

Impacts of the invasive seaweed *Asparagopsis armata* exudate on rockpool invertebrates

Carla O. Silva^{1,*}, Sara C. Novais¹, Amadeu M.V.M. Soares², Carlos Barata³, Marco F.L. Lemos^{1,*}

¹ MARE – Marine and Environmental Sciences Centre, ESTM, Instituto Politécnico de Leiria, 2520-641 Peniche, Portugal

² Department of Biology and CESAM (Centre for Environmental and Marine Studies), University of Aveiro, Aveiro, Portugal

³ Environmental Chemistry Department, IDAEA-CSIC, Jordi Girona 18-26, 08034, Barcelona, Spain

*Authors to whom correspondence should be addressed: Carla Silva or Marco Lemos; Edifício CETEMARES, Avenida do Porto de Pesca, 2520 – 630 Peniche, Portugal; Phone: +351 262 783 607. FAX: +351 262 783 088. E-mail: carla.o.silva@ipleiria.pt or marco.lemos@ipleiria.pt

Abstract

The marine red algae *Asparagopsis armata* is an invasive species which competitive advantage arises from the production and release of large amounts of toxic compounds to the surrounding invaded area, reducing the abundance of native species. The main objective of this study was to evaluate the effects of this invasive seaweed on marine invertebrates by exposing the common prawn *Palaemon elegans* and the marine snail *Gibbula umbilicalis* to the exudate of this macroalgae. The seaweed was collected and placed in tanks, for 12 hours, in the dark in a 1:10 ratio. Afterwards the media containing its secondary metabolites was collected for further testing. Lethal and sublethal effects of *A. armata* were investigated. Biochemical biomarkers responses associated with energy metabolism (lactate dehydrogenase, LDH; electron transport system activity, ETS; content in lipids, proteins and carbohydrates) were analysed. The biomarker responses showed invertebrates' physiological status impairment after exposure to low concentrations of this algae exudate. Highest concentrations of exudate significantly increased lipid content in both organisms. In the shrimp, protein content, ETS, and LDH were also significantly increased. On the contrary, these parameters were significantly decreased in *G. umbilicalis*. A behavioural impairment was also observed in *G. umbilicalis* exposed to *A. armata* exudate, with reduction in feeding consumption. These results represent an important step in the research of natural toxic exudates released to the environment and prospective effects of this seaweed in invaded communities under increasing global change scenarios.

Keywords: Behaviour, Ecotoxicology, Invasive species, *Gibbula umbilicalis*, *Palaemon elegans*; Tidal pools

1. Introduction

Overall rapid globalization, and increasing trends of trade and travel, have been accelerating marine biological invasions, by transporting species to areas outside their native range. These non-indigenous species (NIS) may then be considered invasive, once they establish, spread rapidly, and proliferate and dominate the new habitat without the direct support of humans (Richardson et al., 2011). *Asparagopsis armata* Harvey, 1855 (Bonnemaisoniales, Rhodophyta) is a red seaweed, native to Western Australia, and nowadays distributed throughout Europe in the Atlantic and Mediterranean basin, where it is highly invasive (Otero et al., 2013). This seaweed possesses chemical defence mechanisms that are nuclear to their invasiveness, based on the synthesis and storage of an array of secondary metabolites, which include over 100 halogenated compounds such as haloforms, haloacids, and haloketones (O. McConnell & Fenical, 1977). These halogenated volatile hydrocarbons containing one to four carbons are known antifeedant and cytotoxic compounds, among others (reviewed in Pinteus et al., 2018), and the pungent aroma of these algae is attributed to an essential oil that is composed mainly of bromoform with smaller amounts of other bromine, chlorine, and iodine-containing methane, ethane, ethanol, acetaldehydes, acetones, 2-acetoxypropanes, propenes, epoxypropanes, acroleins, and butenones, stored in vacuoles within gland cells (Burreson et al., 1976). These compounds potent biological effects can induce significant changes in terms of native community composition (Paul et al., 2006a) favoring *A. armata* in a given niche. Due to the enclosed environment during low-tide, rocky pools are ought to be sensitive sites to the increase of released compounds by retained *A. armata*, which may ultimately present adverse effects for other organisms such as seaweed, vertebrates or invertebrates, leading to severe consequences for coastal ecosystems. To investigate potential ecological impairments caused by these compounds, key role species in the structure and functioning of coastal ecosystems were used: marine invertebrates such as the common prawn *Palaemon elegans* and the gastropod *Gibbula umbilicalis*, which inhabit the upper intertidal zone on rocky shores where *A. armata* is often found attached to the substrate, or unattached (drifting), and therefore releasing its chemical exudates. In this

work, the assessment of *A. armata* exudate effects over these marine invertebrates was performed addressing survival and sublethal effects through behavioural and biochemical responses.

Several behavioural parameters have been chosen as indicators of invertebrate health status. Feeding activity have been shown to be sensitive tools to assess the impact of contaminants at concentrations far below lethal levels, which often is related to a decrease of fitness and capacity to respond to contaminants and also often reflects alterations in movement capability, and that will reduce energetic input and metabolism (Cabecinhas et al., 2015; C. O. Silva et al., 2019; Dell’Omo, 2002; Fong & Molnar, 2013). Enzymes involved in energy production have been frequently used as biomarkers to assess the effects of stressors, since exposed organisms usually need additional energy to maintain physiological/biochemical functions (Aderemi et al., 2018; Kühnhold et al., 2017; Silva et al., 2016). Thus, biomarkers such as total content in energy reserves, Lactate Dehydrogenase (LDH) and Electron Transport System (ETS) activities may provide valuable information on the physiological status of the studied organisms. Therefore, the purpose of this research was to address the effects of *A. armata* exudated secondary metabolites on the survival, behaviour, and energetic metabolism of two marine invertebrates inhabiting rock pools, giving further ecotoxicological insight on this seaweed invasive strategy.

2. Material and methods

2.1. Test organisms

The common prawn *Palaemon elegans* and the sea snail *Gibbula umbilicalis* were collected from Carreiro de Joannes, a rocky beach in Peniche, central Portugal (39°21'18.0"N, 9°23'40.6"W), with no known sources of chemical contamination. The organisms were maintained during 7 d in the laboratory in natural seawater at 20 ± 1 °C, with a 16 h:8 h (light:dark) photoperiod in aerated aquaria. Shrimps were fed with mussel and snails fed

with *Ulva lactuca* until used in experiments. Prior to testing, organisms were kept fasting for 24 h.

2.2 *Asparagopsis armata* collection and preparation of exudates

Asparagopsis armata was collected in Berlenga Island, Peniche, Portugal (39°25'03.0"N, 9°30'23.6"W), a marine protected area, by SCUBA. In the lab, after cleaned and sorted, four aquaria with 5kg of *A. armata* and 50L of natural filtered seawater (through 0.45 µm cellulose acetate membrane filters) were left in the dark at 20 °C. After 12h, the algae was removed, the water from the different aquaria was pooled and sieved for bigger particles, followed by a filtration through a 0.45 µm cellulose acetate membrane filter. This exudate was then kept in PET bottles at -20°C until further use. This, due to the obvious composition constrains, constitutes the stock solution for all experiments and the 100% concentration.

2.3 Exposure setup

All experiments were conducted in a climatic room at 20 ± 1 °C, with a 16 h:8 h (light:dark), and experimental replicates consisted of glass vials with 60 mL and 750 mL exudate solution (or seawater in controls) for snails and shrimps, respectively, with one organism each. Flasks were covered with a plastic mesh to prevent organism to escape and to assure constant submersion. Exudate solutions were renewed every 24h to avoid excreta accumulation and possible loss of volatile compounds. Exudate concentrations are presented as % of the exudate produced as described in 2.2.

2.3.1 Survival

After a range finding test, sea snails were exposed to increasing concentrations ranging from 1 to 15 % of exudate (1; 1.57; 2.47; 3.87; 6.08; 9.55; and 15%), and shrimp from 4 to 10% of exudate (4; 4.66; 5.43; 6.32; 7.37; 8.58; and 10%). Exposures lasted 96 h and mortality was recorded daily. During exposures, no food was added. Eight and five replicates per treatment were used for sea snails and shrimps respectively, including a control treatment with filtered seawater only.

2.3.2 Sublethal exposure for biomarker analysis

Information on the lethal effects was used to establish maximum concentrations and conditions for each independent sublethal test, using half the LC₁₀ as the highest concentrations tested. Sea snails were exposed to increasing concentrations of exudate ranging from 0.04 to 0.87 % (0; 0.04; 0.07; 0.14; 0.25; 0.47; and 0.87%), and shrimp were exposed from 0.11 to 2.46 % of exudate (0; 0.11; 0.21; 0.39; 0.72; 1.33; and 2.46%). Exposures lasted 168 h and 16 replicates per treatment were used for snails and 8 for shrimps, including a control treatment with filtered seawater only. At the end of the exposure period, snail's shell was broken with a vise, and soft tissues removed with forceps, weighed and kept on ice for operculum removal. Shrimps were sacrificed by decapitation and dissected. The abdominal muscle was rapidly isolated on ice and weighed. Tissues were maintained at -80 °C until further analysis.

2.3.3 Sublethal exposure for feeding inhibition testing

Feeding activity

For the feeding activity assay, concentrations of the exudate were the same as used for the biomarkers exposure, using 8 replicates per treatment for both invertebrates and exposed for 96h. Organisms were fed with discs of *Ulva lactuca* with c.a. 10 cm² previously dried at 60°C for 48 h, weighed and re-hydrated just before adding to the medium (one disc per replicate). At the end of the 96 h exudate-exposed feeding test, the discs were rinsed in clean water, dried again, weighed, and the feeding was assessed by subtracting the algae final weight to its initial dry mass (mg).

2.4 Biomarkers analysis

2.4.1 Tissue preparation

Snails were processed as pools of two individually exposed organisms, with each pool being considered as one biological replicate for the biomarker analysis (N=8). For shrimps, the muscle tissue of each organism was processed individually and considered as one biological replicate (N=8). The replicate tissues of each invertebrate species were homogenized in potassium phosphate buffer (0.1 M, pH 7.4) at a proportion of 1:12 for *G. umbilicalis* and 1:10 (m:v) for *P. elegans*. The homogenate was then separated into different microtubes for the analysis of total protein, carbohydrate, and lipid content. The remaining homogenate was separated into two fractions centrifuged respectively at 1000 g for 5min (4 °C) for ETS measurement and at 3000g for 5 min (4 °C) for LDH measurement. All aliquots were stored at -80 °C until further analysis.

2.4.2 Energy reserves

Carbohydrate, lipid, and total protein contents were measured according to the approaches outlined by De Coen and Janssen (1997, 2003). The total carbohydrate content was determined in a reaction with phenol 5% and H₂SO₄ (95–97%), using glucose as standard and measuring absorbance at 490 nm (De Coen & Janssen, 1997). Lipid content was determined according to Bligh and Dyer (1959), using tripalmitin as standard and measuring absorbance at 400 nm. The total protein content was determined using the Bradford method (1976), with bovine serum albumin as standard, measuring absorbance at 600 nm. Following De Coen and Janssen (1997, 2003), all energy reserves were transformed into their energetic equivalents (39.5 kJ g⁻¹ lipid, 24 kJ g⁻¹ protein, 17.5 kJ g⁻¹ glycogen).

2.4.3 Energy metabolism related enzymes

Electron transport system (ETS) activity was determined following the method described by De Coen & Janssen (1997). The ETS activity was measured spectrophotometrically by adding NADPH solution and INT (*p* iodo-nitro-tetrazolium) to the sample and absorbance was read at 490 for 3 minutes. The oxygen consumption was then calculated using the

stoichiometric relationship: 2 μmol of formazan formed = 1 μmol of oxygen consumed. The oxygen consumption rate was then converted into the energetic equivalent of 484kJ/mol O_2 for average carbohydrate, lipid, and protein consumption combinations (Gnaiger, 1983). The activity of LDH was measured following Vassault (1983) with adaptations of Diamantino et al. (2001). The process is based on the efficiency of LDH to convert pyruvate to lactate, in the presence of NADH, which results in NADH oxidation and consequent decrease in absorbance. The absorbance was read at 340 nm for 5 min. A molar extinction coefficient of $6.3 \times 10^3 \text{ M cm}^{-1}$ was used, and results were expressed as $\text{nmol min}^{-1} \text{ mg protein}^{-1}$.

2.5 Statistical analysis

Significant differences between each treatment for biomarker analyses and behavioural parameters were studied using one-way analysis of variance (ANOVA) and differences to control were addressed by Dunnett's post hoc test. Normality was checked by Shapiro-Wilk test and homoscedasticity by Levene test. In case of non-normally distributed data, the Kruskal–Wallis test was applied followed by Dunn's post hoc test. Statistical analyses for biochemical and behaviour endpoints were performed with the software SigmaPlot (Systat Software, San Jose, CA) and LCs and the correspondent 95% confidence intervals and global fitting were done on GraphPad Prism version 7 for Mac (GraphPad software, San Diego, CA).

3. Results

3.1 Survival

Acute toxicity tests revealed that *A. armata* exudate affects both species, with *P. elegans* being more tolerant than *G. umbilicalis* with significantly higher LC_{50} ($F_{2,100}=53.03$, $p<0.001$)(Fig.1). *Gibbula umbilicalis* has a 96 h LC_{50} [95% CI] of 2.79% [1.66-4.69] and *P. elegans* a 96 h LC_{50} [95% CI] of 5.04% [4.84-5.25].

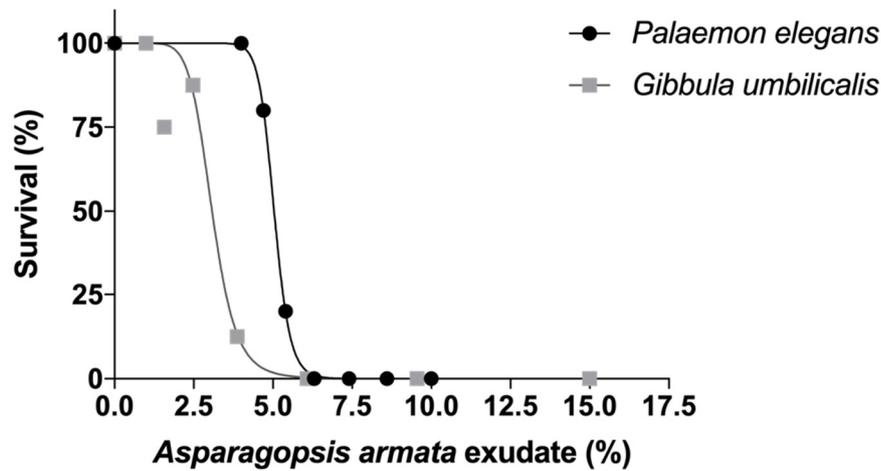


Figure 1 - Survival rate of two marine invertebrates after 96 h exposure to *Asparagopsis armata* exudate. Black circle: *Palaemon elegans*; Gray square: *Gibbula umbilicalis*.

3.2 Behavioural responses - Feeding activity

Feeding activity was affected in *G. umbilicalis* ($F_{6,49}=5.304$, $p<0.001$; Fig. 2A) when exposed to *A. armata* exudate at 0.07% (Dunnett's $p=0.022$), 0.47% (Dunnett's $p<0.001$), and 0.87% (Dunnett's $p<0.001$). No significant differences were found in *P. elegans* feeding activity ($F_{6,36}=0.178$, $p=0.981$; Fig. 2B).

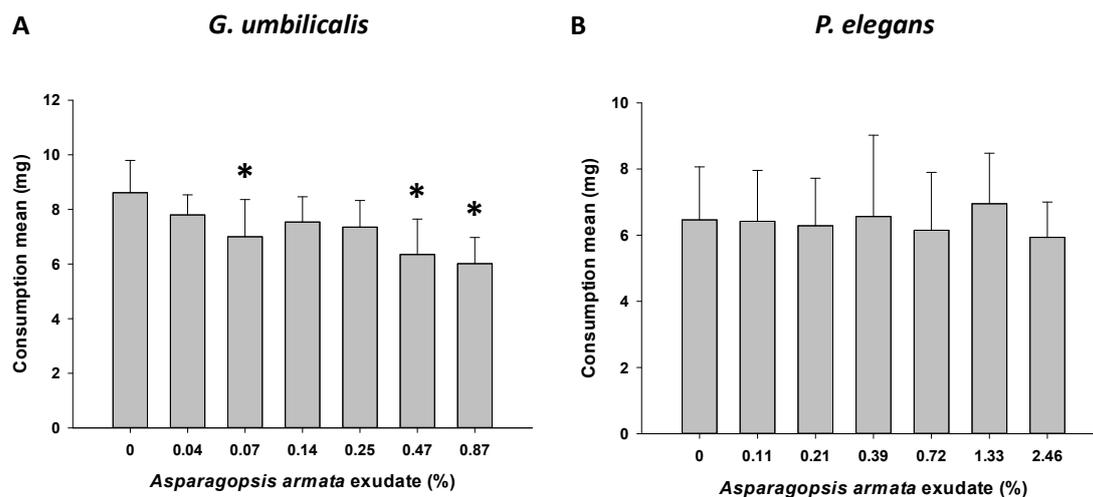


Figure 2 – Feeding activity behaviour of *Gibbula umbilicalis* (A) and *Palaemon elegans* (B) exposed to *Asparagopsis armata* exudate for 96 h. Results are expressed as mean values \pm SE; Asterisks indicate significant differences to the control treatment (0%).

3.3 Energy metabolism related biomarkers

Regarding *G. umbilicalis* exposure to *A. armata* exudate, no significant differences were observed in the carbohydrate and protein contents (Fig. 4a,c). However, there was an increase in accumulation of reserve lipids ($F_{6,47}=8.099$; $p<0.001$; Fig.4b) in 0.07% (Dunnett's $p=0.031$) and 0.87% (Dunnett's $p<0.001$) exudate exposures. ETS activity showed a significant decrease at 0.14% treatment ($H_6=19.784$, Dunn's $p=0.003$; Figure 4d) while LDH was also significantly inhibited ($F_{6,44}=4.041$, $p=0.003$; Fig. 4e) at 0.14% (Dunnett's $p=0.002$) and 0.87% (Dunnett's $p=0.022$).

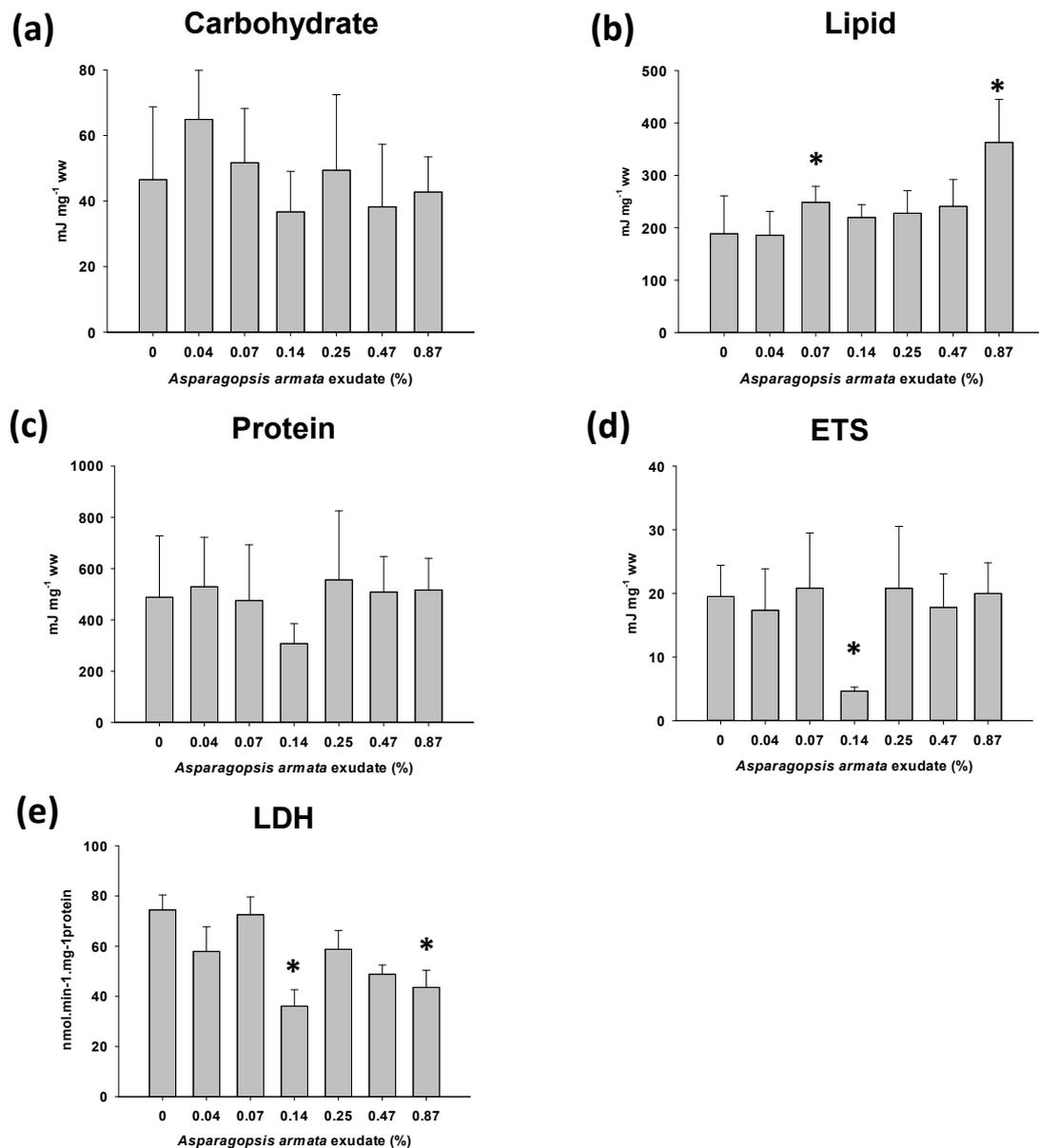


Figure 4 - Energy related parameters in *Gibbula umbilicalis* when exposed to *Asparagopsis armata* exudate for 168 h: (a) total carbohydrate content; (b) total lipid content; (c) total protein content; (d) electron transport system (ETS) activity; (e) lactate dehydrogenase (LDH) activity. Results are expressed as mean values \pm SE; * Significant differences from the control (Dunnett's or Dunn's, $p < 0.05$).

As for the energy reserves measured in the muscle tissue of *P. elegans*, no effects were observed for carbohydrates ($H_6=9.431$, $p=0.151$) after exudate exposure (Fig. 5a) but there

was an increase in total lipids ($F_{6,44}=5.580$, $p<0.001$; Fig. 5b) at the highest tested concentration (Dunnett's $p=0.020$). Mean protein levels were also significantly increased at all concentrations ($F_{(6,38)}=32.667$, $p<0.001$; Fig. 5c) except at the lowest 0.11% (Dunnett's $p=0.272$). ETS followed the same pattern as protein accumulation, with an increase in activity for concentrations equal or higher than 0.21% of exudate ($F_{6,45}=5.757$, $p<0.001$; Fig. 5d). LDH, on the other hand, only showed an increment in activity ($F_{6,46}=3.106$, $p=0.012$; Fig. 5e) at the intermediary concentrations 0.21% (Dunnett's $p=0.009$) and 0.39% (Dunnett's $p=0.014$) and 0.72% (Dunnett's $p=0.036$).

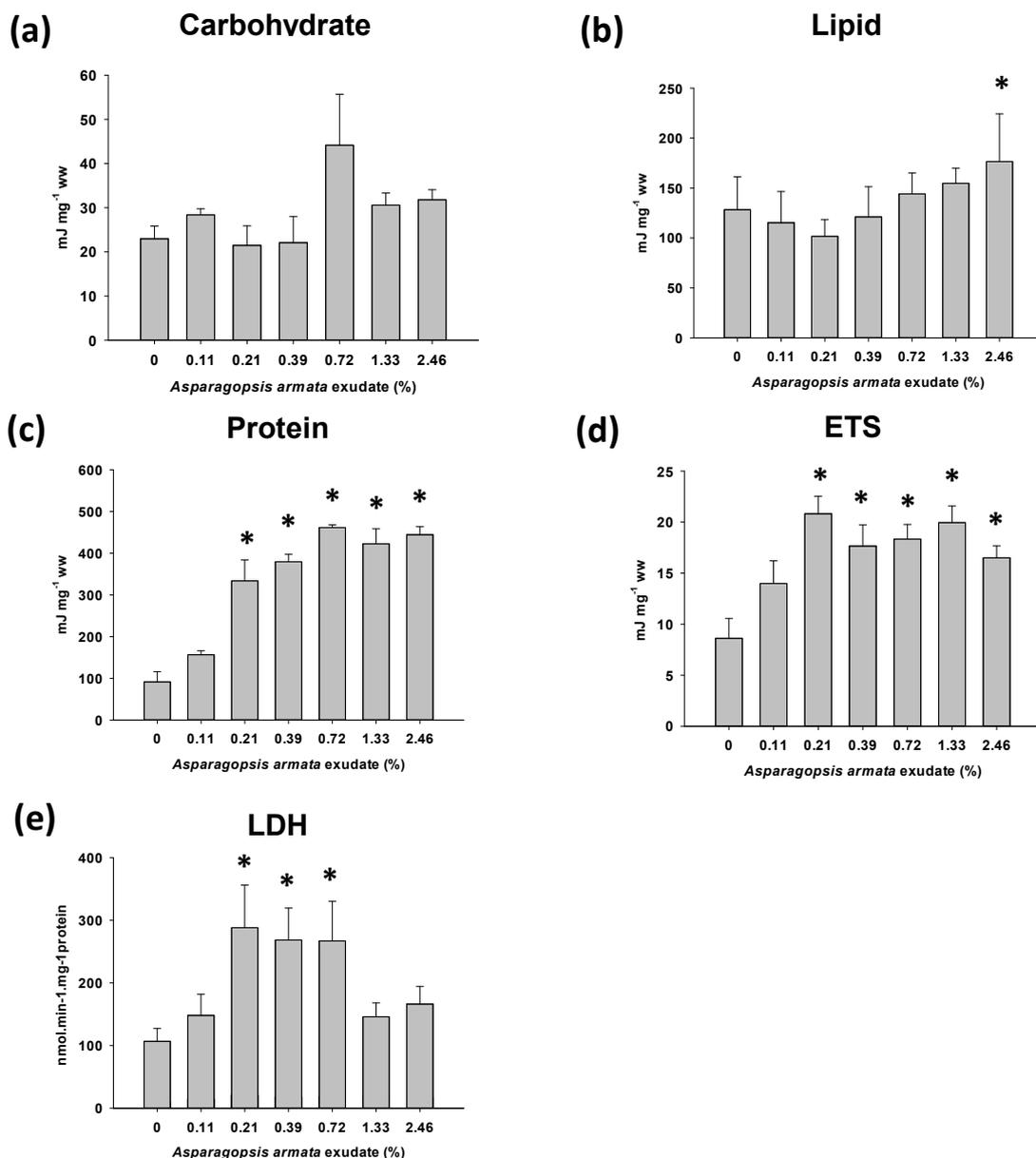


Figure 5 - Energy related parameters in *Palaemon elegans* when exposed to *Asparagopsis armata* exudate for 168 h: (a) total carbohydrate content; (b) total lipid content; (c) total protein content; (d) electron transport system (ETS) activity; (e) lactate dehydrogenase (LDH) activity. Results are expressed as mean values \pm SE; * Significant differences from the control (Dunnett's or Dunn's, $p < 0.05$).

4. Discussion and conclusions

There are some studies addressing the toxicity of seaweeds and its detrimental effects to invertebrates, but very few address toxicity of seaweed secondary metabolites, such as in the study of the effects of *Ulva* sp. exudate on the gastropods *Littorina littorea* and *L. obtusata* (Peckol & Putnam, 2017). The present study shows the potential of *A. armata* exudates to affect marine invertebrates such as *G. umbilicalis* and *P. elegans*. The acute tests demonstrated that the exudate could induce mortalities at very high dilution from the initial seaweed exudate, with a 96 h LC₅₀ of 2.93% for *G. umbilicalis* and of 5.05 % for *P. elegans*. The reasons for such low *A. armata* exudate tolerance in both invertebrates are of some concern due to the well documented importance of these species to the functioning of rocky shore communities as principal microalgal consumers of seagrass biofilm (Orth & Van Montfrans 1984).

The comparison between 96 h dose-response curves revealed that *P. elegans* were more tolerant than *G. umbilicalis* with significantly higher LC₅₀. In fact, the high sensitivity of *G. umbilicalis* to this exudate is less obvious, given the well-documented tolerance of this species to extreme environmental conditions (Southward, 1958). Additionally, *G. umbilicalis* have been found to be relatively tolerant to other contaminants (organophosphate pesticides and metals) (Cabecinhas et al., 2015; Silva et al., 2017; Silva et al., 2019).

Alteration of normal behavioural patterns, such as feeding, caused by exposure to contaminants may pose serious risks to the success of the community species that co-

inhabit in intertidal rockpools, like shrimps and snails such as the ones tested here. Here, feeding activity was impaired by the seaweed exudate at sublethal concentrations, which agrees with the literature showing that behavioural endpoints are sensitive tools to evaluate sub-lethal effects of contaminants (Amiard-Triquet et al., 2012). In the literature, there are few examples of feeding inhibition by macroalgae compounds. Sea urchin (*Lytechinus variegatus*) feeding was inhibited with caulerpenyne, an oxygenated sesquiterpene extracted from *Caulerpa prolifera*, and cymopol, a monoterpene-bromohydroquinone from *Cymopolia barbata*, both green invasive macroalgae (McConnell et al., 1982). Other studies evidenced that halogenated monoterpenes isolated from the red algae *Plocamium lepitophyllum* inhibit food consumption by sea-urchin and gastropods (Sakata et al., 1991) and *A. armata* dichloromethane extracts have also revealed feeding deterrence (Paul et al., 2006b). Feeding is generally one of the first responses to environmental perturbations and its inhibition can cause a reduction in an organism's energy assimilation resulting in a reduction in resource allocation to growth, reproduction, and survival (McLoughlin et al., 2000; Sokolova et al., 2012). In this work, the exudate derived from *A. armata* deterred the feeding of the marine gastropod *G. umbilicalis*, which is potentially due to the chemical defence characteristics of *A. armata*, documented in the literature as possessing compounds, mostly halogenated, with the primary function to deter herbivory (Borell et al., 2004; Paul et al., 2006b). Often, feeding inhibition derives from movement reduction due to toxic exposure, and consequent less capacity to find food and to process it (Cabecinhas et al., 2015). In sum, behavioural impairments, in general, reveal disturbances that may be associated with differences in energy uptake and allocation, which may have important ecological consequences (Lemos et al., 2010). The secondary metabolites (i.e. allelochemicals) produced by *A. armata* act as chemical defences against competitors and predators (Hay & Fenical, 1988; Paul & Ritson-Williams, 2008), however, few studies have examined the effects of macroalgal allelochemicals on both biochemical and behavioural responses, with most research focused on their defensive functions against herbivory. In this work, it was observed that *A. armata* exudate not only interfere with the feeding behaviour of one of the species but also induce changes in several energy metabolism related biochemical parameters of both invertebrates.

Regarding effects on *G. umbilicalis*, *A. armata* induced a significant increase in total lipids, along with decreased activities of LDH and ETS. The primary source of energy are the carbohydrates followed by lipids and then proteins, and their mobilization is often used to counter toxic stress. Notwithstanding, in this case, the high lipid content may be related to the fact that the invertebrates maintain the energy reserves as they alter their behaviour, decreasing their activity, and thus with less expenditure. In fact, the sea snail has become less active, as seen in the proxy feeding at higher concentrations of exudate. This may ultimately lead to an energetic shift with a lesser energy expenditure, despite the probable higher energetic demands for detoxification mechanisms. This trend has been reported in the literature for other compounds as is the case of the study of Verslycke and co-authors (2004) with mysids, where a decrease in feeding was observed along with increasing lipids levels after exposure to chlorpyrifos.

LDH is an important glycolytic enzyme in biological systems and its activity is an indication of increased energy demand to cope with exposure to toxicant challenge (Wu & Lam, 1997; Diamantino et al., 2001). The significant decrease in LDH verified in the higher exudate concentrations, indicative of a reduction in anaerobic capacity, may reflect systemic toxicity impairing the organisms to fight the toxic stress, which might later on have further severe lethal consequences – knowing the last concentration is almost lethal range. This decrease in general cellular metabolism is also in line with ETS results, where the decrease in the aerobic capacity is also suggested by the significant reduction of ETS in marine snails exposed to the medium concentration of 0.14%. This non-monotonic response may derive from the complexity in the media and differentiated mechanisms of action of different individual compounds and their different concentrations in the mixture at a given exudate dilution, which constitutes an extra challenge to the interpretation of results of such nature.

In exposed *Palaemon elegans* there was an increase in lipids and proteins along with the increase of ETS and LDH activities. The increase in lipids was similar as discussed previously for the sea snails, but for *P. elegans*, the exudate also exerted a significant increase of protein content. This increase may also reflect an induction in protein synthesis for detoxification processes and other defence mechanisms (Smolders et al., 2003). This is also

supported by the metabolic reactions assessed that, contrary to the sea snails, indicate an enhancement of the cellular metabolism, with LDH and ETS activities being increased at the same concentrations. This indicates that the organisms are spending energy both anaerobically and aerobically to fight stress caused by *A. armata* exudate. This pattern indicates that the amount of energy available for growth, molting, reproduction and other biological functions might be compromised (Sokolova et al., 2012).

These results indicate that organisms have different energy requirements to deal with the stress caused by the macroalgae exudate. This comparative analysis may provide important insights into the heterogeneous effects of the *A. armata* exudate, driven by species-specific metabolic susceptibility patterns.

The present work was performed using exudates which, to obtain naturally, demand that the seaweed is placed in seawater at a given ratio and defined conditions, which will mostly represent what is exudated by *A. armata* in the nature. Ratios of 1:10 and over are commonly found in rock pools, which may remain enclosed from minutes to several hours during a tidal period. Other factors which may induce additional stress and compound release may influence more toxicity such as the case of temperature or hydrodynamics (Gschwend et al., 1985). Also, the exudates were prepared in the dark, and the production of volatile halogenated organic compounds (VHOCs) production rate tends to decrease under dark conditions (Bondu et al., 2008). Despite the difficulties to benchmark this stressor preparation and impacts with a real case scenario, the very high toxicity of this seaweed might not even reflect the worst case scenario of exposure to exudates from this macroalgae, especially in bloom events, in summer, in tide pools, where a body of water separates from the sea for hours during a tidal cycle, where seaweed concentrations are high and water dilution is little.

The present study is an important step in the research of natural toxic exudates released into the environment and the mechanisms on how they can affect the surrounding organisms and their mode of action in the invaded ecosystems.

To the present knowledge, this represents the first work to study the exudate *per se* and its impacts in costal invertebrates. Although, as stated, this exudate contains a myriad of

compounds, its toxicity is attributed mainly to the halogenated compounds (secondary metabolites) produced and stored in vacuoles within *A. armata*'s gland cells (Burreson et al., 1976). Additionally to the results and toxic effects seen here at high dilutions of the prepared exudate, to better understand the real impact on coastal environments, and specially in more exposed tidal pools, further studies should be made to understand the concentrations found and its variation, considering seaweed density and other biotic and abiotic factors to better address this seaweed chemical defences true impact in coastal environments. These impacts may probably be more extent compared to the ones here found due to the referred increased stress and conditions that may lead to an increased production of secondary metabolites and also densities that can be much higher, especially when considering seasonal seaweed stranding, and all other factors affecting these organisms in a high stress burden ecosystem.

5. Acknowledgements

This study had the support of Fundação para a Ciência e a Tecnologia (FCT) through the Strategic Project UID/MAR/04292/2013, project MARINE INVADERS – The impact and mechanisms of success of the invasive seaweed *Asparagopsis armata* on coastal environments (POCI-01-0145-FEDER-031144), and grant awarded to Carla Silva (SFRH/BD/87030/2012). The project was also partially funded by the Integrated Programme of SR&TD “SmartBioR” (reference Centro-01-0145-FEDER-000018) cofunded by Centro 2020 program, Portugal2020, European Union, through the European Regional Development Fund. Authors also thank Sofia Silva (MARE, Portugal) for help in laboratorial work.

References

1. Aderemi, A.O., Novais, S.C., Lemos, M.F.L., Alves, L.M., Hunter, C., & Pahl, O. (2018). “Oxidative stress responses and cellular energy allocation changes in microalgae following

- exposure to widely used human antibiotics." *Aquatic Toxicology*, 203, 130-139.
2. Amiard-Triquet, C., Amiard, J.C., & Rainbow, P.S. (2012). "Ecological Biomarkers: Indicators of Ecotoxicological Effects". CRC Press, Boca Raton, 464 pp.
 3. Bligh, E. G., & Dyer, W. J. (1959). "A rapid method of total lipid extraction and purification." *Canadian Journal of Biochemistry and Physiology*, 37(8), 911–917.
 4. Borell, E.M., Foggo, A. & Coleman, R.A. (2004). "Induced resistance in intertidal macroalgae modifies feeding behaviour of herbivorous snails". *Oecologia*, 140, 328–334.
 5. Bondu, S., Cocquemot, B., Deslandes, E., & Morin, P. (2008). "Effects of salt and light stress on the release of volatile halogenated organic compounds by *Solieria chordalis*: a laboratory incubation study". *Botanica Marina*, 51(6), 485-492.
 6. Bradford M.M. (1976). "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding." *Analytical Biochemistry*, 2(1-2), 248-254.
 7. Burreson, B. J., Moore, R. E., & Roller, P. P. (1976). "Volatile Halogen Compounds in the Alga *Asparagopsis taxiformis* (Rhodophyta)." *Journal of Agricultural and Food Chemistry*, 24(4), 856–861.
 8. Cabecinhas, A. S., Novais, S. C., Santos, S. C., Rodrigues, A. C. M., Pestana, J. L. T., Soares, A. M. V. M., & Lemos, M. F. L. (2015). "Sensitivity of the sea snail *Gibbula umbilicalis* to mercury exposure--linking endpoints from different biological organization levels." *Chemosphere*, 119, 490–497.
 9. De Coen, W. M., & Janssen, C. R. (1997). "The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular Energy Allocation: A new methodology to assess the energy budget of toxicant-stressed *Daphnia* populations." *Journal of Aquatic Ecosystem Stress and Recovery*, 6(1), 43–55.
 10. De Coen, W.M., & Janssen, C. R. (2003). "The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics." *Environmental Toxicology and Chemistry*, 22(7), 1632-1641.
 11. Dell'Omo, G. (Ed.). (2002). "Behavioural ecotoxicology". John Wiley & Sons, 492pp.
 12. Diamantino, T.C., Almeida, E., Soares, A.M.V.M., & Guilhermino, L. (2001). "Lactate dehydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* straus." *Chemosphere*, 45(4-5), 553-560.
 13. Fong, P. P., & Molnar, N. (2013). "Antidepressants cause foot detachment from substrate

- in five species of marine snail." *Marine environmental research*, 84, 24-30.
14. Gnaiger, E., 1983. "Calculation of energetic and biochemical equivalents of respiratory oxygen consumption." In: Gnaiger, E., Forstner, H. (Eds.), *Polarographic Oxygen Sensors. Aquatic and Physiological Applications*. Springer, Berlin, Heidelberg, New York, pp. 337–345.
 15. Gschwend, P. M., MacFarlane, J. K., & Newman, K. A. (1985). "Volatile halogenated organic compounds released to seawater from temperate marine macroalgae." *Science*, 227(4690), 1033-1035.
 16. Hay, M.E. and Fenical, W. 1988. "Marine plant–herbivore interactions: the ecology of chemical defense." *Ann. Rev. Ecol. Syst.* 19(1), 111-145.
 17. Kühnhold, H., Kamyab, E., Novais, S., Indriana, L., Kunzmann, A., Slater, M., & Lemos, M.F.L. (2017). "Thermal stress effects on energy resource allocation and oxygen consumption rate in the juvenile sea cucumber, *Holothuria scabra* (Jaeger, 1833)." *Aquaculture*, 467, 109-117.
 18. Lemos, M.F.L., Soares A.M.V.M., Correia, A., Esteves, A.C. (2010). "Proteins in ecotoxicology - how, why and why not?" *Proteomics*, 10, 873-887.
 19. McConnell, O., & Fenical, W. (1977). "Halogen chemistry of the red alga *Asparagopsis*." *Phytochemistry*, 16(3), 367–374.
 20. McConnell, O. J., Hughes, P. A., Targett, N. M., & Daley, J. (1982). "Effects of secondary metabolites from marine algae on feeding by the sea urchin, *Lytechinus variegatus*." *Journal of Chemical Ecology*, 8(12), 1437–1453.
 21. McLoughlin, N., Yin, D.Q., Maltby, L., Wood, R.M., & Yu, H.X. (2000). "Evaluation of sensitivity and specificity of two crustacean biochemical biomarkers". *Environ. Toxicol. Chem.* 19, 2085–2092.
 22. Orth, R. J., & Van Montfrans, J. (1984). "Epiphyte-seagrass relationships with an emphasis on the role of micrograzing: a review". *Aquatic Botany*, 18(1-2), 43-69.
 23. Otero, M., Cebrian, E., Francour, P., Galil, B., & Savini, D. (2013). "Monitoring marine invasive species in Mediterranean marine protected areas (MPAs): a strategy and practical guide for managers." Malaga, Spain: IUCN, 136.
 24. Paul, N.A., Cole, L., De Nys, R., & Steinberg, P. D. (2006a). "Ultrastructure of the gland cells of the red alga *Asparagopsis armata* (Bonnemaisoniaceae)." *Journal of Phycology*, 42(3), 637–645.
 25. Paul, N.A., De Nys, R., & Steinberg, P. D. (2006b). "Seaweed-herbivore interactions at a

- small scale: Direct tests of feeding deterrence by filamentous algae." *Marine Ecology Progress Series*, 32: 1-9.
26. Paul, V.J., & Ritson-Williams, R. (2008). "Marine chemical ecology". *Natural Product Reports*, 25(4), 662-695.
 27. Peckol, P., & Putnam, A. B. (2017). "Differential toxic effects of *Ulva lactuca* (Chlorophyta) on the herbivorous gastropods, *Littorina littorea* and *L. obtusata* (Mollusca)." *Journal of Phycology*, 53(2), 361-367.
 28. Pinteus, S., Lemos, M.F.L., Alves, C., Neugebauer, A., Silva, J., Thomas, O. P., Gaspar, H., Botana, L. M., & Pedrosa, R. (2018). "Marine Invasive Macroalgae: turning a real threat into a major opportunity - the biotechnological potential of *Sargassum muticum* and *Asparagopsis armata*." *Algal Research*, 34, 217-234.
 29. Richardson, D.M., Pyšek, P., & Carlton, J. T. (2011). "A compendium of essential concepts and terminology in invasion ecology." *Fifty years of invasion ecology: the legacy of Charles Elton*, Wiley-Blackwell, 409-420.
 30. Sakata, K., Iwase, Y., Ina, K., & Fujita, D. (1991). "Halogenated terpenes isolated from the red alga *Plocamium leptophyllum* as feeding inhibitors for marine herbivores." *Nippon Suisan Gakkaishi*, 57(4), 743-746.
 31. Silva, C.S.E., Novais, S.C., Lemos, M.F.L., Mendes, S., Oliveira, A.P., Gonçalves, E. J., & Faria, A.M. (2016). "Effects of ocean acidification on the swimming ability, development and biochemical responses of sand smelt larvae." *Science of the Total Environment*, 56: 89-98.
 32. Silva, C.O., Simões, T., Novais, S.C., Pimparel, I., Granada, L., Soares, A.M., Barata, C., & Lemos, M. F. (2017). "Fatty acid profile of the sea snail *Gibbula umbilicalis* as a biomarker for coastal metal pollution". *Science of the Total Environment*, 586, 542-550.
 33. Silva, C.O., Novais, S.C., Alves, L., Soares, A.M.V.M., Barata, C., & Lemos, M.F.L. (2019). "Linking cholinesterase inhibition with behavioural changes in the sea snail *Gibbula umbilicalis*: Effects of the organophosphate pesticide chlorpyrifos." *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 225: 108570.
 34. Smolders, R., De Boeck, G., & Blust, R., (2003). "Changes in cellular energy budget as a measure of whole effluent toxicity in zebrafish (*Danio rerio*)." *Environ. Toxicol.Chem*, 22.4: 890-899.
 35. Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A. (2012). "Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates." *Mar. Environ. Res.* 79:1-15

36. Vassault, A. (Ed.) (1983). "Methods of enzymatic analysis." Academic Press, New York, pp.118–126.
37. Verslycke, T., Roast, S. D., Widdows, J., Jones, M. B., & Janssen, C. R. (2004). "Cellular energy allocation and scope for growth in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) following chlorpyrifos exposure: a method comparison". J. Exp. Mar. Biol. Ecol., 306(1), 1-16.
38. Wu, R.S.S., & Lam, P.K.S. (1997). "Glucose-6-phosphate dehydrogenase and lactate dehydrogenase in the green-lipped mussel (*Perna viridis*): possible biomarkers for hypoxia in the marine environment." *Water Res.* 31, 2797–2801