# Biochemical and physiological responses induced in Mytilus galloprovincialis after a chronic exposure to salicylic acid

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## Abstract

A vast variety of substances currently reaches the aquatic environment, including newly developed chemicals and products. Lack of appropriate analytical methods for trace determinations in aquatic ecosystem compartments and lack of information regarding their toxicity explains existing regulation gaps. However, suspicion of their toxicity assigned them as Contaminants of Emerging Concern (CECs). Among CECs are Pharmaceuticals including Salicylic Acid (SA), which is the active metabolite of acetylsalicylic acid (ASA; aspirin). The aim of the present study was to evaluate the potential effects of SA on the mussel Mytilus galloprovincialis. For this, organisms were exposed for 28 days to different concentrations of SA (0.005; 0.05; 0.5 and 5 mg/L), resembling low to highly polluted sites, after which different physiological and biochemical parameters were evaluated to assess organism's respiration rate, neurotoxic, metabolic and oxidative stress status. Our results clearly showed that SA strongly reduced the respiration capacity of mussels. Also, SA inhibited the activity of superoxide dismutase (SOD) and catalase (CAT) enzymes, but increased the activity of glutathione peroxidase (GPx) and glutathione-S-transferases (GSTs), which prevented the occurrence of lipid peroxidation (LPO). Nevertheless, oxidative stress was confirmed by the strong decrease of the ratio between reduce glutathione (GSH) and oxidized (GSSG) glutathione in contaminated mussels. Moreover, neurotoxicity was observed in mussels exposed to SA. Overall, this study demonstrates the metabolic, neurotoxic and oxidative stress impacts of SA in M. galloprovincialis, which may result in negative consequences at the population level.

## 1. Introduction

Pharmaceutical drugs are ubiquitous substances (Fent et al., 2006), identified as contaminants of emerging concern (CECs), as they have been in the environment for many years but concern of their possible effects has been raised much more recently (Sauvé and Desrosiers, 2014) mainly because they were not detected previously due to the lack of highly sensitive and appropriate instruments (Ferrer and Thurman, 2012; Leendert et al., 2015). The presence of pharmaceuticals in the environment is mainly attributed to the discharges of treated effluents (Brun et al., 2006; Sehonova et al., 2017; 2019) as secondary processes of waste water treatment plants (WWTPs) are not designed to remove these substances, resulting in their discharge to receiving surface waters reaching coastal areas (Daughton, 2001; Heberer, 2002; Kümmerer, 2008). Although the amount of pharmaceuticals released into the environment is generally lower in comparison with other pollutants (Fent et al., 2006; McEneff et al., 2014), concerns of their impacts to the environment rise from their continuous release and persistence in the aquatic systems with concentrations reaching levels from µg/L to mg/L (Daughton and Ternes, 1999; Halling-Sørensen et al., 1998; Nunes et al., 2015).

Among the most abundant pharmaceuticals in the environment are antidepressants (Sehonova et al., 2018), anti-inflammatories, lipid regulators, anticonvulsants and various antibiotics (Zhang et al., 2008), with concentrations ranging from ng/L to mg/L (Cahill et al., 2004; Calisto et al., 2011; Castiglioni et al., 2004; Fatta-Kassinos et al., 2011; Gaw et al., 2014; Kümmerer, 2004; Zuccato et al., 2006). These compounds persist in ecosystems (Glassmeyer et al., 2008) and even if present at extremely low levels they are able to induce toxic effects in non-target organisms (Brooks et al., 2003a, b; Gunnarsson et al., 2008; Gust et al., 2009; Johnson et al., 2005).

Salicylic acid (SA) is the main primary metabolite of acetylsalicylic acid (ASA) (Nyúl et al., 2018) that represents the most widely consumed anti-inflammatory agent in the world, and belongs to the class of non-steroidal anti-inflammatory drugs as a result of its antipyretic, analgesic and anti-rheumatic properties (Buntenkötter et al., 2016; Maclagan, 1876). In dermatology, it is used as a keratolytic agent, possessing also bacteriostatic and fungicidal properties. Its mechanism of action involves an irreversible block of the enzyme cyclooxygenase (COX) (Grosser, 2006; Vane, 1998), an enzyme that catalyses the conversion of arachidonic acid into endoperoxides, thus causing the inhibition of prostaglandin synthesis (important inflammatory mediators), consequently reducing inflammation and pain (Zivna et al., 2015). Although several studies already evaluate the effects of SA in vertebrate species (Doi et al., 2002; Nunes et al., 2015; Zivna et al., 2016, 2013) very few assessed the impacts of this substance in invertebrates (Gómez-Oliván et al., 2014), especially in marine species (Nunes, 2019) and, to our knowledge, no studies have examined the effects of this drug in marine bivalves, such as the mussel Mytilus galloprovincialis.

Therefore, the aim of the present study was to determine the effects of a chronic exposure (28 days) at ecologically relevant concentrations of SA (0.00–5.00 mg/L) (Ferrer et al., 2001; Heberer, 2002; Metcalfe et al., 2003) to the mussel M. galloprovincialis. This species is among the aquatic organisms most threatened by the presence of contaminants in estuarine systems, as they are sessile and filtering organisms, capable of accumulating pollutants in their tissues (Capillo et al., 2018). These animals possess physiological and subcellular mechanisms that allow them to cope with the toxic effects of various pollutants (Burgos-Aceves and Faggio, 2017; Faggio et al., 2016, 2018; Pagano et al., 2016; Savorelli et al., 2017; Torre et al., 2013) and in the present study several physiological and biochemical markers were evaluated to understand the impacts of SA on M. galloprovincialis respiration rate, metabolic capacity, oxidative stress and neurotoxic status.

## 2. Materials and methods

### 2.1. Experimental setup

Mytilus galloprovincialis specimens were collected in the Ria de Aveiro (northwest Atlantic coast of Portugal), in February 2018. In order to minimize the effect of body weight on the SA accumulation and biochemical responses of organisms, animals with similar weight (8.9 ± 1.0 g fresh weight, FW) were selected. M. galloprovincialis has been widely used as

bioindicator for the monitoring of environments polluted by pharmaceuticals and personal care products (among others, Franzellitti et al., 2013; Freitas et al., 2019; Gonzalez-Rey and Bebianno, 2014; Pagano et al., 2016; Schmidt et al., 2011; Trombini et al., 2016).

After sampling, organisms were transported to the laboratory where they were maintained for fifteen days in artificial seawater, in order to release metals and micro-organisms (Freitas et al., 2012; Maffei et al., 2009) and to acclimate to laboratory conditions. Seawater was prepared by the addition of artificial sea salt (Tropic Marin® Sea Salt) to reverse osmosis water. During this period, organisms were maintained at 17.0 ± 1.0 °C; pH 8.0 ± 0.1, salinity 30 ± 1, 12 light: 12 h dark photoperiod and continuous aeration. Seawater was renewed every two days. During this period, mussels were every two-three days fed with AlgaMac Protein Plus, Aquafauna Bio-Marine, Inc (150 000 cells/animal).

After this period, organisms were distributed into different glass aquaria (7 L seawater, salinity 30), with 7 individuals per container and 3 containers per condition: CTL (0.0 mg/L), 0.005; 0.05; 0.5 and 5.0 mg/L of SA. Salicylic acid (2-Hydroxybenzoic acid sodium salt) used in the experiment was obtained from Sigma-Aldrich, Milan, Italy; chemical purity ≥ 99,5%; molecular weight (MW) 160.10. For the experimental assay, organisms were exposed for 28 days.

The 0.005 and 0.05 mg/L concentrations of SA used in this study were prepared from a stock solution with a concentration of 50 mg/L; the 0.5 mg/L concentration of SA used in this study was prepared from a stock solution with a concentration of 500 mg/L, while the 5.0 mg/L concentration of SA used in this study was prepared from a stock solution with a concentration of 5000 mg/L. The concentrations selected were based on published literature that showed concentrations in the 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 effects in marine species testing concentrations up to 40 mg/L (Claessens et al., 2013; Nunes et al., 2015; Nunes, 2019; Zivna et al., 2016). Also, the concentrations used aimed to: i) test a wide range of contamination levels, and ii) determine the threshold concentrations that may affect M. galloprovincialis mussels.

During the exposure period containers were continuously aerated, temperature and salinity were daily checked with a thermometer and a refractometer, respectively. When necessary, temperature and salinity were adjusted adding water to the containers, according to the above conditions. Mortality was daily checked and organisms were considered dead when their shells gaped and failed to shut again after external stimulus. During the entire exposure period (28 days) 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 (10 mL) were collected every week from each aquarium and used for SA quantification analysis to compare nominal and real exposure concentrations.

After exposure (28 days) six individuals per condition (two per aquarium/replicate) were used to determine the respiration rate (RR). The remaining organisms were immediately frozen and 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 determination of biochemical parameters.

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

Concentrations of SA were measured in water and soft tissues by using a 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 samples (1.5 g) were dehydrated and sonicated at 50 °C 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, 150 x 4.60 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 0.005 mg/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: physiological parameters

#### 2.3.1. Mortality

For each condition, the mortality was determined by dividing the total number of dead individuals at the end of the exposure period (28 days) by the total number of individuals used at the start of the experiment.

#### 2.3.2. Respiration rate

The respiration rate (RR) was measured at the end of the experimental period (28 days). Measurements were performed by simple static respirometry, using two organisms of the

same aquarium per respirometer chamber. Each of these chambers, which were equipped with an oxygen sensor spot glued to its inner wall using silicon paste, was filled with the same seawater used during the experimental period. Organisms were placed in these chambers under dark and fully-oxygenated conditions, and they were allowed to acclimate for 30 min to avoid the influence of manipulation on RR. After this period, chambers were filled to their maximum capacity (1 L) to avoid the formation of air bubbles and were then sealed air-tightly. Measurements started in fully oxygenated medium and RR was recorded as a function of declining O2 concentration (mg/L) over time every 15 min during 2 h, with a multi-channel fiber optic oxygen meter (Multi channel oxygen meter, PreSens GmbH, Regensburg, Germany) for simultaneous read-outs. Data were recorded using the software PreSens Measurement Studio 2. Twenty-two measurements were carried out at a time (including a blank, i.e. chamber containing no organisms to account for background respiration). Organisms were posteriorly dried and weighed. Respiration rate was expressed in mg O2 consumed per h per g dry weight (DW).

### 2.4. Biological responses: biochemical parameters

Extraction was performed with specific buffers (see Freitas et al., 2019, 2017, 2012) to determine: the activity of the electron transport system (ETS), the glycogen (GLY), andprotein (PROT) concentrations, the activity of the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferases (GSTs) and acetylcholinesterase (AChE), lipid peroxidation levels (LPO), reduced (GSH) and oxidized (GSSG) glutathione content. These samples were simultaneously disrupted using a TissueLyser II, for 60 s at 4ºC. After this procedure samples were centrifuged for 25 min at 10 000 g (10 min at 3 000 g for ETS activity) at 4 °C. Supernatants were stored at −80 °C or directly used to measure the above-mentioned biomarkers. All the measurements of biochemical parameters were performed in duplicate.

#### 2.4.1. 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 during 10 min in 25 s intervals. The amount of formazan formed was calculated using the molar extinction coefficient (ε) 15,900 M−1 cm− 1 and 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.

### 2.4.2. Antioxidant and biotransformation defences

The activity of SOD was determined based on the method of Beauchamp and Fridovich (1971). SOD activity was measured at 560 nm. Absorbance values were read after 20 min of incubation at room temperature. One unit of enzyme activity corresponds to a reduction of 50% of nitroblue tetrazolium (NBT). A calibration curve was performed with SOD standards (0.25–60 U/mL). 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 those 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 in a microplate reader. 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 at 10 s intervals during 5 min and 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 activity of enzyme causing the formation of 1.0 µmol NADPH oxidized per min.

The activity of GSTs was determined according to the method of Habig et al. (1976). It was measured spectrophotometrically at 340 nm, using the molar extinction coefficient (ε) 9.6 mM−1− cm-1. GSTs catalyze the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, forming a thioether. Results were expressed in U per g FW where U is defined as the activity of enzyme that catalyses the formation of 1 µmol of dinitrophenyl thioether per min.

### 2.4.3. Indicators of cellular damage and redox balance

FLPO was measured according to Ohkawa et al. (1979). LPO levels were calculated.by the quantification of malondialdehyde (MDA), a by-product of lipid peroxidation. 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.

### 2.4.4. Neurotoxicity

Acetylthiocholine iodide (ATChI 5 mM) substrates were used for the determination of AChE following the method of Ellman et al. (1961) with modification by Mennillo et al. (2017). Enzyme activities were recorded continuously for 5 min at 412 nm and expressed in nmol per min per g FW, using the molar extinction coefficient (ε) 13,600 nM−1 cm−1.

## 2.5. Data analysis

Results from RR, ETS activity, PROT and GLY concentrations, activities of SOD, CAT, GPx, GSTs and AChE, LPO levels and GSH/GSSG values 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 hypothesis tested was: SA had no impacts on mussels, with no significant differences among tested conditions. Significant differences among exposure concentration were represented with different letters.

# 3. Results

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

The concentrations of SA measured in water samples collected immediately after spiking confirmed the nominal concentrations and for each of the exposure conditions no significant differences were observed among the four weeks of exposure (Table 1).

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.

| SA concentrations (mg/L) | Water (mg/L) | | | Mussels tissues (μg/g) |
|---|---|---|---|---|
| 1st week | 2nd week | 3rd week | 4th week | 4th week |
| CTL | <LOD | <LOD | <LOD | <LOD |
| 0.005 | 0.005 ± 7.0E-5 | 0.005 ± 1.5E-4 | 0.005 ± 7.0E-5 | 0.005 ± 2.1E-4 <LOD |
| 0.05 | 0.05 ± 0.01 | 0.041 ± 0.00 | 0.04 ± 0.00 | 0.05 ± 0.00 <LOD |
| 0.5 | 0.46 ± 0.01 | 0.41 ± 0.04 | 0.43 ± 0.06 | 0.46 ± 0.06 10.78 ± 10.92 |
| 5.0 | 4.40 ± 0.61 | 4.37 ± 0.25 | 4.23 ± 0.04 | 3.78 ± 0.32 58.9 ± 30.03 |

The concentrations of SA in mussels exposed to 0.005 and 0.05 mg/L were below the detection limit (5.0 μg/g) (Table 1). Mussels exposed to 0.5 mg/L of SA presented concentrations that ranged between 33 μg/L and values lower than the detection limit (LOD = 5.0 μg/g), while when exposed to the highest SA exposure concentration (5.0 mg/L) mussels presented SA concentrations that ranged between 120 and 23 μg/L.

Bioconcentration factor significantly decreased from 25.6 at 0.5 mg/L SA exposure concentration to 11.8 for organisms exposed to the highest concentration (5.0 mg/L).

### 3.2. Physiological parameters

#### 3.2.1. Mortality

Mytilus galloprovincialis exposed to SA for 28 days showed a 100% survival under all tested concentrations.

#### 3.2.2. Respiration rate

The respiration rate (RR) evaluated in mussels is presented in Fig. 1. M. galloprovincialis significantly decreased the RR when exposed to SA, with no significant differences among organisms exposed to different SA concentrations.

Fig. 1

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Fig. 1. Respiration Rate (RR) in Mytilus galloprovincialis exposed to different concentrations of salicylic acid (SA) (CTL-0.0, 0.005, 0.05, 0.5 and 5.0 mg/L of SA). Results are the means + standard deviation. Significant differences ($p < 0.05$) among concentrations are identified with different lower case letters.

### 3.3. Biochemical parameters

#### 3.3.1. Metabolic capacity and energy reserves

Metabolic capacity: The results on electron transport system activity (ETS) showed significantly higher values in mussels exposed to SA in comparison to control organisms, with highest values at the two lowest concentrations (0.005 and 0.05 mg/L) (Fig. 2A).

Fig. 2

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Fig. 2. A: Electron transport system (ETS) activity, B: Glycogen (GLY) and C: Protein (PROT) concentrations in Mytilus galloprovincialis exposed to different concentrations of

salicylic acid (SA) (CTL-0.0, 0.005, 0.05, 0.5 and 5.0 mg/L of SA). Results are the means + standard deviation. Significant differences ($p < 0.05$) among concentrations are identified with different lower case letters.

Energy reserves: Glycogen (GLY) content significantly increased in contaminated mussels, with the highest values obtained at the highest SA concentration (5.0 mg/L) (Fig. 2B).

The organisms exposed to SA significantly increased the protein (PROT) content at the highest exposure concentration (5.0 mg/L), with no significant differences among the remaining conditions (Fig. 2C).

3.3.2. Antioxidant and biotransformation defences

The activity of superoxide dismutase (SOD) significantly decreased in mussels exposed to SA, except for the lowest concentration (0.005 mg/L) where no significant differences were observed in comparison to the control (Fig. 3A).

Fig. 3

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Fig. 3. Activities of A: Superoxide dismutase (SOD); B: Catalase (CAT); C: Glutathione peroxidase (GPx) and D: Glutathione-S-transferases (GSTs) in Mytilus galloprovincialis exposed to different concentrations of salicylic acid (SA) (CTL-0.0, 0.005, 0.05, 0.5 and 5.0 mg/L of SA). Results are the means + standard deviation. Significant differences ($p < 0.05$) among concentrations are identified with different lower case letters.

The activity of catalase (CAT) was significantly lower in contaminated organisms in comparison to control mussels (Fig. 3B).

The activity of glutathione peroxidase (GPx) increased in mussels exposed to SA, with significant differences between organisms exposed to control and concentrations of 0.005, 0.05 and 0.5 mg/L of SA (Fig. 3C).

The activity of glutathione S-transferases (GSTs) increased significantly in organisms exposed to 0.05, 0.5 and 5.0 mg/L in comparison to organisms exposed to control and the lowest SA concentration (Fig. 3D).

3.3.3. Indicators of cellular damage

No significant differences were observed in terms of lipid peroxidation (LPO) levels among tested conditions (Fig. 4A).

Fig. 4

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Fig. 4. A: Lipid peroxidation (LPO) levels and B: reduced/oxidised glutathione (GSH/GSSG) ratio in Mytilus galloprovincialis exposed to different concentrations of salicylic acid (SA) (CTL-0.0, 0.005, 0.05, 0.5 and 5.0 mg/L of SA). Results are the means + standard deviation. Significant differences ($p < 0.05$) among concentrations are identified with different lower case letters.

The ratio of reduced (GSH) to oxidized (GSSG) glutathione showed significantly lower values in organisms exposed to 0.5 and 5.0 mg/L SA in comparison to the remaining conditions (Fig. 4B).

3.3.4. Neurotoxicity

Acetylcholinesterase activity significantly decreased in mussels exposed to SA in comparison to control organisms, with no significant differences among contaminated mussels (Fig. 5).

Fig. 5

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Fig. 5. Acetylcholinesterase (AChE) activity in Mytilus galloprovincialis exposed to different concentrations of salicylic acid (SA) (CTL-0.0, 0.005, 0.05, 0.5 and 5.0 mg/L of SA). Results are the means + standard deviation. Significant differences ($p < 0.05$) among concentrations are identified with different lower case letters.

4. Discussion

The presence of neuroactive, anti-inflammatory and cardiovascular drugs in marine coastal ecosystems and the induction of negative effects in organisms inhabiting these areas increases the need to better understand this environmental risk. The present study demonstrated the bioaccumulation of SA and consequent toxic impacts in Mytilus galloprovincialis especially when exposed to higher concentrations (0.5 and 5.0 mg/L), with alterations on mussels' respiration rate, metabolic capacity, oxidative stress and neurotoxic status.

The toxic effects of salicylates have been fully described in mammals, including the induction of oxidative stress (Klessig et al., 2016). Similar effects were described in non-target

organisms including vertebrates as fish (Choi et al., 2015; Klessig, 2016; Nunes et al., 2015) and invertebrates, namely polychaetes (Nunes, 2019). Nevertheless, effects on bivalves are unknown, namely in what regards to impacts on their physiological behaviour such as respiration capacity, but also alterations on their biochemical performance including metabolic, oxidative and neurotoxic impairments.

The present findings clearly demonstrated that in the presence of SA mussels strongly reduce their respiration rate (RR), with lower values at higher SA concentrations. This strategy may indicate that by reducing their respiration rate mussels were trying to avoid SA accumulation, which is in accordance with the decrease observed in the bioconcentration factor along with the increasing exposure gradient. Similar strategies were already demonstrated by other authors when submitting bivalves to pollutants, including the reduction in clearance rate shown by Ruditapes philippinarum exposed to the drug carbamazepine (Almeida et al., 2015). Nevertheless, the present study also revealed that the reduction of mussels' RR was not accompanied by a metabolic depression indicating that, although experiencing stressful conditions, mussels were able to activate their metabolism, here identified by increased electron transport system (ETS) activity. A similar metabolic activation was already shown by other marine invertebrate species, including bivalves exposed to copper (Ivanina et al., 2016), caffeine (Cruz et al., 2016) and to cetirizine (Teixeira et al., 2017), but also in polychaetes exposed to mercury (Freitas et al., 2017), indicating that up to certain levels of stress organisms are able to activate their metabolism to fuel up defence mechanisms.

The present findings further revealed that although mussels increased their metabolism they were able to prevent the expenditure of their energy reserves that even increased at higher SA exposure concentration, especially noticed for protein (PROT) content. Such increase in PROT concentration may be related to higher production of enzymes involved in defence mechanisms. This behaviour can indicate that mussels were trying to fight against the stress induced by SA by increasing the amount of enzymes involved in antioxidant and biotransformation defence capacity. Former studies already demonstrated the capacity of SA to cause oxidative stress in a diversity of non-target species, including vertebrate (Zivna et al., 2013) and invertebrate (Nunes, 2019) species, with overproduction of reactive oxygen species (ROS) and consequent activation of antioxidant defences. In the present study although the enzymes superoxide dismutase (SOD) and catalase (CAT) were significantly inhibited in mussels exposed to SA, the enzymes glutathione peroxidase (GPx) and glutathione-S-transferases (GSTs) showed a significant increase in activity especially at higher SA concentrations. The inhibition of the activity of CAT and SOD in M. galloprovincialis exposed to SA may be related to high production of ROS due to the presence of this drug, a response pattern already described as a consequence of high stress levels, namely in the clam R. philippinarum exposed to ibuprofen (Milan et al., 2013). Under oxidative stress conditions CAT and GPx are likely to degrade similar substrates, and in the present study it seems that after exposure to SA only GPx was activated. It is known that GPx is involved in the metabolism of several peroxides to their corresponding alcohols. This oxidation-reduction reaction is dependent on one cofactor, the reduced glutathione (GSH). In the present study the increase on GPx activity was accompanied by a decrease on GSH

content and increased oxidized glutathione (GSSG) content, as shown by the decrease on GSH/GSSG along the increasing SA exposure concentration. Similar results, with increasing GPx activity after SA exposure, were already observed in polychaetes (Nunes, 2019) and fish (Nunes et al., 2015).

From the present study, the observed increase of GSTs activity indicates that this group of enzymes is involved in the detoxification of SA in M. galloprovincialis. GSTs are mainly related to the conjugation and excretion of toxic compounds by the activation of conjugation reactions with endogenous substrates, in particular GSH. It has been shown that a wide diversity of drugs modulates the activity of GSTs, including diclofenac, ibuprofen and paracetamol (among others, Freitas et al., 2019; Nunes et al., 2017; Parolini et al., 2010, 2011) and it was also demonstrated that SA induces the activation of SA (Marrs, 1996).

As a consequence of antioxidant and especially biotransformation capacity, lipid peroxidation (LPO) levels were not significantly increased in exposed mussels but the lowest GSH/GSSG values at the highest SA concentrations still indicates that organisms were experiencing oxidative stress at these conditions. Although previous studies already demonstrated the capacity of SA to induce LPO in vertebrates (Doi and Horie, 2010), the observed absence of a clear pattern of cellular damage may result from the capacity of M. galloprovincialis mussels to successfully eliminate this drug, evidencing that the protective behaviour of this species was enough to prevent damage of the lipid membrane. Also Nunes et al. (2019) demonstrated that no LPO was induced in the polychaete species Hediste diversicolor exposed to SA.

The present study further demonstrated the neurotoxic capacity of SA, with a direct relationship between increasing SA concentration and decreasing acetylcholinesterase (AChE) activity. Also Nunes et al. (2019) demonstrated the neurotoxic impacts of SA in H. diversicolor polychaetes.

Overall, based on the results obtained in the present study we may hypothesise that M. galloprovincialis mussels are injured after prolonged exposure to SA, revealing a concentration-dependent response pattern. Moreover, the present findings highlight the oxidative stress and neurotoxic status of organisms with consequences to population maintenance, showing that environmentally realistic concentrations of SA negatively affect M. galloprovincialis, corroborating the use of this species as model to investigate the impact of SA on bivalves. The information gathered in this paper it is a step forward the knowledge on the biochemical and physiological responses of mussels towards environmental pollutants exposure.

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