to natural stressors such as predation and strong wave action onto the shores. In this sense, this study suggests that the presence of *A. armata* might increase the risk of mussels' dislodgment, making them more vulnerable to tides, waves and potential predators, since the byssal thread formation was impaired in individuals exposed to the alga exudate. Mussels produced less functional byssus which in turn had weaker attachment strength when compared to the non-exposed individuals. Consequently, *M. galloprovincialis* beds may be particularly endangered, which raises important concerns as they provide vital services such as coastline protection and complex habitat, providing food and shelter to other coastal species [88]. This impact can thus lead to potential damage to economic activities depending on this species [88].

5. Conclusions

In conclusion, antioxidant defences were induced in *M. galloprovincialis* in response to the exudate of *A. armata*, coupled with increased energy consumption, with a consequent

decline in cellular energy allocation. The exudate also impaired mussels' clearance rate and byssal thread production, compromising their feeding activity and ability to stay attached to the subtracts, which are essential for their survival. This study emphasises the problem of invasive seaweeds in the native ecosystems, namely the release of potential secondary metabolites that might be toxic to native species, leading to potential deleterious ecological and economic consequences in coastal environments.

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