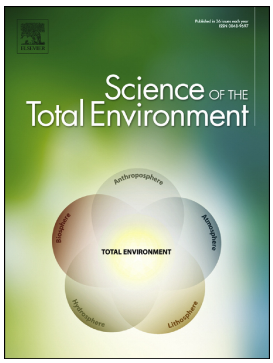

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Combined effects of salinity changes and Salicylic Acid exposure in *Mytilus galloprovincialis*

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ABSTRACT

Pharmaceuticals and Personal care products (PPCPs) are frequently released into several marine matrices, representing significant environmental and ecotoxicological risks. Among the widest spread PPCPs in aquatic systems is Salicylic acid (SA), with known negative effects on marine and freshwater species. Nevertheless, the toxicity resulting from these emerging pollutants, including SA, together with climate change has still received little attention up to date. Among climate change related factors salinity is one that most affects aquatic organisms. To better understand the combined impacts of SA and salinity, the present study evaluated the biochemical alterations induced in *Mytilus galloprovincialis* mussels exposed to SA and different salinity levels, acting individually and in combination. The effects observed clearly highlighted that cellular damages were mainly observed at higher salinity (35), with no additive or synergistic effects derived from the combined presence of SA. Higher antioxidant capacity of mussels in the presence of SA may prevented increased LPO levels in comparison to uncontaminated mussels. Nevertheless, in the presence of SA mussels revealed loss of redox balance, regardless of the salinity level. Furthermore, mussels exposed to SA at control salinity showed increased metabolic capacity which decreased when exposed to salinities 25 and 35. These findings may indicate the protective capacity of mussels towards higher stressful conditions, with lower energy reserves expenditure when in the presence of SA and salinities out of their optimal range. Although limited cellular damages were observed, changes on mussel's redox balance, antioxidant mechanisms and metabolism derived from the combined exposure to SA and salinity changes may compromise mussel's growth and reproduction. Overall, the present study highlights the need to investigate the impacts induced by pollutants under present and future climate change scenarios, towards a more realistic environmental risk assessment.

Keywords: Climate change; Salicylic Acid; Oxidative Stress; Biomarkers; Metabolic capacity; mussels

1. INTRODUCTION

Coastal systems are increasingly exposed to a huge diversity of pollutants, resulting from population growing around these areas, newly developed technologies and processes that generate higher amount of unknown wastes, but also due to generalized and easier access to a diversity of materials and substances. Among the widest spread pollutants in aquatic systems are pharmaceuticals and personal care products, including Salicylic acid (SA). This organic acid is widely used in topical cosmetic and dermatological consumer products, being commonly used to treat skin diseases like acne or psoriasis. Furthermore, SA is the principal metabolite of acetylsalicylic acid (ASA, aspirin), a non-steroidal anti-inflammatory drugs (NSAIDs), which is a common and widely used analgesic, anti-pyretic and anti-inflammatory drug, with an estimated 40,000 tonnes (50 to 120 billion pills) consumed each year (Godersky et al., 2018; Jones, 2015; Wong, 2019). As a result of the wide use of SA and ASA, concentrations of SA in different aquatic systems range from few ng/L in freshwater and marine environments to several µg/L in wastewater influents and effluents (among others, Biel-Maeso et al., 2018; Fent et al., 2006; Paíga et al., 2016; Sim et al., 2011; Wang et al., 2010). The increasing knowledge on the wide occurrence of SA in diverse aquatic environments worldwide distributed has rose concerns on possible toxic impacts towards non-target species inhabiting these aquatic systems. In this regard, still scarce information is available on the toxic effects of SA towards aquatic organisms, especially marine invertebrates, with recent studies showing oxidative stress impacts in freshwater species, namely in *Lemna minor* plants (Alkimin et al., 2019), the crustacean *Daphnia magna* (Gómez-Oliván et al. 2014) and the fish *Salmo trutta* (Nunes et al. 2015), but also in marine and estuarine species such as fish *Mugil cephalus* (Fazio et al., 2013*)*, mussels (*Mytilus galloprovincialis*, Freitas et al., 2019) and polychaetes (*Nereis diversicolor*, Nunes, 2019). Studies conducted by Freitas et al. (2019a) further demonstrated that the exposure to SA can cause increase of metabolic capacity in *M. galloprovincialis* after exposure to an increasing gradient of SA concentrations.

Besides pollutants, environmental alterations associated with climate change related factors and extreme weather events have shown to negatively affect aquatic organisms. The acceleration in the global rainfall and evaporation cycle can dramatically change salinity patterns, especially in coastal and estuarine areas (Coughlan et al., 2009). This situation may be of major concern since seawater salinity has been identified as one of the most important factors impacting estuarine and coastal areas (Cardoso et al., 2008; IPCC 2013), with known effects on species richness and abundance, organisms growth and reproduction, and population spatial distribution (Lapresta-Fernández et al., 2012; Matozzo and Marin 2011; Verdelhos et al. 2015; Gosling, 2008; Telesh and Khlebovich, 2010). Under laboratory conditions different authors also demonstrated the negative impacts caused by salinity changes on marine species, namely on bivalves, including alterations on their metabolic profiles (Carregosa et al., 2014a), metabolism and oxidative status (Sarà et al., 2008; Coughlan et al., 2009; Carregosa et al., 2014b; Gonçalves et al., 2017; Hamer et al., 2008; Moreira et al., 2016a; Velez et al., 2016), and also on bivalves' immune responses (Bussell et al., 2008; Matozzo and Marin, 2011; Reid et al., 2003).

Under environmental conditions the above mentioned stressors often act in combination, with the presence of pollutants in aquatic systems being coincident with the occurrence of salinity alterations. Nevertheless, information on the impacts resulting from the combination of these stressors are still scarce, with limited information concerning changes on pollutants toxicity due to salinity alterations as well as regarding changes induced by salinity on organisms' sensitivity to pollutants. It is known that salinity changes may alter pollutants chemical speciation, mobilization from sediments, solubility and adsorption, factors that may influence bioaccumulation and toxicity (Zwolsman et al., 1997; Kumar et al., 2015; Riba et al., 2004). In what concerns to changes on species pollutants sensitivity due to salinity alterations, Zanette et al. (2011) showed that salinity enhances the negative effects caused by the diesel oil in the oyster *Crassostrea gigas* oxidative status. Studies assessing the impacts of As under different salinity levels revealed that *Ruditapes philippinarum* clams exposed to the combination of both stressors experienced higher cellular

damage and greater antioxidant defences inhibition than each stressor acting alone (Freitas et al., 2016). On the other hand, the exposure of *Mytilus galloprovincialis* to Triclosan and Diclofenac under different salinity levels revealed higher lipid peroxidation levels when mussels were simultaneously exposed to each of the contaminants and stressful salinities (25 and 35) (Freitas et al., 2019c).

Nevertheless, although the advance on the understanding on how pollutants effects may differ under different salinity levels, current knowledge on the combined effects of salinity and PPCPs is still in its infancy. For this reason, the present study aimed to evaluate the effects of SA in the mussels species *M. galloprovincialis*, exposed to different salinity levels, resembling drought and rainy periods, associated with extreme weather that have been increasing both in intensity and frequency.

2. MATERIALS AND METHODS

2.1. Experiment set up

Mytilus galloprovincialis specimens were collected in the Ria de Aveiro (northwest Atlantic coast of Portugal), in February 2018. Individuals with similar size (condition index: dry tissue weight / dry shell weight = 7.7±0.5) were selected to avoid differences in bioconcentration levels and biochemical responses.

In the laboratory mussels were acclimated to laboratory conditions for fifteen days in artificial seawater. Seawater was prepared by the addition of artificial sea salt (Tropic Marin® Sea Salt) to reverse osmosis water. During this period, seawater was maintained at 17.0 ± 1.0 °C; pH 8.0 ± 0.1, salinity 30 ± 1, 12 light: 12 h dark photoperiod and continuous aeration, while mussels were fed with AlgaMac Protein Plus (150 000 cells/animal) every 2-3 days after water renewal.

After this period, organisms were distributed into different salinities (30, 25 and 35), in the absence and presence of Salicylic acid (SA): CTL (salinity 30 and absence of SA); salinity 30 in the presence of SA (4.0 mg/L); salinity 25 in the presence and absence of SA; salinity 35 in the presence and absence of SA. Mussels were distributed among different conditions, with 3 glass aquaria (7 L seawater) per condition and 7 individuals per aquarium. Salicylic acid (2-Hydroxybenzoic acid sodium salt) used in the experiment was obtained from Sigma-Aldrich, Milan, Italy; chemical purity ≥ 99,5%; molecular weight 160.10. For the experimental assay organisms were exposed for 28 days.

The concentration of SA used (4.0 mg/L) was selected based on published literature that showed concentrations in the aquatic environment ranging from 0.004 mg/L to 20.00 mg/L (Ferrer et al., 2001; Heberer, 2002; López-Serna et al., 2012; Paíga et al., 2016; Ternes et al., 2001) and impacts in aquatic organisms testing concentrations up to 40 mg/L (Claessens et al., 2013; Nunes et al., 2015; Nunes, 2019; Zivna et al., 2016).

During the exposure period (28 days), aquaria were continuously aerated, temperature (17 ºC) and salinity were daily checked and adjusted if necessary. Mortality was daily checked and organisms were considered dead when their shells gaped and failed to shut again after external stimulus. During the entire experiment, animals were fed with Algamac protein plus (150.000 cells/animal) three times per week. The exposure medium was renewed weekly, after which SA concentration was re-established. Immediately after medium renewal water samples (50 mL) were collected every week from each aquarium and used for SA quantification analysis to compare nominal and real exposure concentrations.

To assess SA stability in the water medium during the experimental period, a set of aquaria without animals but under the same conditions (SA concentration, salinities 30, 25 and 35; temperature 17 ºC) was prepared. For this, 3 aquaria per condition were prepared and, each week, water samples were collected to quantify SA concentrations after 7 days of exposure, the period of time between medium renewal.

During the experimental period no mortality was observed and *M. galloprovincialis* specimens per condition (two individuals per aquarium/replicate) were immediately frozen. The whole body of three frozen organisms per aquarium/replicate (nine per condition) was pulverized individually with liquid nitrogen and divided into aliquots of 0.5 g FW, which were used for SA quantification and biochemical parameters determination. One individual from each aquarium was lyophilized for lipids content determination.

2.2. Salicylic acid quantification in water and mussel's tissues

Concentrations of SA were measured in water and soft tissues by using an high performance liquid chromatography-ultraviolet (HPLC-UV) detection method. Water samples were analyzed by using the method of Baranowska and Kowalski (2012) with modifications. Water samples were filtered and extracted with solid phase extraction (Oasis HLB 6cc 150 mg solid-phase extraction

cartridges, Waters), followed by HPLC analysis. Soft tissues samples were analyzed by using the method of Madikizela et al. (2017) with modifications. Tissue sample (1.5 g) were dehydrated and sonicated at 50 ºC for 1 min using 5 mL of acetonitrile (10 mL) as the extraction solvent. The supernatant was collected after centrifugation and diluted using Milli-Q grade water and then purified with solid phase extraction as reported for water samples (Oasis HLB 6cc 150 mg solid-phase extraction cartridges, Waters). The chromatographic system consisted of a Series 200 PerkinElmer gradient pump coupled to a Series 200 PerkinElmer variable UV detector, which was set at 230 nm. The mobile phase consisted of acetonitrile, methanol and 25 mM phosphate buffer, at a ratio of 5:5:90 (v:v). A 100 μL injection was used each time. The reversed-phase column was a Haisil, LC column (5 μm, 150x4.60 mm, Higgins). The column was kept at room temperature. Turbochrome software was used for data processing. The recovery was >70% for water samples and >75% for soft tissues. The detection limit (LOD), calculated as a signal-to-noise ratio of 3:1, was 0. 5 μg /L for water samples and 5.0 μg/g for soft tissues. For calculations, a value corresponding to LOD/2 was assigned to all samples that exhibited values <LOD, accordingly to Glass and Gray (2001).

Bioconcentration factor (BCF) was calculated by dividing the mean concentration of SA found in organism's tissues by the mean concentration of SA measured in the water medium.

2.3. Biological responses

Extraction was performed with specific buffers (see Freitas et al., 2019, Pirone et al., 2019) to determine: lipid peroxidation (LPO) levels, reduced (GSH) and oxidized (GSSG) glutathione content; the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx); electron transport system (ETS) activity and the concentrations of glycogen (GLY), protein (PROT) and lipid (LIP). These samples were sonicated for 15 s at 4 °C and centrifuged for 10 min at 10 000g (3 000g for electron transport system activity) at 4 °C. Supernatants were stored at −80

°C or directly used to measure the above mentioned biomarkers. All the biochemical parameters were performed in duplicate.

2.3.1. Indicators of cellular damage and redox balance

For LPO quantification supernatants were extracted using 20% (v/v) trichloroacetic acid (TCA). LPO was measured according to Ohkawa et al. (1979). LPO levels were calculated.by the quantification of malondialdehyde (MDA). Absorbance was read at 535 nm using the molar extinction coefficient (ε) 156 mM^{-1} cm^{-1}. LPO was expressed in nmol of MDA formed per g FW.

Reduced (GSH) and oxidized (GSSG) glutathione content were determined according to Rahman et al. (2006), using reduced and oxidized glutathione standards (0-60 µmol/L) to produce a calibration curve. Absorbance was measured at 412 nm, for both assays. GSH and GSSG concentrations were expressed in nmol per g FW. GSH/GSSG was calculated dividing the GSH values by 2x the amount of GSSG.

2.3.2. Antioxidant defences

The activity of SOD was determined based on the method of Beauchamp and Fridovich (1971). A calibration curve was performed with SOD standards (0.25-60 U/mL). SOD activity was measured at 560 nm. Results were expressed in U *per g* FW where U corresponds to a reduction of 50% of nitroblue tetrazolium (NBT).

The activity of CAT was quantified according to Johansson and Borg (1988) and the modifications were performed following that of Carregosa et al. (2014). Standards of formaldehyde (0-150 µM) were prepared to produce a calibration curve. The absorbance was read at 540 nm. CAT activity was expressed in *U per g* FW. One unit (U) is defined as the amount of enzyme that caused the formation of 1.0 nmol of formaldehyde per min.

The activity of GPx was quantified following Paglia and Valentine (1967) protocol. The absorbance was measured at 340 nm. The enzymatic activity was determined using the molar

extinction coefficient (ε) 6.22 $mM^{-1}cm^{-1}$. The results were expressed as *U per g* FW, where U represents the number of enzymes that caused the formation of 1.0 μmol NADPH oxidized per min.

2.3.3. Metabolic capacity and energy reserves

The ETS activity was measured following King and Packard (1975) and modifications performed by De Coen and Janssen (1997). The absorbance was measured at 490 nm. The amount of formazan formed was calculated using the molar extinction coefficient (ε) 15,900 M^{-1} cm^{-1}. The results expressed in nmol per min per g FW.

The GLY content was quantified according to sulphuric acid method (Dubois et al., 1956), using glucose standards (0-5 mg/mL) to produce a calibration curve. Absorbance was measured at 492 nm. Concentrations of GLY were expressed in mg per g FW.

Total PROT content was determined according to the Biuret spectrophotometric method (Robinson and Hogden, 1940), using bovine serum albumin (BSA) as standards (0–40 mg/mL) to produce a calibration curve. Absorbance was measured at 540 nm. Concentrations of PROT were expressed in mg per g FW.

Lipids (LIP) extraction and content determination followed the methods developed by Folch et al. (1957) and Cheng et al. (2011), respectively. A standard curve was determined using cholesterol standards (0–100%). The absorbance was measured at 520 nm. The results were expressed in percentage of lipids per mg dry weight (DW).

2.4. Data analysis

Results from LPO levels, GSH/GSSG, and SOD, CAT and GPx activities, LIP, PROT and GLY concentrations, as well as ETS activity, were submitted to hypothesis testing using permutational multivariate analysis of variance with the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008). The t-statistics in the pairwise comparisons were evaluated in terms of significance. Values lower than 0.05 were considered as significantly different. The null hypotheses

tested were: i) when in the absence of SA, different salinity levels had no effects on mussels; significant effects among salinity levels were represented in figures with lower case letters; ii) when in the presence of SA, different salinity levels had no effects on mussels; significant differences among salinity levels were represented in figures with upper case letters; iii) for each salinity level, SA had no effects on mussels; for each salinity level, significant differences between mussels with and without SA were represented with an asterisk.

3. RESULTS

3.1. Salicylic acid concentration in water and mussel's tissues

Concentrations of SA measured each week after 7 days of exposure in aquaria without mussels demonstrated the stability of this compound with values of SA measured for each test salinity presenting no significant differences to the nominal concentration (4 mg/L): salinity 30 – 4.5±0.6; salinity 25 - 4.6±0.7; salinity 35 - 4.2±0.01 mg/L. These results validate the spiking procedure and demonstrates that during 7 days, the period between seawater renewal, SA was in the medium.

The concentrations of SA measured in water samples collected immediately after spiking confirmed the nominal concentrations at salinities 30 and 25 (Table 1). However, at salinity 35 mean values of SA concentrations observed after each spiking were below the nominal concentration (Table 1).

The concentrations of SA in mussels exposed to different tested conditions differed, with lower values in mussels exposed to control salinity (30). Nevertheless, no significant differences were found between mussels exposed to salinities 25, 30 and 35 (Table 1).

If we consider mean concentration value obtained at each condition, higher BCF was obtained at salinity 35, while the lowest value was found at control salinity (Table 1).

3.1. Biological responses

3.1.1. Indicators of cellular damage and redox balance

For non-contaminated mussels, LPO levels showed significant differences between salinities 30 and 35, while in mussels exposed to SA significant differences were observed between the two lower salinities (30 and 25) and the highest one (35) (Figure 1A). No significant differences were observed between non-contaminated and contaminated mussels, regardless the salinity level.

The ratio between reduced (GSH) and oxidized (GSSG) glutathione showed, for non-contaminated mussels as well as organisms exposed to SA, no significant differences among different tested salinities (Figure 1B). Differences between contaminated and non-contaminated mussels were noticed for all tested salinities, with significantly higher values in non-contaminated organisms (Figure 1B).

3.3.2 Antioxidant defences

In non-contaminated mussels the activity of superoxide dismutase (SOD) was significantly higher at salinities 25 and 35 compared to salinity control (30), with no significant differences between mussels exposed to salinities 25 and 35 (Figure 2A). A similar pattern was observed in contaminated mussels, with significant differences among salinities and higher SOD activity at salinity 35 (Figure 2B). Significantly higher SOD activity was found in contaminated mussels compared to non-contaminated ones at salinities 25 and 35 (Figure 2A).

For non-contaminated and contaminated mussels no significant differences occurred in catalase (CAT) activity among tested salinities (Figure 2B). When comparing non-contaminated and contaminated mussels, regardless the salinity, significantly higher activity was observed in non-contaminated organisms (Figure 2B).

The activity of glutathione peroxidase (GPx) was significantly higher in non-contaminated mussels exposed to salinity 35 in comparison to mussels exposed to control (30) and salinity 25 (Figure 2C). No significant differences were found among salinities in SA exposed mussels (Figure 2C). No significant differences were observed between contaminated and non-contaminated mussels, for all the tested salinities (Figure 2C).

3.3.3. Metabolic capacity and energy reserves

For non-contaminated mussels, the results on electron transport system activity (ETS) showed no significant differences among salinity conditions (Figure 3A). Mussels exposed to SA showed

significantly higher ETS activity at salinity 30 compared to salinities 25 and 35 (Figure 1A). Differences between contaminated and non-contaminated mussels were observed only at salinity 30, with higher values in mussels exposed to SA (Figure 3A).

For non-contaminated mussels, the glycogen (GLY) content was significantly higher at salinity 35 compared to mussels exposed to salinities 30 and 25 (Figure 3B). Mussels exposed to SA showed no significantly different GLY content among salinity conditions (Figure 3B). For all tested salinities, significantly higher GLY content was observed in non-contaminated mussels (Figure 3B).

Non-contaminated mussels as well as organisms exposed to SA showed no significant differences in terms of Protein (PROT) content among different tested salinities (Figure 3C). Differences between contaminated and non-contaminated mussels were only noticed at salinity 35, with significantly higher values in non-contaminated organisms (Figure 3C).

Non-contaminated mussels showed significantly higher lipids (LIP) content at salinity 35 compared to salinities 30 and 25 (Figure 3D). No significant differences were observed in the LIP content among SA contaminated mussels exposed to different salinities levels (Figure 3D). Differences between non-contaminated and contaminated mussels showed significantly higher LIP content in non-contaminated mussels at salinity 35 (Figure 3D).

4. DISCUSSION

The toxic effects of salicylates are well-known for mammals, with induction of oxidative stress being commonly identified as a biological response (Buntenkötter et al., 2016; Doi et al., 2002; Doi and Horie, 2010; Needs and Brooks, 1985). Recent studies showed similar impacts in non-target organisms including aquatic vertebrates, as fish (Choi et al., 2015; Klessig, 2016; Nunes et al., 2015), but still few studies have been conducted on aquatic invertebrates (Freitas et al., 2019a; Nunes, 2019). A part from this, up to now, no studies investigated the influence salinity shifts on organism's responses to SA exposure which may constitute one of the most probable climate scenarios. In fact, considering that coastal and in particular estuarine species are increasingly exposed to pollutants and that shifts on salinity levels are increasingly frequent as a result of extreme weather events, combined exposure to pollutants and salinity levels corresponds to an already occurring realistic scenario. The obtained results are therefore innovative, highlighting changes on organism's sensitivity towards pollutants due to climate change related factors

Since SA presents logKow value (2.26) it may be considered hydrophilic and moderately soluble in water, ensuring the presence of this compound in seawater during the experimental period. This is corroborated by the fact that results obtained showed that, in the absence of mussels, the concentration of SA in the water was maintained along 7 days exposure periods (corresponding to water renewals) regardless tested salinities, revealing no effects of salinity on SA concentration in the medium. Furthermore, the presence of SA in seawater allowed the accumulation of this compound by mussels. Although a slight increase of SA in mussels tissues was observed at stressful salinity conditions (25 and 35), with higher BCF values also at these conditions, the absence of significant differences in SA tissues concentrations among different salinities prevents any further conclusion on SA accumulation related with salinity levels. Such results may be related with the fact that tested salinities did not influence SA solubility and availability and, on the other hand, did not significantly influence mussel's filtration capacity or detoxification ability. Nevertheless, the

results obtained indicate that metabolic capacity of contaminated mussels was diminished at salinities 25 and 35 compared to control salinity (30), indicating that mussels could be under reduced filtration activity which was not sufficient to limit accumulation of SA at such stressful conditions.

Although similar SA concentrations were found in mussels exposed to different salinities, impacts on mussels biochemical performance differed among tested conditions, namely in what regards to oxidative stress. Oxidative stress is defined as a disturbance in the balance between the production and elimination of reactive oxygen species (ROS). The generation of ROS is a natural process in all aerobic species, occurring during mitochondrial electron transport of aerobic respiration or by oxidoreductase enzymes and metal catalysed oxidation. When subjected to environmental changes, such as increases/decreases in salinity and/or the presence of pollutants, organisms often increase their ROS formation with consequent changes on their antioxidant mechanisms of defence. Previous studies already demonstrated that the presence of pollutants such as pharmaceuticals but also changes on salinity levels may induce oxidative stress in marine invertebrates, with alteration on antioxidant enzymes activities, occurrence of cellular damage and loss of redox balance (among others, Franzellitti et al., 2013; Zuccato et al., 2006; Gonzalez-Rey, et al., 2014; Freitas et al., 2019b, 2019c). In the particular case of SA, previous studies already described increases of antioxidant enzymes and lipid peroxidation levels, suggesting its potential pro-oxidative effect (Doi et al. 2002; Doi and Horie 2010), also in non-target organisms (Nunes et al., 2015; Freitas et al., 2019a). Nevertheless, no studies are known on the combined effects of SA exposure and salinity stressful conditions.

In the absence of SA, the obtained results clearly demonstrated that increased salinity levels (35) induced cellular damage in mussels, identified by higher lipid peroxidation (LPO) values. However, at lower salinity (25) no differences were found compared to organisms under control salinity (30). These results highlight that a decrease on salinity may be less stressful to *M. galloprovincialis* individuals than an increase or, on the other hand, at lower salinities mussels may

better trigger their defence mechanisms against the excess of ROS. Similar findings were observed previously by Freitas et al. (2019b) for the same species exposed to similar salinity changes.

As for the uncontaminated mussels, when in the presence of SA cellular damage was only observed at higher salinity (35), with no significant differences between contaminated and non-contaminated mussels, regardless the salinity level, showing no synergistic or additive effects between both stressors in terms of cellular damage. Such results may thus indicate that concentrations tested were not high enough to induce cellular damage in mussels and, moreover, salinity levels tested were not stressful enough to increase sensitivity of mussels towards SA exposure with no changes on the effects of salinity when in the presence of SA. Previous studies conducted by Freitas et al. (2019a) already showed no significant differences in LPO levels among a range of SA concentrations (0.005, 0.05, 0.5 and 5.0 mg/L). Also Nunes (2019) showed that in the polychaete *Hediste diversicolor* SA was not able to generate a full state of oxidative stress, since no peroxidative damage occurred, verified by the absence of LPO. These authors highlighted that the protective adaptive response of the SA exposed individuals were sufficient to minimize the damage caused by reactive oxygen species (ROS) generated from the metabolism of SA.

Nevertheless, although LPO levels were similar between contaminated and non-contaminated mussels regardless the salinity level, the ratio GSH/GSSG clearly revealed negative effects in SA exposed mussels with significantly lower GSH/GSSG values. In particular, although uncontaminated mussels showed no differences in terms of GSH/GSSG values, with no significant effects of salinity on mussels redox balance, the presence of SA significantly influenced mussels redox balance at all the tested salinities. The increasing amount of GSSG observed in the presence of SA is a clear indication of oxidative stress, which was already observed in the same mussel species exposed to other drugs, Triclosan and Diclofenac (Freitas et al., 2019c). These authors also showed lower GSH/GSSG values in contaminated mussels, regardless the salinity levels tested.

Regarding antioxidant defences, mussels exposed to salinities outside control tended to increase their antioxidant enzymes activities, namely in terms of SOD and GPx, with higher activity

values at the highest tested salinity. These results corroborate the hypothesis that salinity 25 may induce lower stress to mussels than salinity 35, which presented higher LPO levels despite their increased antioxidant capacity. Similarly, hypersalinity was accompanied by upregulation of antioxidant enzymes such as SOD in the flatworm *Macrostomum lignano* (Rivera-Ingraham et al., 2016).

In the presence of SA at control salinity (30) mussels showed no changes on SOD and GPx activity compared to non-contaminated mussels, while CAT was inhibited in the presence of SA. These results may indicate low effect of SA on mussels SOD and GPx activity while greatly affecting the activity of CAT. Freitas et al. (2019a) showed lower SOD and CAT activities in mussels exposed to SA concentrations higher than 0.005 mg/L. Nevertheless, studies conducted by Nunes (2019) demonstrated that GPx increased along the increasing exposure gradient of SA (50.0, 75.0, 112.5, 168.75, 253.125 μg/L) in the polychaete *H. diversicolor*, while CAT was only activated at the lowest SA activity. Nevertheless, as revealed in the present study, such antioxidant defences were able to protect organisms against cellular damage generated by ROS and/or the concentrations tested were not enough to induce LPO. Contrasting results obtained from different studies may be related with the tested concentrations that above certain limits of organism's tolerance antioxidant enzymes are inhibited or the response to SA is species-dependent. The present study further demonstrated that at salinities outside the control, contaminated mussels showed a clear activation of SOD, especially at salinity 35, which may be associated with greater oxidative stress experienced by mussels at these conditions. Studying the effect of SA in maize plants under saline stress, Fahad and Bano (2012) also reported that SA treatment in plants grown in saline field had increased SOD activity. Although CAT and GPx presented a similar behaviour in SA exposed mussels regardless the salinity tested, the enhance on SOD activity at salinities 25 and 35 may indicate an interactive effect of SA and salinity over this enzyme.

When exposed to different salinity levels mussels metabolic capacity showed no differences among tested salinities, revealing no clear impacts of salinity on mussels ETS activity.

Nevertheless, in terms of energy reserves higher GLY and LIP concentrations were found at salinity 35, indicating lower expenditure of energy reserves at this salinity. Sokolova et al. (2017) proposed that energy-related biomarkers can be used to predict ecological consequences of environmental stressful conditions. Thus, we may postulate that the behaviour observed in the present study may indicate a mussels protective behaviour as a response to increased stress levels, with mussels reducing the expenditure of energy reserves under an unfavourable situation. However, this response may occur up to certain stress levels, after which organisms have the need to use their energy reserves. Also studies conducted by Freitas et al. (2019c) demonstrated that *M. galloprovincialis* specimens exposed to different salinities increased their GLY content under stressful salinity conditions (25 and 35). Marigómez et al. (2017) also showed that, in comparison with healthy mussels, stressed populations showed a high energy storage that partially contributes to alleviate thermal stress.

In the presence of SA, at control salinity ETS activity was increased compared to non-contaminated organisms, a response that was not followed by enzymes activation under this condition and thus it seems that increased metabolism was not used to fuel up defence mechanisms. At salinities 25 and 35 mussels decreased their ETS activity compared to control salinity showing the limited capacity of SA exposed mussels to increase their metabolism under the combined effects of salinity and SA. With the decrease of ETS activity at these conditions (salinities 25 and 35, under SA exposure) mussels prevented the use of their energy reserves corroborating once again the hypothesis that under a certain stress levels organisms are able to avoid the expenditure of their energetic reserves. The decrease of ETS under these conditions may also reduce the generation of ROS, which could also contribute to limit the occurrence of cellular damage.

CONCLUSIONS:

Overall, the present findings highlight the impacts of increased salinity levels in mussels oxidative status, with higher injuries under salinity 35 compared to salinity 25 and control (30). The

results obtained further demonstrated that, in general, organisms showed no significant differences between contaminated and non-contaminated mussels at each salinity level, indicating the absence of an additive or synergistic effect induced by the combination of both stressors. However, the increase of SOD activity in mussels exposed to SA and salinities 25 and 35 may indicate a join effect of both stressors on this enzyme, since at control salinity no differences were observed in SOD activity between contaminated and non-contaminated mussels. Also, the decrease on mussel's metabolic capacity in the presence of SA and salinities 25 and 35 compared to mussels exposed to control salinity may indicate an inhibitory effect of salinity when mussels are exposed to SA.

The present study highlights the risks derived from the presence of SA in coastal systems, revealing increased impacts especially under salinity shifts, which may easily result from a prolonged warming or raining periods. In particular, the realistic tested concentration (4.0 mg/L) associated with current increasing frequency and intensity of extreme weather events, including heat waves and drought periods, makes this study of major relevance. The present study represents a first step towards a more realistic environmental risk assessment, considering more than one stressor acting individually, highlighting the possible increased injuries derived from the combination of stressors, which my compromise not only mussels biochemical processes but also population growth and reproduction as metabolism differed in the presence of two or one stressor.

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Conflict of Interest

The Authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affi liations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Figure captions

Figure 1. A: Lipid peroxidation (LPO) and B: reduced/oxidised glutathione (GSH/GSSG) ratio, in *Mytilus galloprovincialis* exposed to different salinity levels (30-control, 25 and 35) in the absence and presence of salicylic acid (SA). Results are the means ± standard deviation. Significant differences ($p<0.05$) among concentrations are identified with different lower case letters (non-contaminated mussels) or uppercase letters (contaminated mussels). For each salinity level, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Figure 2. A: Superoxide dismutase (SOD); B: Catalase (CAT) and C: Glutathione peroxidase (GPx) and Glutathione-S-transferases (GSTs) activities, in *Mytilus galloprovincialis* exposed to different salinity levels (30-control, 25 and 35) in the absence and presence of salicylic acid (SA). Results are the means ± standard deviation. Significant differences ($p<0.05$) among concentrations are identified with different lower case letters (non-contaminated mussels) or uppercase letters (contaminated mussels). For each salinity level, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Figure 3. A: Electron transport system (ETS) activity, B: Glycogen (GLY), C: Protein (PROT) and D: Lipids (LIP) content, in *Mytilus galloprovincialis* exposed to different salinity levels (30-control, 25 and 35) in the absence and presence of salicylic acid (SA). Results are the means ± standard deviation. Significant differences ($p<0.05$) among concentrations are identified with different lower case letters (non-contaminated mussels) or uppercase letters (contaminated mussels). For each salinity level, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Table 1- Salicylic acid (SA) concentrations in water (mg/L), collected immediately after spiking at the 1st, 2nd, 3rd and 4th weeks of exposure, and in mussel's tissues (µg/g dry weight) at the end of the experimental period (28 days). LOD for water samples 0.005 mg/L; LOD for tissue samples 5 µg/g.

Conditions	Water	Tissues
CTL	<LOD	<LOD
30	3.8±0.6[a,b]	30. 5±11.4
25	3.9±0.2[a]	42.6±20.6
35	3.3±0.3[b]	42.8±18.7

- Higher cellular damage was observed at salinity 35
- Loss of redox homeostasis in the presence of SA
- Increased SOD activity in the presence of SA, especially under salinity 35
- Inhibition of CAT in the presence of SA
- Lower metabolic capacity in mussels exposed to the combination of both stressors

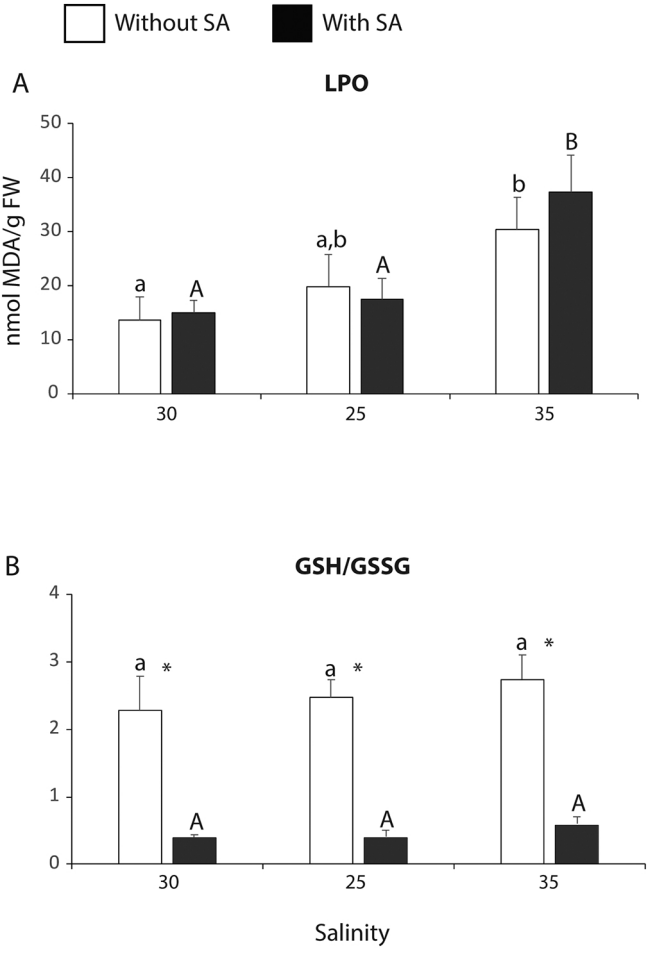
Without SA
With SA
A
LPO
nmol MDA/g FW
50
40
30
20
10
0
a
A
a,b
A
b
B
30
25
35
B
GSH/GSSG
4
3
2
1
0
a *
A
a *
A
a *
A
30
25
35
Salinity

Figure 1

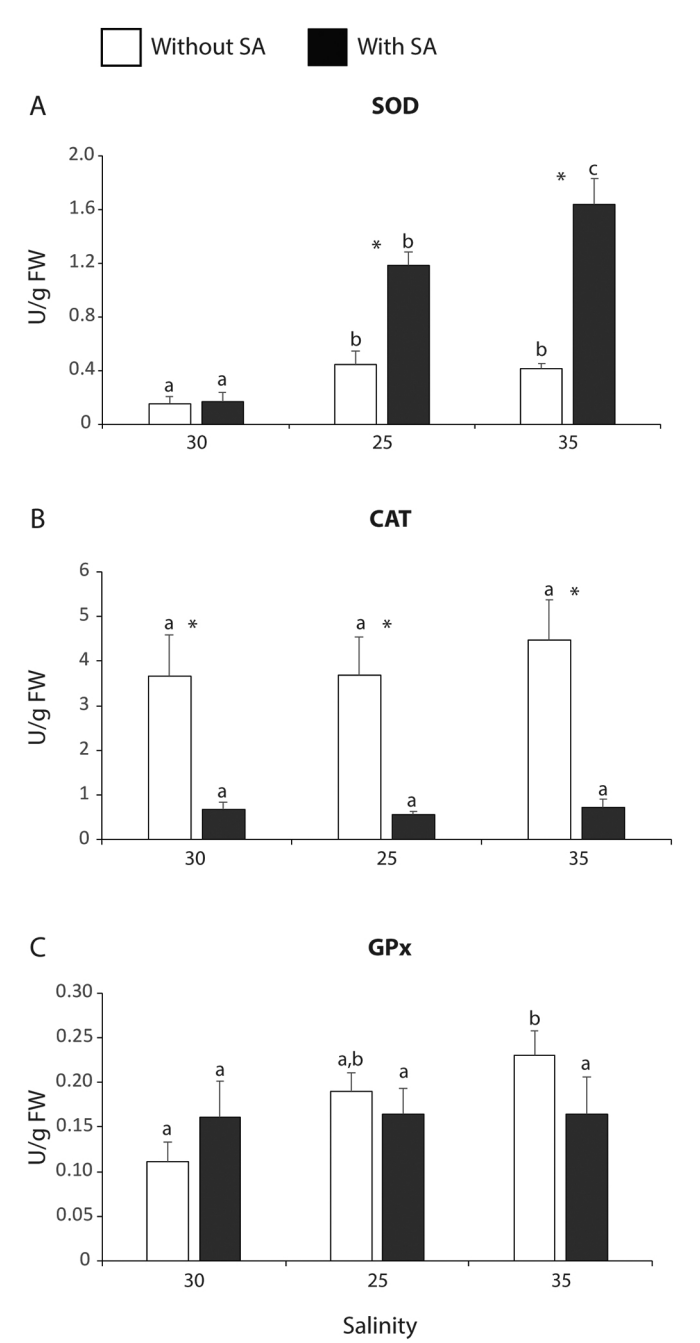
Without SA
With SA
A
SOD
U/g FW
2.0
1.6
1.2
0.8
0.4
0
a
a
b
*
b
b
*
c
30
25
35
B
CAT
U/g FW
6
5
4
3
2
1
0
a
*
a
a
*
a
a
*
a
30
25
35
C
GPx
U/g FW
0.30
0.25
0.20
0.15
0.10
0.05
0
a
a
a,b
a
b
a
30
25
35
Salinity

Figure 2

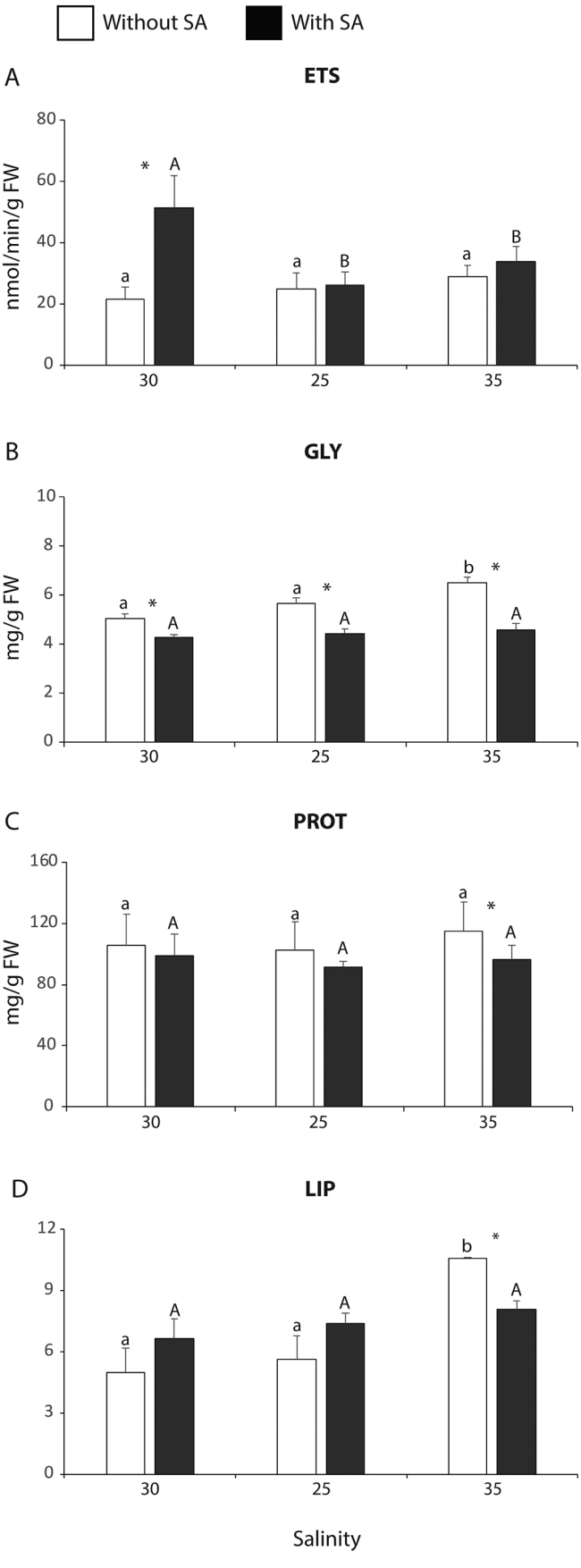
Without SA
With SA
A
ETS
nmol/min/g FW
80
60
40
20
0
a
*
A
a
B
a
B
30
25
35
B
GLY
mg/g FW
10
8
6
4
2
0
a
*
A
a
*
A
b
*
A
30
25
35
C
PROT
mg/g FW
160
120
80
40
0
a
A
a
A
a
*
A
30
25
35
D
LIP
12
9
6
3
0
a
A
a
A
b
*
A
30
25
35
Salinity

Figure 3