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Asparagopsis armata exudate cocktail: the quest for the mechanisms of toxic action of an invasive seaweed on marine invertebrates

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Abstract

The red seaweed *Asparagopsis armata* exhibits a strong invasive behaviour and is included in the list of the "Worst invasive alien species threatening biodiversity in Europe". This seaweed has been shown to produce a large diversity of halogenated compounds with effective biological effects, deeply affecting rockpool species. Therefore, the present study aimed to investigate the biochemical responses to sublethal concentrations of *Asparagopsis armata* exudate on two coastal organisms, the marine snail *Gibbula umbilicalis* and the rockpool shrimp *Palaemon elegans*. Antioxidant defences superoxide dismutase (SOD) and glutathione-S-transferase (GST), oxidative damage endpoints lipid peroxidation (LPO) and DNA damage, the neuronal parameter acetylcholinesterase (AChE), as well as the fatty acid profile were evaluated. Results revealed different metabolic responses between species, indicating that *A. armata* exudate affected the organisms through different pathways. Despite previous studies indicating that the exudate effected *G. umbilicalis*' survival and behaviour, this does not seem to result from oxidative stress or addressed neurotoxicity. On the other hand, for *P. elegans*, an inhibition of AChE and the decrease of antioxidant capacity concomitant with the increase of LPO, suggests neurotoxicity and oxidative stress as mechanisms of exudate toxicity for this species. For fatty acids, there were different profile changes between species, also more pronounced for *P. elegans* with a general increase in PUFA with exudate exposure, which commonly means a defence mechanism protecting from membrane disruption. Nonetheless, the omega-3 PUFAs ARA and DPA were increased in both invertebrates, indicating a common mechanism regulation of inflammation and immunity responses to this stress. This work provides further insight on the mechanisms of invertebrate response and tolerance to an expanding coastal environmental stress as is the marine invader *A. armata.*

Keywords: Biomarkers, Fatty acid profile, Halogenated compounds, Oxidative stress, Red macroalgae, Secondary metabolites

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1. Introduction

More than 3800 halogenated compounds are known to exist [1], and many are known to be present in the environment, having both biogenic and anthropogenic sources. The largest source of biogenic organohalogens are seaweeds, sponges, corals, tunicates and bacteria [1]. Seaweeds produce an array of organohalogens, which exhibit important and vital ecological roles as defence compounds [2].

Asparagopsis armata, a species of the family Bonnemaisoniaceae is known to form specialized cells, known as vesicle or gland cells, which are sources of these halogenated products including halomethanes, haloalkanes, haloacids, and haloketones [3], reported to have potent biological effects to protect themselves from attacks by herbivores and pathogens [4, 5], which may ultimately influence rockpool diversity [6].

The eutrophication and the occurrence of algal blooms may result in negative ecological consequences to the aquatic ecosystem. Algal blooms of some seaweeds, such as *A. armata*, can be retained in the rockpools and release high concentrations of halogenated compounds, which can be harmful and compromise inhabiting biota [6, 7]. Limited information is available on effects of macroalgae exudated secondary metabolites in aquatic environments. However, some negative effects of these compounds on aquatic ecosystem can be found in the literature [e.g., 7, 8]. Besides suppressing the growth of other algae [6, 9], macroalgae exudates can affect the development and grazing of invertebrates [4] and even vertebrates [10].

In this study, *Gibbula umbilicalis* (da Costa 1778) and *Palaemon elegans* (Rathke 1837), abundant species on rocky shores and having wide geographical distributions [11], were used as testing model species to assess the impacts of *A. armata* on coastal communities through a biomarker mechanistic approach.

Once these invertebrates are exposed to pollutants such as *A. armata* exudate, these compounds go through biotransformation reactions, stimulating the production of reactive oxygen species (ROS) which can damage cellular macromolecules [12], in the form of lipid peroxidation (LPO) and DNA strand breaks. Key antioxidant enzymes that protect cells against ROS include superoxide dismutase (SOD), representing the primary defence against oxygen toxicity, being responsible for the transformation of $O2^{\bullet-}$ into H_2O_2 [13].

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Glutathione-S-transferase (GST) plays a role in the second phase of the detoxification process, where it facilitates the excretion of xenobiotics [14]. Environmental stressors may also promote neurotoxicity. Acetylcholinesterase, involved in the synaptic transmission of nerve impulse through the hydrolysis of neurotransmitter acetylcholine into choline and acetate, is known to be inhibited by contaminants such as pesticides [15, 16, 17].

Fatty acid profile (FAP) has also been used as a biochemical response to pollutant exposure [18, 19, 20]. Fatty acids (FAs) are amongst the main constituents of the cell membrane and are involved in a wide range of biological pathways, from the production and permeability of cell membrane to lipids main components, while also being signalling mediators and used as fuel in all metabolic systems [21].

This biomarker approach allows for a mode-of-action assessment of *Asparagopsis armata* exudates impact in organisms and eventual repercussions in higher levels of biological organization, such as population or even community levels [22].

This work aimed to evaluate biochemical responses of the common species *Palaemon elegans* and *Gibbula umbilicalis* after exposure to impactful concentrations of *Asparagopsis armata* exudate, by assessing oxidative damage (lipid peroxidation and DNA damage), antioxidant and detoxification enzymes (superoxide dismutase and glutathione S-transferase), neuronal activity (acetylcholinesterase) and fatty acid profile changes.

2. Material and methods

2.1. Test organisms

The collection of *Palaemon elegans* and *Gibbula umbilicalis* was performed in an intertidal rocky shore (Carreiro de Joannes), in Peniche, central Portugal (39°21'18.0"N, 9°23'40.6"W). Their maintenance in laboratory was carried out with temperature kept at 20 ± 1 °C, with a photoperiod of 16 h: 8 h (light:dark), and constant aeration. During this period, every two days, the organisms were fed *ad libitum* with small fragments of mussels for the shrimps and *Ulva lactuca* for snails.

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2.2. Experimental setup

Asparagopsis armata collection, preparation of exudates, and experimental design followed previous work by Silva et al. [7]. Briefly, after collected in Berlenga Island (Peniche), by SCUBA, the seaweed was cleaned and 5kg of *A. armata* was placed in aquaria containing 50L of filtered seawater during 12h in the dark at 20 °C. Then, the seaweed was removed, and the water was filtered and kept at -20ºC until further use (exudate). As in Silva et al. [7], the produced exudate constitutes the stock solution for all experiments, and experimental concentrations are presented as % of the exudate produced.

After the acclimation period (7 days), the organisms were randomly transferred to glass sampling flaks, and the concentrations of exudate used were: 0, 0.04, 0.07, 0.14, 0.25, 0.47, and 0.87% for the sea snails; and 0, 0.11, 0.21, 0.39, 0.72, 1.33, and 2.46% for shrimp [highest concentration for both ranges based on half the LC_{10}; as in Silva et al. [7]]. Media was obtained by adding corresponding exudate percentages to natural seawater (v/v). Exposures lasted for 168h and 16 and 8 replicates were used per treatment for sea snails and shrimps, respectively. At the end of the exposure period, organisms were sacrificed and kept at -80 °C until further analysis.

2.1. Biochemical analysis

2.1.1 Tissue preparation

Test organisms were sacrificed, and soft tissues were removed and dissected on ice. Pools of two snails (each pool considered as one biological replicate) were homogenized in a proportion of 1:12 (m:v) of potassium phosphate buffer (0.1 M, pH 7.4). The homogenate was then divided into 3 microtubes and kept at -80ºC until further analysis of DNA damage, FAP and lipid peroxidation (LPO). For the latter, sampling microtubes contained BHT (2.6-dieter-butyl-4-metylphenol) 4% in methanol to prevent tissue oxidation. The rest of the

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homogenate was separated into two microtubes that followed different centrifugations: 1) centrifuged for 5 min at 3000g (4 °C) for the analysis of AChE activity on the resulting supernatant stored at -80 °C; 2) centrifuged for 20 min at 10000 g (4 °C) to obtain the post-mitochondrial supernatant (PMS), which was stored at -80 °C for posterior analysis of SOD and GST activities.

For the *P. elegans* samples, the homogenate was separated by different tissues into microtubes, following different centrifugations: eyes were homogenized on 300 µL of potassium phosphate buffer (0.1 M, pH 7.4) and centrifuged for 5 min at 3000g (4 °C) for the analysis of AChE activity; hepatopancreas was also homogenized in potassium phosphate buffer in a proportion 1:10 (w:v) and divided into different microtubes for the analysis of LPO, DNA damage and FAP; the remaining homogenate was centrifuged for 20 min at 10000 g (4 °C) to obtain the PMS for posterior analysis of SOD and GST activities. All aliquots were kept at -80ºC until further analysis.

2.1.2 Antioxidant and detoxification defences

GST activity was determined by the method of Habig et al. [23] adapted to microplate, following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm for 3 min. GST activity was calculated, using a molar extinction coefficient of 9.6×10^3 M cm^{-1}, and expressed in nmol min^{-1} mg^{-1} of protein.

SOD activity was measured according to McCord & Fridovich [24], adapted to microplate, using the xanthine/xanthine oxidase mediated reduction of cytochrome C. The decrease of the cytochrome C reduction was followed at 550 nm and SOD activity was expressed in U mg^{-1} of protein using a SOD standard of 1.5 U ml^{-1}, where 1 U represents the amount of enzyme required to inhibit the rate of reduction of cytochrome c by 50%

2.1.3 Oxidative damage

The LPO levels were determined by measuring the content of thiobarbituric acid reactive substances (TBARS), following Ohkawa et al. [25] and Bird & Draper [26]. After the reaction

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with TBA (2-thiobarbituric acid), absorbance was read at 535 nm and results were expressed in nmol TBARS g^{-1} ww (wet weight), using a molar extinction coefficient of $1.56x10^5$ M cm^{-1}.

DNA damage (strand breaks) analysis was based in the DNA alkaline precipitation assay [27] with adaptations from de Lafontaine et al. [28]. After the precipitation of nucleoproteins and intact DNA, the DNA kept in the supernatant was linked with Hoesch dye (1 µg mL^{-1} bis-benzimide, Sigma-Aldrich), allowing the estimation of damage levels by fluorescence, using an excitation/emission wavelength of 360/460 nm. Results were expressed as µg/g ww of DNA using calf thymus DNA as standard to extrapolate DNA concentration.

2.1.4 Neuromuscular biomarker

The AChE activity was determined through the methodology proposed by Ellman et al. [29] adapted to microplate [30]. The absorbance was read at 414 nm for 5 min, monitoring the formation of 5-thio-2-nitrobenzoate anion (TNB), which results from the reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) with thiocholine, a product of the acetylcholine substrate hydrolysis performed by AChE. Results are expressed in nmol min^{-1} mg^{-1} of protein using a molar extinction coefficient of $13.6x10^3$ M cm^{-1}.

2.1.4 Fatty acid profile

The methodologies for FA preparation and analysis were performed according to Silva et al. [19], with minor modifications. Briefly, for the initial saponification step, 150 µL of 2M KOH (diluted in 67% ethanol; v/v) was added to 150µl of homogenate. Samples were then similarly kept at 80°C for 1 hour, cooled to room temperature, diluted with water, acidified (HCl) and FAs isolated with hexane.

To the FA fractions isolated from each sample after saponification, 1.5 mL of acetyl chloride:methanol (1:20 v/v) solution was added for the derivatization step, and samples were kept at 80ºC for 1 hour. After adding, 1 mL of Mili-Q water and 1 mL of hexane for phase separation, the organic layer was recovered to clean GC vials and solvent was

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evaporated in a vacuum concentrator (SpeedvacTM) for 10 minutes. Samples were then resuspended in 50 µL of hexane and methylated nonadecanoic acid (50 µL; 10 mg.mL^{-1}) was added as an internal standard to each sample, prior to gas chromatography analysis. Fatty acid methyl ester mixes (PUFA No1 from Marine source and PUFA No 3 from Menhaden oil) were used as external standards (Supelco, Bellefonte, Pa., U.S.A.). Operating conditions were as described by Silva et al. [19]. Theoretical correction factor (FCT) for FID detectors was applied in FA quantification, according to Guo [31].

2.2 Statistical analysis

All statistical analyses were run using R software (version 3.6.3) in combination with user interface RStudio 1.2.5033. Boxplot graphs were prepared in Graphpad Prism version 7 for Mac (GraphPad Software, San Diego, CA). Both biomarker and fatty acid data were checked for normality with Shapiro-Wilk test ("shapiro.test" function), with most variables having *p*-value<0.05 (indicative of non-normal distribution). Thus, Kruskal–Wallis one-way analysis of variance test was performed ("kruskal.test" function) to determine significant differences between exudate treatments for biomarkers and for every fatty acid detected ($p<0.05$). For significantly different variables, this was followed by a post-hoc Nemenyi-Test ("kwAllPairsNemenyiTest" function), package PMCMRplus [32], to find which specific treatments significantly differed. For the metabolic shift analysis, each significantly different fatty acids' mean values was normalized to the respective mean of the control, and a Bray-Curtis dissimilarity matrix was computed ("vegdist" function), package vegan [33], that was used in the production of heatmaps. To visualize the different levels of expression, a degree of colour was assigned where green represents the lowest amount of fatty acid, passing through black and ending in red, as a higher amount of fatty acid.

3. Results

3.1. Biochemical biomarkers

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Measurements of antioxidant defences, oxidative damage, and neuromuscular biomarkers in gastropod (*G. umbilicalis*) and shrimp (*P. elegans*) are illustrated in Figure 1 and 2, respectively. In general, *A. armata* exudate induced alterations biochemical alterations in both *G. umbilicalis* and *P. elegans* metabolism, yet with different trends of effects between species.

The antioxidant and detoxification enzymes evaluated revealed a significant decrease in GST activity in *G. umbilicalis* at 0.14 (C3; p=0.001) and 0.25% (C4; p=0.015) concentrations of *A. armata* exudate, but for SOD, no significant differences were found (Fig. 1a,b). Concerning the parameters addressing oxidative damage, a significant decrease was observed in peroxidation of lipids at lower exudate concentrations (0.04%), (C1; p=0.041) but with no effects for DNA-strand breaks being registered (Fig.1c,d). Neuromuscular parameter (AChE) had significant higher activities at 0.14 (C3; p=0.0214) and 0.47 % (C5; p=0.0246) of *A. armata* exudate (Fig.1e).

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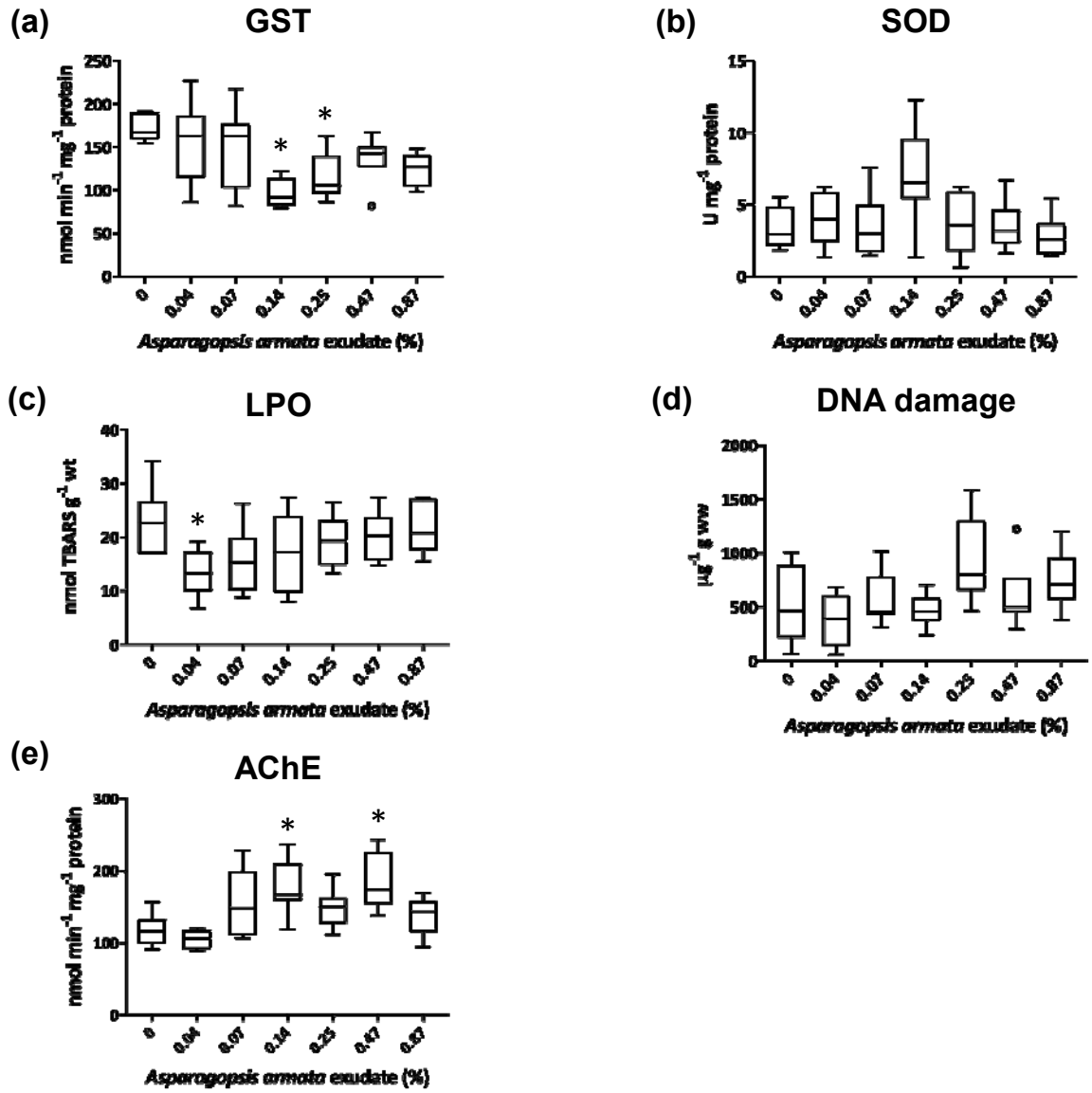

Fig. 1 - Results of detoxification and antioxidant defences (a) and (b), oxidative damage (c) and (d), and neuromuscular (e) biomarkers in *Gibbula umbilicalis* when exposed to *Asparagopsis armata* exudate for 168 h. Results are shown in boxplot (i.e. the median, the first and the third quartiles, the non-outliers range and the outliers); * Significant differences from the control (Nemenyi, $p<0.05$).

The biochemical analysis to the shrimp *P. elegans*, revealed no significant differences detected for GST, but a decrease in SOD was observed at 0.11 % (C1; $p=0.028$) of *A. armata* exudate concentration (Fig.2a,b). Regarding the oxidative damage, significant effects of the

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exudate were observed in LPO at the highest exudate concentration of exposure (C6; p=0.0018), and with a trend to increase through consecutive concentrations (Fig.2c). As observed for *G. umbilicalis*, no damage was observed in DNA-strands for *P. elegans* (Fig. 2d). Also, an inhibition of the neuromuscular parameter AChE was identified at 0.21% of *A. armata* exudate (C2; p= 0.032) (Fig.2e).

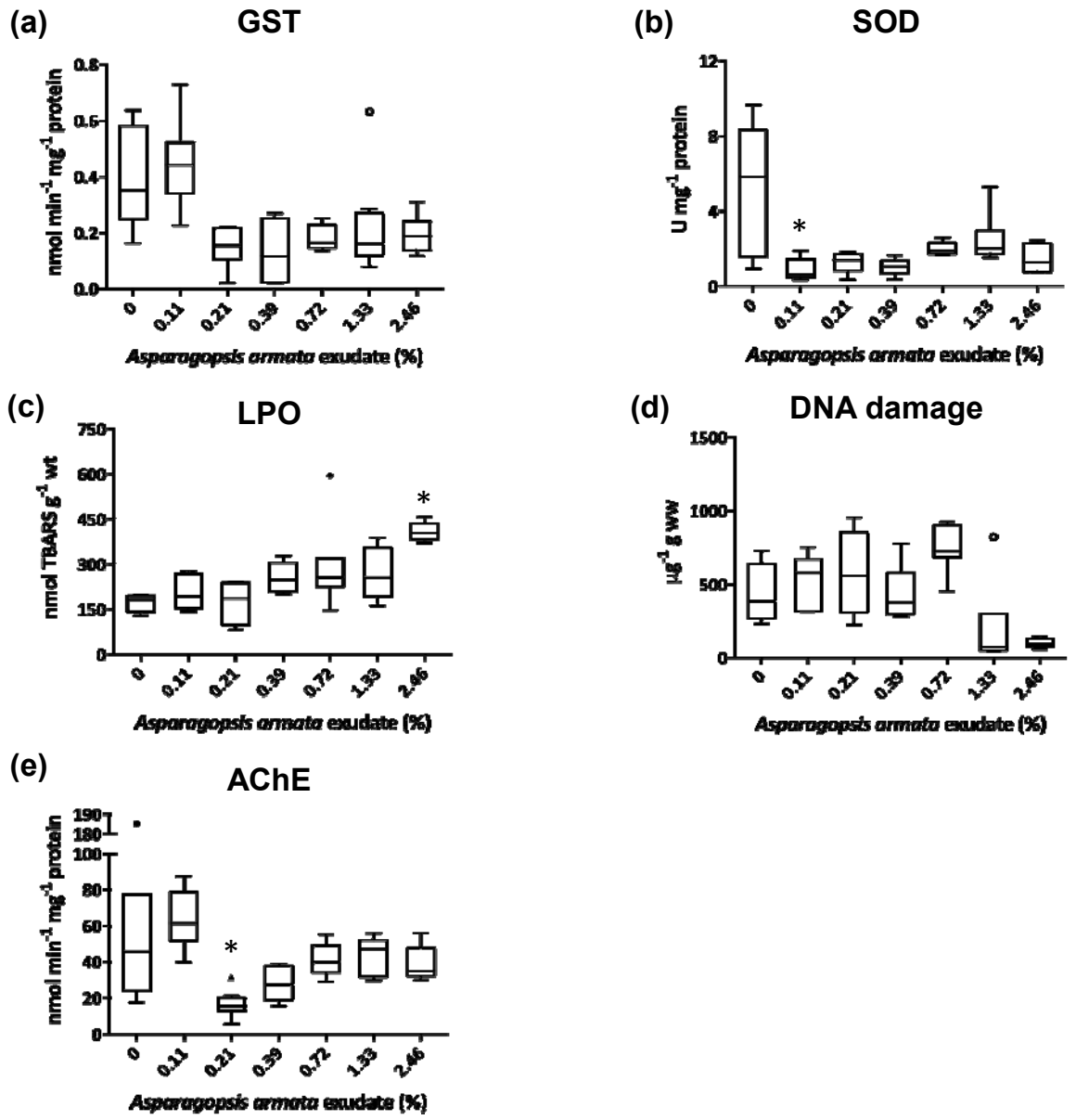

Fig. 2 - Results of detoxification and antioxidant defences (a) and (b), oxidative damage (c) and (d), and neuromuscular (e) biomarkers in *Palaemon elegans* when exposed

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to *Asparagopsis armata* exudate exudate for 168 h. Results are shown in boxplot (i.e. the median, the first and the third quartiles, the non-outliers range and the outliers); * Significant differences from the control (Nemenyi, $p<0.05$).

3.2 Fatty acid profile

Globally, the tissues of the two invertebrates presented FAs ranging from capric acid (10:0) to nervonic acid (24:1 n9), in a total of 43 different FAs detected and identified throughout this study. The complete list of fatty acids found for the different treatments and for both species tested can be consulted in Tables S1 and S2 (supplementary data). After exposure to *A. armata* exudate, both invertebrates presented significant alterations in some particular FAs. Such carboxylic acids from both invertebrates that were considered metabolically important and presenting significant differences in at least one of the treatments (17 FA in total), were selected to be further discussed and are presented in Fig.3. To streamline the overall interpretation, these FAs were clustered according to similarities in their concentration levels across the different exudate treatments.

Heatmaps depicts an overall comparison of 13 significantly different fatty acids for *G. umbilicalis* (Fig. 3A) and 10 for *P. elegans* (Fig. 3B). It should be noted that there is a generalized shift in lipid metabolism between concentration 0.07% (C2) and 0.14% (C3) for *G. umbilicalis* and between concentrations 0.39% (C3) and 0.72% (C4) for *P. elegans,* where especially for *P. elegans*, more unsaturated FAs tend to increase and more saturated FAs tend to decrease. In *G. umbilicalis*, FAs were separated by two major groups. Vaccenic (18:1 n7; $p=0.045$), adrenic (AdA; 22:4 *n*6; $p=0.01$), docosapentaenoic (DPA; 22:5 *n*3; $p=0.00$), arachidonic (ARA; 20:4 *n*6; $p=0.00$), and hexadecenoic (16:1 n5; $p=0.00$) acids comprise the first cluster. When compared to control, there is an increase in metabolic levels of these FAs generally starting from 0.14% (C3) of exudate. The opposite was observed for the second cluster, composed by behenic acid (22:0, $p=0.020$), 22:3 *n*6 ($p=0.007$), myristoleic acid (14:1; $p=0.024$), dihomo-gamma-linolenic acid (DGLA; 20:3 *n*6; $p=0.018$), eicosadienoic acid (EDA; 20:2 *n*6; $p=0.037$), heneicosapentaenoic acid (HPA; 21:5 *n*3; $p=0.032$), eicosenoic

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acid (EA; 20:1 n9; *p*=0.031), and decanoic acid (10:0; *p*=0.008). Here, there is a general decrease in metabolic levels of these FAs and especially after the 0.14% concentration (C3). Exceptionally, *n*6 DGLA and eicosadienoic acids presented an increase in C5 (0.47%; *p*=0.018 and *p*=0.037, respectively), and 22:3 n6 also increase in C3 (0.14%; *p*=0.008).

For *P. elegans,* 3 main clusters were identified, in which the first one is composed by pentadecenoic acid (15:1), remaining invariable throughout treatments, but significantly higher for the higher concentration (C6; *p*=0.002).

In the second cluster, EA (20:1 n9; *p*=0.001) and tridecanoic acid (13:0; *p*=0.000) presented higher concentrations in control and first treatments, with further depletion in higher exudate concentrated treatments. However, the third cluster, which comprises mostly polyunsaturated FAs: DPA (22:5 *n*3; *p*=0.004), docosadienoic acid (22:2 *n*6; *p*=0.008), HPA (21:5 *n*3; *p*=0.003), 22:3 *n*6 (*p*=0.004), alpha-linoleic acid (ALA; 18:3 *n*3; *p*=0.022), ARA (20:4 *n*6; p=0.034), and decanoic acid (10:0; *p*=0.006), demonstrate an inverse behaviour to the previous cluster, by overall increased FA quantities with increasing concentrations of the exudate (from 0.72%, C4).

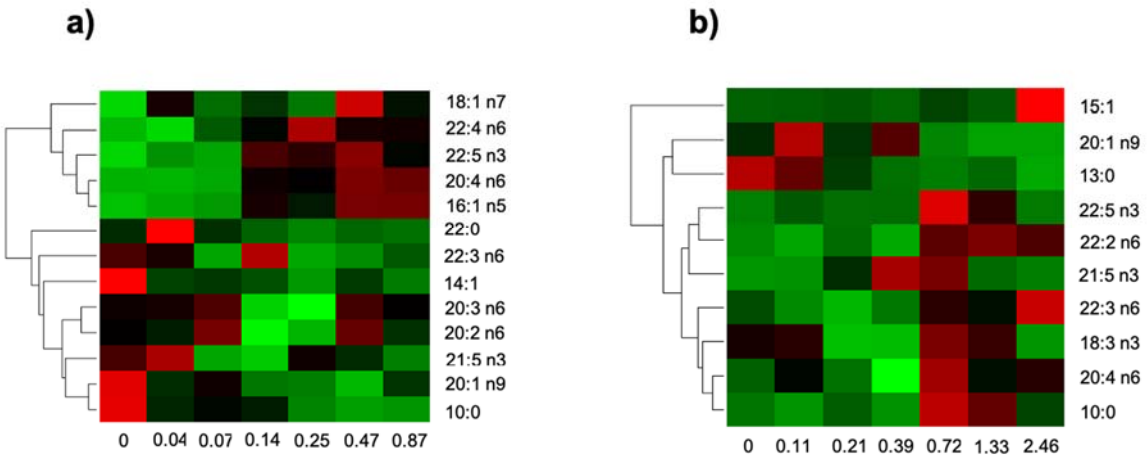

Fig. 3 - Heatmap depicts an overall comparison of the significantly different fatty acids in *Gibbula umbilicalis* (**A**) and *Palaemon elegans* (**B**) based on their sensitivity against different concentrations of *Asparagopsis armata* exudate. Rows are clustered using correlation distance and colour scaling was performed by row.

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4. Discussion

The present study was designed to further address potential mechanisms of action involved in the previously observed toxic effects on two coastal invertebrate species, the marine snail *Gibbula umbilicalis* and the shrimp *Palaemon elegans*, exposed to the exudate of *A. armata* [7]. This was performed by evaluating parameters including neuronal, detoxification and oxidative stress biochemical biomarkers and fatty acid composition. In the present study, *A. armata* exudate induced shifts in the studied endpoints, in both snails and shrimps, yet with different responses between species. The dissimilarities between biomarkers from both species suggest different metabolic processes being affected, the same way previous study [7] indicated different species' sensitivity and energetic impacts. Red algae are a rich source of halogens, predominantly bromine and iodine [8]. In the case of *A. armata*, the major natural products known are numerous halogenated metabolites which possess a wide range of volatility and solubility [3]. The overall toxicity of halogen-containing compounds seems to be derived from their abilities as alkylating agents [3] or by inducing ROS production [34]. Also, the alkylating agents, such as haloacetones found in *A. armata*, are well-known enzyme inhibitors, able of cross-linking serine and histidine residues in various proteins [3].

Here, the activity of the enzyme GST on the marine snail was in fact significantly decreased in mid exudate concentrations. An inhibition of GST is often found as result of an increased level of produced ROS, which among other damage also inhibits enzymes, and has previously been reported for other marine snails [e.g., 35]. In the present case, this inhibition may also be due to the aforementioned direct action of the exudate compounds on the enzyme [3], while some allelochemicals of plants are also known to act as GST inhibitors [36]. Owing their toxicity to a myriad of secondary metabolites, including more than 100 halogenated compounds [3], exudate dilutions representing a cocktail of different compounds, present in different concentrations, are due to trigger differentiated mechanisms of action and thus non-monotonic dose-responses [7], a factor that increases the need for a less straightforward analysis of organism responses along concentrations.

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Regarding oxidative stress parameters, for *G. umbilicalis*, the non-observed effects in antioxidant defence enzymes are concomitant with the non-observed damage in both DNA and lipids. In fact, LPO levels decreased with the snail exposure to lower concentrations of the exudate. The rationale for this decrease, also seen in a vast number of other studies [e.g., 37, 38], is yet not clear, and entails further research and careful analysis.

Regarding the neuronal parameter, AChE is involved in the regulation of the transmission of nerve impulses, and contaminants such as chlorpyrifos has been demonstrated to inhibit AChE activity in *G. umbilicalis* [17]. However, in this work, a significant induction of AChE was observed in *G. umbilicalis* exposed to 0.14 and 0.47% exudate concentrations. Reddy et al. [39] also found an increase in the enzyme activity on crab *Barytelphusa guerini* after 4d of exposure to fluoride, a halogenated compound. The induction of AChE activity after macroalgae exudate exposure could be due to a phenomenon of overcompensation as proposed by Badiou et al. [40].

Regarding the shrimp *P. elegans*, and similar to *G. umbilicalis*, an over-production of ROS [40] and/or exudate compounds' enzyme inhibitory action [3] might have resulted in the seen inhibition of SOD activity and the trend for a decreased level of GST. Further on, this inhibition might have led to the accumulation of ROS, which in turn led to an increase of LPO. These results are also in agreement with the study of Box et al. [42] where the invasive red macroalgae *Lophocladia lallemandii* induced the increase of MDA levels generated by lipid peroxidation, to the bivalve *Pinna nobilis*). Notwithstanding, and although alkylating agents are present in some *A. armata* extracts and have been proved to possess genotoxic properties [3], damage on DNA was not found in the present study.

In *P. elegans*, AChE was inhibited at 0.21% of exudate exposure. In literature, methanolic extracts from *Sargassum* sp. and *Gracilaria gracilis* showed to inhibit the fish *Nile tilapia* ChEs [43]. Moreover, Custódio et al. [44] has shown that extracts of *A. armata* had potent inhibitory capacity on AChE (58.4% at 10 mg mL^{-1}) of human cells. Typically, this enzyme inhibition is known as an early sign of behavioural impairments. Although no effects were seen for feeding activity after the same exudate exposure for 96h [7], this enzyme inhibition may disclose potential higher-level behavioural effects in longer exposures, not addressed in the present study.

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Additionally to the different species sensitivity and differentiated mechanisms of toxic response, one has to note that the choice of sub-lethal concentrations were made considering the survival effects (half the LC_{10} as top concentration) [7]. This implied having an appreciably higher concentration range for the shrimp in this study, which may explain the more explicit oxidative and neurotoxic effects here seen.

Differentiated fatty acids profiles shifts due to exudate exposure were also observed between species. The obtained results demonstrated 13 and 10 differentiating metabolites in *G. umbilicalis* and *P. elegans*, respectively, indicating *A. armata* exudate altered fatty acid biosynthesis and metabolism in the organisms, although through a different way. Many studies have shown that pollution can change the composition of FAs from organisms in the aquatic environment [20, 45, 46, 47].

In a broad observation, and especially for *P.elegans*, an increase in PUFA could be observed, while saturated and monounsaturated FAs tended to decrease. The increase in PUFA content can be considered a defence mechanism, protecting the membranes from oxidation disruption [45]. For *G. umbilicalis*, an increase in the first cluster including ARA and DPA can be seen. Further, in the literature, an increase of these two PUFA was also found for the bivalve *Mizuhopecren yessoensis* after being exposed to cadmium [48]. It is known that 22:5 *n*3 (DPA) can be retro-converted to eicosapentaenoic acid (EPA; 20:5 *n*3) and that it reacts with lipoxygenases to form distinctive oxylipins, such as the specialized pro-resolving mediators involved in the resolution of inflammation [49]. Also, omega-3 PUFAs like DPA serve as precursors of eicosanoids (prostaglandins, thromboxanes, leukotrienes, etc.), which have a wide range of physiological functions in immune system, inflammatory response, neural function, reproduction, and improve the organisms' adaptation to environmental stress [50]. The increase of ARA (20:4 n6) in both invertebrates is an indication that this FA was possibly required for activation of eicosanoid synthesis for the regulation of inflammation and immunity responses, triggered by the exudate [51]. This increase maybe an adaptive response of treated organisms to face the detrimental effects. This fact is in accordance with a study of Silva et al. [19], where levels of ARA in *G. umbilicalis* were observed to increase after metal exposure. Additionally, DGLA (20:3 n6) and EDA (20:2 n6), although clustered in a different group, presented an increase,

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particularly at 0.47% (C5). These particular FAs are also involved in eicosanoid synthesis, and DGLA is also desaturated to form ARA, thus explaining their increase.

In *P. elegans*, a metabolic shift between the third and fourth treatments was observed in terms of FA profiling, where a general increase in PUFA was denoted in 0.74% (C4) of *A. armata* exudate. This may be attributed to an inflammatory response due to the increase of *n*3 and *n*6 FAs, as previously reported by Simopoulos [52], and more evident for the shrimp than for the snail. These class of PUFA serve as potent anti-inflammatory and immunomodulatory agents. HPA (21:5 *n*3) is a stronger inhibitor of the conversion of α-linoleic acid and dihomo-γ-linolenic acid to ARA and inactivates prostaglandin H synthase as rapidly as do ARA [53].

Although there is a trend to an increase in PUFA with increasing exudate concentrations in *P. elegans*, in the higher concentration (2.46%, C6) there is a decrease of *n*3 PUFA. This may be due to the loss of ability of *P. elegans* to cope with the stressor at higher concentrations, which might have led to the observed increase in lipid peroxidation levels. However, and most likely, the pro-oxidant effect of exudate altered membrane integrity and fluidity in the shrimp membrane cells, due to decreased adaptation and activity of the membrane-bound enzymes and pumps that block the membrane permeability. At the same concentration, despite the overall PUFA reduction, *n*6 and short chain FAs increased. This event is suggestive of a post-inflammatory response [54]. This behaviour, where *n*-3 PUFA decrease and *n*-6 PUFA increase, was also previously reported in literature for *Venus verrucosa*, exposed to lead [55], and for *Mytilus edulis* under cadmium and copper exposure [45].

Comparing the responses in the two species we can highlight the fatty acids DPA and ARA with more similar responses, where there was an evident increase with the exposure. Overall, *A. armata* exudate exposure had an influence on membrane functioning by means of disturbing the membrane of the organisms, which could be significantly injurious to membrane-bound enzymes.

2. Conclusion

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In the present study, potential neurotoxicity, oxidative stress mechanisms of action and fatty acid profile changes, behind the seen *A. armata* exudate impacts, were addressed in two rockpool invertebrates. In *P. elegans*, oxidative stress and neurotoxic effects were found as potential higher levels of biological toxicity drives, while for *G. umbilicalis* these routes do not seem to relate to seen impacts (i.e. feeding behaviour).

Fatty acids also revealed different metabolic responses between species, indicating that *A. armata* exudate may in fact be affecting the organisms through different pathways, despite the increase of PUFAs ARA and DPA, in both invertebrates, which points towards common inflammatory and immunity regulation response.

Given the paramount rate of expansion of this invasive species across aquatic environments, where different species are present, a greater body of research is necessary to investigate how *A. armata*, and even other potentially harmful algae blooms, impact rockpools and other environments and even how other global change drivers may act and even enhance this problem.

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