Chemosphere 252 (2020) 126563

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Toxic impacts of *rutile* titanium dioxide in *Mytilus galloprovincialis* exposed to warming conditions

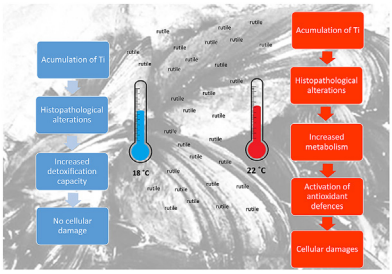
Carla Leite [a], Francesca Coppola [a], Rui Monteiro [b, f], Tania Russo [c], Gianluca Polese [c], Mariana R.F. Silva [d], Mirtha A.O. Lourenço [d, e], Paula Ferreira [d], Amadeu M.V.M. Soares [a], Eduarda Pereira [b], Rosa Freitas [a, *]

[a] *Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193, Aveiro, Portugal*
[b] *Departamento de Química & CESAM/LAQV-REQUIMTE, Universidade de Aveiro, 3810-193, Aveiro, Portugal*
[c] *Dipartimento di Biologia, Universitá degli studi di Napoli Federico II, 80126, Napoli, Italy*
[d] *CICECO- Instituto de Materiais de Aveiro, Departamento de Engenharia de Materiais e Cerâmica, Universidade de Aveiro, 3810-193, Aveiro, Portugal*
[e] *Istituto Italiano di Tecnologia, Center for Sustainable Future Technologies, Via Livorno, 60, 10144, Torino TO, Italy*
[f] *CIIMAR, Universidade do Porto, 4050-123 Porto, Portugal*

HIGHLIGHTS

- *Mytilus galloprovincialis* bio-accumulated titanium regardless the temperature.
- Histopathological alterations were induced by Rutile NPs regardless the temperature.
- Rutile exposed mussels at lower temperature activated detoxification that prevented cell damage.
- Rutile exposed mussels at higher temperature activated antioxidant defences but showed cell damage.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 23 January 2020
Received in revised form
17 March 2020
Accepted 18 March 2020
Available online 23 March 2020

Handling Editor: Prof Willie Peijnenburg

Keywords:
Climate changes
Mussels
Titanium dioxide
Oxidative stress
Metabolism
Histopathology

ABSTRACT

Climate change is leading to a gradual increase in the ocean temperature, which can cause physiological and biochemical impairments in aquatic organisms. Along with the environmental changes, the presence of emerging pollutants such as titanium dioxide (TiO_2) in marine coastal systems has also been a topic of concern, especially considering the interactive effects that both factors may present to inhabiting organisms. In the present study, it has been assessed the effects of the presence in water of particles of *rutile*, the most common polymorph of TiO_2, in *Mytilus galloprovincialis,* under actual and predicted warming conditions. Organisms were exposed to different concentrations of *rutile* (0, 5, 50, 100 μg/L) at control (18 ± 1.0 °C) and increased (22 ± 1.0 °C) temperatures. Histopathological and biochemical changes were evaluated in mussels after 28 days of exposure. Histopathological examination revealed similar alterations on mussels' gills and digestive glands with increasing *rutile* concentrations at both temperatures. Biochemical markers showed that contaminated mussels have an unchanged metabolic capacity at 18 °C, which increased at 22 °C. Although antioxidant defences were activated in contaminated organisms at 22 °C, cellular damage was still observed. Overall, our findings showed that histopathological impacts occurred after *rutile* exposure regardless of the temperature, while biochemical alterations were only significantly noticeable when temperature was enhanced to 22 °C. Thus, this study

* Corresponding author. Departamento de Biologia, Universidade de Aveiro Campus Universitário de Santiago, 3810-193, Aveiro, Portugal.
E-mail address: rosafreitas@ua.pt (R. Freitas).

https://doi.org/10.1016/j.chemosphere.2020.126563
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demonstrated that temperature rise may significantly enhance the sensitivity of bivalves towards emerging pollutants.

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1. Introduction

Since the beginning of the industrial revolution, the atmospheric concentration of carbon dioxide (CO_2) has been increasing (IPCC, 2014) and unless CO_2 emissions are reduced, it is expected that the concentration in the atmosphere reach up to ~1000 ppm until the end of this century (Pörtner et al., 2014). Nearly 30% of the atmospheric CO_2 is absorbed by the oceans, resulting into seawater chemical changes, including a decrease of seawater's pH level (IPCC, 2014). Besides changes in seawater's properties, the increase of CO_2 combined with other "greenhouse" gases has also triggered the rise in the average atmospheric temperature as well as ocean temperature, with predictions forecasting that global ocean warming, between 0.5 °C (RCP2.6) and 1.5 °C (RCP8.5), will reach by the end of the century a depth of about 1 km (Collins et al., 2013; IPCC, 2013). Recent studies already showed that seawater warming is expected to induce major shifts in species spatial distribution, growth and reproductive patterns (Clarke, 2003; Hoffmann et al., 2003; Pörtner et al., 2007; Grilo et al., 2011; Santos et al., 2011; Verdelhos et al., 2011), with alterations on species physiological (Boukadida et al., 2016; Pörtner and Knust, 2007) and biochemical (Andrade et al., 2019; Freitas et al., 2017; Nardi et al., 2017; Velez et al., 2017; Verlecar et al., 2007) performance. Furthermore, temperature rise may also change organisms' responses when exposed to pollutants, as demonstrated previously (Banni et al., 2014; Boukadida et al., 2016; Coppola et al., 2017, 2018; Izagirre et al., 2014; Manciocco et al., 2014; Nardi et al., 2017).

Among several emerging pollutants, titanium dioxide (TiO_2), a naturally occurring mineral, is one of upmost concern. TiO_2 exhibits properties such as: bright white color, long-term stability, super hydrophilicity, ability to block UV light, antimicrobial activity as well as availability at relatively low cost (Cho et al., 2013; Hoffmann et al., 1995; Su et al., 2006; Wang et al., 2009). Due to these properties TiO_2 has extensively been used as pigment in paints, plastics, and paper (Winkler, 2003; Kaegi et al., 2008; Amorim et al., 2018); in personal care products as sunscreen, creams and toothpastes (Wahie et al., 2007; Johnson et al., 2011; Lu et al., 2015; de la Calle et al., 2017; Sureda et al., 2018); and in food packaging where it acts as an antibacterial agent (Cui et al., 2016; Zhu et al., 2018). *Rutile* is the most common natural form of TiO_2, and it is usually used in optical elements, because it has the highest refractive index at visible wavelengths of any known crystal. It also exhibits a particularly large birefringence and high dispersion (Iswarya et al., 2016, 2018). Thus, the release of TiO_2, particularly *rutile*, into the aquatic environment becomes unavoidable due to its worldwide usage, notably in consumer products, which led to their unintentional discharge into the environment, especially to aquatic bodies (Colvin, 2003; Lecoanet and Wiesner, 2004; Guzman et al., 2006; Nowack and Bucheli, 2007). Nevertheless, dissolved Ti is usually present at very low concentrations in aquatic systems, with concentrations between 0.01 and 5.5 μg/L (Skrabal, 2006; Yan et al., 1991; Yokoi and van den Berg, 1991), but due to the increasing use of TiO_2, its concentration has been rising in aquatic systems (Batley et al., 2013; Gondikas et al., 2014). TiO_2 particles are found in domestic sewage, wastewater, industrial effluents and surface runoff from the paints on building facades (Brar et al., 2010; Kaegi et al., 2008; Kiser et al., 2009; Weir et al., 2012). Kiser et al. (2009) found out that raw sewage contains 100–3000 μg/L of Ti, and as the wastewater treatment plants are not able to retain all TiO_2 particles, a small fraction ends up in the natural water systems (Shi et al., 2016). Bivalves like mussels, in particular *Mytilus galloprovincialis*, play a significant ecologic and economic role in marine ecosystems, being commonly used as bioindicators of pollutants effects, including TiO_2 (Barmo et al., 2013; Canesi et al., 2010; D'Agata et al., 2014; Mezni et al., 2018). However, to the best of our knowledge, in the literature available the toxicity on this species induced by TiO_2 crystalline forms combined with high temperature has not been studied yet. However, previous studies conducted by Monteiro et al. (2019a,b) demonstrated that the estuarine mussel *M. galloprovincialis* was affected by titanium (Ti), with alterations in the organism's metabolic capacity, oxidative damage and defence mechanisms. Wang et al. (2019) found that, in general, TiO_2 particles affected several hemocyte parameters (total hemocyte count, hemocyte mortality, phagocytosis activity, lysosomal content, esterase activity, mitochondrial number, mitochondrial membrane potential and reactive oxygen species content) in the mussels *M. coruscus*. Huang et al. (2018) demonstrated that TiO_2 particles cause negative effects in gills resulting in a rise of malondialdehyde (MDA) levels in *M. coruscus* mussels.

Since in most aquatic environments, especially coastal systems, both climate change related factors and pollution act in combination, it is important to understand how temperature may change the effects induced by pollutants as well as the sensitivity of organisms to them. Thus, the present study aimed to evaluate the impact of TiO_2 *rutile* polymorph in marine bivalves under actual and predicted warmer conditions. Therefore, the present study evaluates the impact that different concentrations of *rutile* induce in *M. galloprovincialis* when exposed to two distinctive temperatures (18 and 22 °C), in order to understand the effect that the predicted temperature rise would have on the toxicity of *rutile* and sensitivity of mussels exposed to this pollutant. For this, metabolic capacity (electron transport system activity), energy reserves content (glycogen and protein concentrations), oxidative status (antioxidant and biotransformation enzymes activities, lipid peroxidation levels) markers in mussels whole soft tissue and histopathological alterations in gills (lipofuscin aggregates, loss of cilia, enlarged central vessel and hemocyte infiltration) and digestive tubules (lipofuscin aggregates, hemocyte infiltration, atrophy and necrosis) were evaluated.

2. Materials and methods

2.1. Experimental conditions

Mytilus galloprovincialis specimens were collected during low tide in September 2018 in the Ria de Aveiro coastal lagoon (Portugal). In order to avoid differences in biological responses, mussels with similar size (mean length of 6.0 ± 0.6 cm, mean width of 3.4 ± 0.4 cm) were selected.

The bivalves were transported in plastic containers from the field to the laboratory, where they were placed, for two weeks, in different aquaria for depuration and acclimation. During this period, conditions in the laboratory were: temperature 18.0 ± 1.0 °C; pH 8.0 ± 0.1 (resembling conditions at the sampling

area) with 12 h light and 12 h dark as a photoperiod and continuous aeration. Organisms were maintained in artificial seawater (salinity 30 ± 1), prepared with reverse osmosis water with commercial salt (Tropic Marin® SEA SALT). Seawater was renewed every day for the first three days and every three days until the end of the two weeks. During the first three days animals were not fed and after this initial period mussels were fed with Algamac protein plus (150.000 cells/animal) three times per week.

After depuration/acclimation, organisms were distributed into two climatic rooms to maintain organisms at two different air and seawater temperatures: 18 ± 1 °C (control, considering the average temperature measured at the sampling site) and 22 ± 1 °C (control + 4 °C, resembling predicted warmer conditions (Collins et al., 2013; IPCC, 2014)). Within each temperature mussels were divided into different aquaria, with increasing *rutile* TiO_2 concentrations. The tested conditions in concentrations of titanium (Ti) were: CTL (control)) 0 μg/L; C1) 5 μg/L; C2) 50 μg/L; and C3) 100 μg/L. Per condition three replicates (three aquaria of 3 L) were used with five mussels per aquaria. This range of Ti concentrations was selected according to the values reported in previous works for pristine and contaminated aquatic systems (Gondikas et al., 2014; Kiser et al., 2009; Yan et al., 1991; Yokoi and van den Berg, 1991; Skrabal, 2006). For the assay, TiO_2 particles (*rutile*, Alfa Aesar), in a powder form, were dispersed in ultrapure water using a bath sonicator (60 Hz), for 10 min, to obtain stock solutions of 60 and 600 mg/L of Ti. From these dispersions, dilutions were done to obtain spiking conditions.

The experimental period was 28 days and during the entire period the containers were continuously aerated, with a 12 h light: 12 h dark photoperiod. Temperature (18 ± 1 or 22 ± 1 °C), pH (8.0 ± 0.1) and salinity (30 ± 1) were daily checked and adjusted if necessary. Mortality was also daily checked. During the 28 days, organisms were fed with Algamac protein plus (150.000 cells/animal) three times a week. During the experiment, seawater was renewed weekly, after which the respective *rutile* concentration was re-established. Immediately after the seawater renewal and *rutile* spiking into the water, water samples (5 mL) were collected from each aquarium. This sampling was for further quantification of Ti, to obtain the real exposure concentrations and compare with nominal ones, and for characterization of the Ti.

At the end of the exposure, with the exception of one mussel per aquarium (three per condition) used for histopathological analyses, the organisms were individually frozen with liquid nitrogen and stored at −80 °C, until manual homogenization with a mortar and pestle under liquid nitrogen. Each homogenized organism was divided into aliquots of 0.5 g of fresh weight (FW) for Ti quantification and biomarkers analyses.

All procedures related with *rutile* characterization, Ti quantification, histopathological and biochemical analyses are detailed in the supplementary material.

Histopathological analyses were conducted in gill and digestive tubules of three mussels per condition. Histopathological indexes (I_h) were measured (see supplementary material for details).

Biochemical analyses were conducted in nine organisms per condition and included the quantification of electron transport system activity (ETS), glycogen (GLY) and total protein (PROT) content, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferases (GSTs) activities, and lipid peroxidation levels (LPO) (see supplementary material for details).

2.2. Statistical analyses

Results on Ti concentrations, histopathological indexes (I_hG, I_hDG), biochemical markers (ETS, GLY, PROT, SOD, CAT, GPx, GSTs, LPO) were submitted to a statistical hypothesis testing using permutational analysis of variance, employing the PERMANOVA + add-on in PRIMER v6 (Anderson et al., 2008). The pseudo-F *p*-values were evaluated in terms of significance and values lower than 0.05 ($p < 0.05$) were considered as significantly different. The null hypotheses tested were: i) for each temperature and biological response (Ti accumulation, histopathological and biochemical markers) , no significant differences existed among exposure concentrations (0, 5, 50 and 100 μg/L), with significant differences represented in figures with different lowercase letters for 18 °C and uppercase letters for 22 °C; ii) for each exposure concentration (0, 5, 50 and 100 μg/L) and biological response (Ti accumulation, histopathological and biochemical markers) , no significant differences existed between temperatures (18 and 22 °C), with significant differences represented in figures with an asterisk.

The matrix gathering the histopathological and biochemical descriptors as well as the concentrations of Ti in mussel's tissues, per condition, was used to calculate the Euclidean distance similarity matrix. This similarity matrix was simplified through the calculation of the distance among the centroids based on the condition, which was then submitted to ordination analysis, performed by Principal Coordinates (PCO). Pearson correlation vectors of biochemical descriptors (correlation > 0.75) were superimposed on the top of the PCO graph.

3. Results

3.1. Rutile characterization

In order to understand the impact of TiO_2 *rutile* polymorph particles in marine bivalves, it is important to understand the chemical and structural features of the particles as well as their behaviour in the exposure medium. The X-ray diffraction pattern displayed at Fig. 1S of the Supplementary data confirmed the presence of monophasic particles with *rutile* crystallographic phase. Dashlines indicate the position of the *rutile* reflections according to the JCPDS no. 04-013-6225, which are very similar to the observed in the measured sample, confirming the presence of *rutile* tetragonal phase with lattice parameters of $a = b = 4.5922$ Å and $c = 2.9578$ Å. The density of the particles was determined from XRD pattern using the lattice parameters and had a value of 4.27 g/cm^3. Raman spectroscopy also confirmed the characteristic vibrational mode of the pure crystallographic phase of *rutile* (Fig. 2S). The diameter of particles was measured on 50 particles of the SEM micrograph which is shown at Fig. 3S. The particles present undefined morphology with aggregates of relatively large size. The specific surface area (S_{BET}) of the particles was calculated to be 3 m^2/g. The particles present low specific surface area probably due to the agglomeration level.

To understand the size of the aggregates, DLS measurements were performed at 25 °C. The size distribution of the *rutile* suspensions in the exposure medium was followed along 24 h and results are displayed at Table 1S. The particles seem to have a strong tendency to agglomerate during the first hour in the exposure medium. This behaviour should be related to the period necessary to the particles to be stabilized by the interparticle repulsive forces. After 1 h, a Z-Average size value of 4066 ± 268 nm was measured. This value decreased to 2139 ± 71 nm after 24 h of exposure. This decrease was due to the sedimentation of larger particles, living in suspension only the smallest ones in a smaller proportion. It should be noted that the last DLS values acquisitions were very difficult to perform and did not fit the quality criteria of the equipment. These problems should be associated to two cumulative effects related to

the presence of deposited particles at the bottom of the cell and low concentration of *rutile* particles stabilized in suspension.

3.2. Titanium concentrations in waters and mussels

In all the collected water samples Ti concentration was below the detection limit (2 μg/L).

Under both temperatures, Ti concentration in *M. galloprovincialis* tended to increase along the exposure gradient, but significantly higher values were only observed between the highest exposure concentration (100 μg/L) and the remaining conditions (Table 1). When comparing the temperatures, significant differences ($p = 0.001$) were only obtained at the highest tested concentration, with higher values at 18 °C (Table 1).

3.3. Histopathological parameters

Mussels' gills and digestive tubules showed several histopathological alterations. The exposure to *rutile* at different concentrations led to an increase of damage severity in a dose dependent manner in mussels' gills, under both temperatures (Fig. 1 upper image). At 18 °C, exposed mussels' gills displayed a progressive increase of hemocytes infiltration, lipofuscin aggregates and enlargement of the central vessel (see supplementary material for details, Table 2S). At 22 °C, there was a progressive increase of hemocytes infiltration and lipofuscin aggregates. Also, at the warming scenario, both the cilia loss and the enlargement of the central vessel were more evident at the highest concentration (see supplementary material for details, Table 2S). Under both temperatures, the I_h obtained for mussels' gills (Fig. 2A) significantly increased along the exposure gradient (except for the lowest concentration), with the uppermost values in mussels exposed to the highest concentration. Regarding temperature impact, I_h was found to be significantly higher at 22 °C than at 18 °C in organisms exposed to a concentration of 50 μg/L.

The analysis of the digestive tubules (Fig. 1 lower image) showed that mussels under 18 °C, presented a progressive increase of hemocytes infiltration, atrophy and accumulation of lipofuscin. No necrosis was found at any concentration at 18 °C (see supplementary material for details, Table 2S). At 22 °C there was also a progressive increase of hemocytes infiltration and atrophy. The necrosis alterations appeared at concentrations of 50 and 100 μg/L at 22 °C (see supplementary material for details, Table 2S). Under both temperatures the I_h obtained for mussels' digestive gland (Fig. 2B) was significantly higher in the mussels exposed to the highest concentration of *rutile*. Comparing temperatures, I_h was significantly higher at 22 °C in non-contaminated organisms.

Table 1
Concentrations of Ti (μg/g) in mussels' soft tissues after 28 days of exposure to each condition (CTL, 5, 50 and 100 μg/L) and both temperatures (18 and 22 °C). Values are mean ± standard deviation. Significant differences ($p < 0.05$) among concentrations for each temperature (18 and 22 °C) are represented with different letters (lower case letters for 18 °C, upper case letters for 22 °C); while significant differences between temperatures for each tested concentration are represented with an asterisk. Quantification limit for Ti was 0.25 μg/g.

	Exposure concentrations (μg/L)	[Ti] (μg/g)
18 °C	0	2.1 ± 0.3 ^{a}
	5	2.4 ± 1.0 ^{a}
	50	2.5 ± 0.4 ^{a}
	100	4.5 ± 0.3 ^{b*}
22 °C	0	1.8 ± 0.7 ^{A}
	5	2.3 ± 0.6 ^{A}
	50	2.2 ± 0.6 ^{A}
	100	3.3 ± 0.4 ^{B*}

3.4. Biochemical parameters

3.4.1. Metabolic capacity and energy reserves

At 18 °C no significant differences were observed in terms of ETS values among concentrations, while at 22 °C significantly higher values were observed in mussels exposed to 5 and 50 μg/L in comparison to those non-contaminated ones or exposed to the highest exposure concentration (100 μg/L). Significant differences between temperatures were only observed in mussels exposed to the lowest exposure concentration (5 μg/L), with the highest values being recorded in the ones kept at 22 °C (Fig. 3A).

The GLY content in mussels exposed to 18 °C was significantly superior in contaminated mussels comparatively to non-contaminated ones. At the warmest conditions significantly higher values were observed at the maximum exposure concentration (100 μg/L), with no differences to organisms exposed to 5 μg/L. No significant differences were observed between both temperatures for each of the tested concentrations, while in non-contaminated mussels significantly higher GLY content was observed in organisms kept at 22 °C (Fig. 3B).

In terms of PROT content, at 18 °C significant differences were only observed between control and 5 μg/L exposed organisms. At 22 °C, mussels exposed to *rutile* presented significantly lower PROT content than non-contaminated organisms. No significant differences were observed between temperatures for each of the tested conditions (Fig. 3C).

3.4.2. Antioxidant and biotransformation defences

Values of the activity of SOD in mussels exposed to 0 μg/L, at 18 °C were inferior to those exposed to 5 μg/L (18 °C) but were superior to those exposed to higher concentrations (50 and 100 μg/L, 18 °C). At the warmest conditions, SOD activity significantly increased along the exposure gradient, with the highest values in mussels registered at the highest exposure concentration. Comparing both temperatures, SOD activity was significantly higher at 18 °C in non-contaminated organisms as well as in organisms exposed to the lowest tested concentration. However, an opposite pattern was observed at the highest concentrations (significantly higher activity in mussels at 22 °C exposed to 50 and 100 μg/L) (Fig. 4A).

The CAT activity showed no significant differences among tested conditions at 18 °C. At 22 °C contaminated mussels tended to decrease their CAT activity with the lowest value displayed in organisms exposed to 50 μg/L. Furthermore, significantly higher CAT values were observed in organisms contaminated with 5 and 100 μg/L at 22 °C than at 18 °C (Fig. 4B).

At 18 °C, contaminated mussels showed significantly higher GPx activity than non-contaminated mussels. At 22 °C, no significant differences were observed among conditions. Differences between temperatures were only observed in organisms exposed to 50 μg/L, with the highest activity found at 22 °C (Fig. 4C).

Mussels maintained at 18 °C showed significantly higher GSTs activity at the highest exposure concentration. At 22 °C a significant decrease was observed in organisms exposed to 50 μg/L in comparison to those exposed to 5 μg/L. Mussels maintained at 18 °C, showed significantly higher GSTs values, all except at 5 μg/L, compared to the ones at 22 °C (Fig. 4D).

3.4.3. Cellular damage

Organisms at 18 °C tended to maintain their LPO levels, with significantly lower values only at 5 μg/L compared to control. At 22 °C contaminated mussels showed lower LPO levels than non-contaminated ones, but significant differences to the control were only observed at 5 μg/L. Significantly higher LPO levels were observed in mussels exposed to 22 °C in comparison to the ones

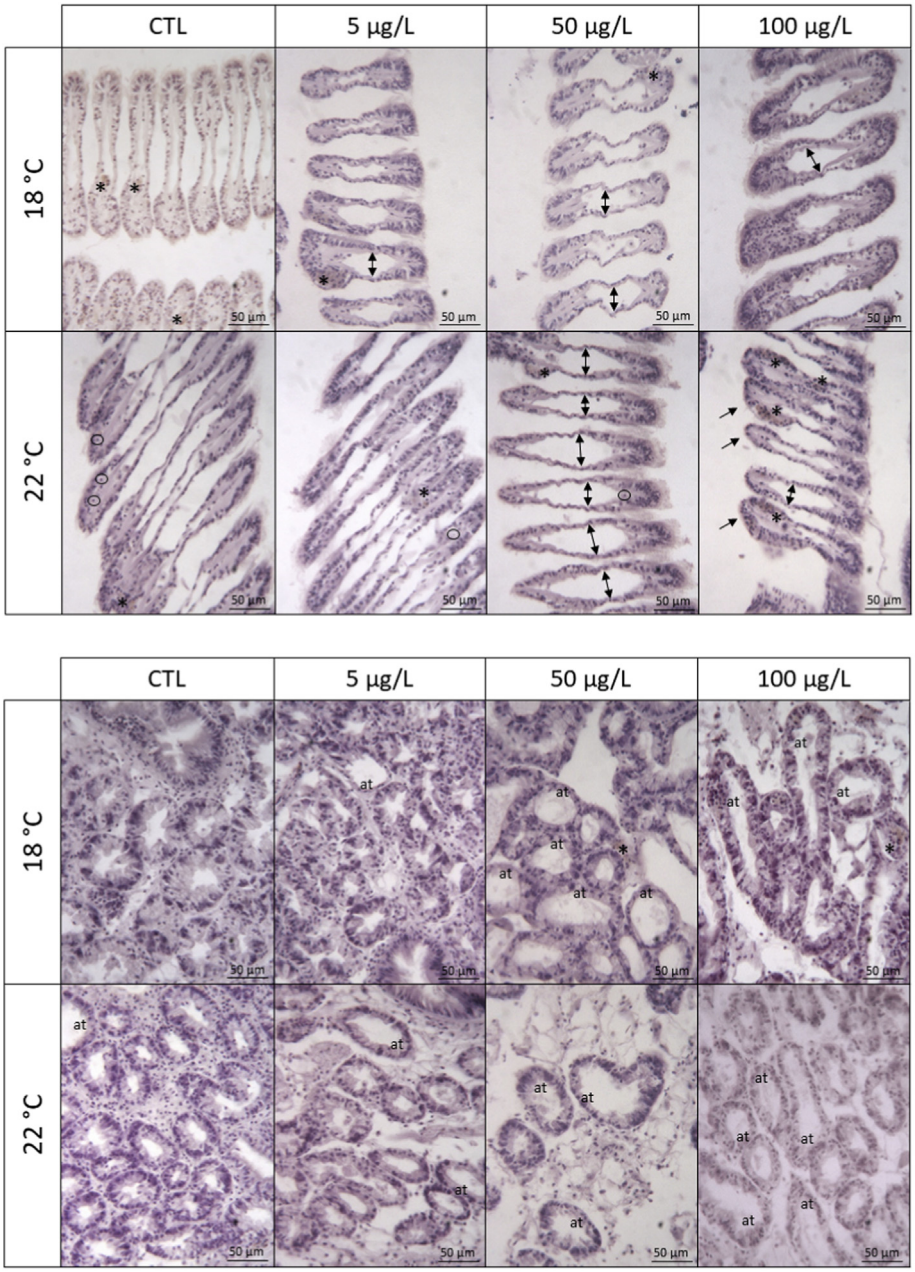

Fig. 1. Upper image: Micrographs of histopathological alterations in the gills; Lower image: Micrographs of histopathological alterations observed in the digestive tubules, of *Mytilus galloprovincialis* exposed to each condition (CTL-0, 5, 50 and 100 µg/L) and both temperatures (18 and 22 °C), stained with hematoxylin: lipofuscin aggregates (*); enlargement of the central vessel; hemocytes infiltration (circles) and loss of cilia (arrows) (n = 36 per organ). Scale bar 50 µm.

maintained at 18 °C (Fig. 5).

3.5. Multivariate analysis

Results from the PCO analysis are presented in Fig. 6. The first principal component axis (PCO1), which represents 33.8% of the variability separates non-contaminated organisms (control conditions at both temperatures) and mussels exposed to 5 and 50 µg/L under 22 °C (in the positive side), from the remaining conditions (in the negative side). PCO2 axis explained 29.4% of the variability, clearly separating organisms exposed to the temperature of 18 °C (positive side) from organisms exposed to 22 °C (negative side). PROT was positively associated to PCO1 positive axis, while Ti concentration in mussels' tissues was highly correlated with PCO1 in the negative axis. CAT and LPO levels were close associated with organisms exposed to 22 °C and lower test concentrations (5 and 50 µg/L) while histopathological indices (I_h Gills and I_h DG), GPx, and GLY were close related with organisms exposed to the highest test concentration (100 µg/L) at 22 °C. GSTs were closely related to organisms exposed to *rutile* at 18 °C.

4. Discussion

As studied by SEM microscopy and DLS measurements, the *rutile* TiO_2 particles were very aggregated. Upon dispersion in the exposure medium, it was observed an initial increase on the Z-average size of the particles, which after 1 h starts to decrease. This decrease on the aggregates size in suspension was assigned to the

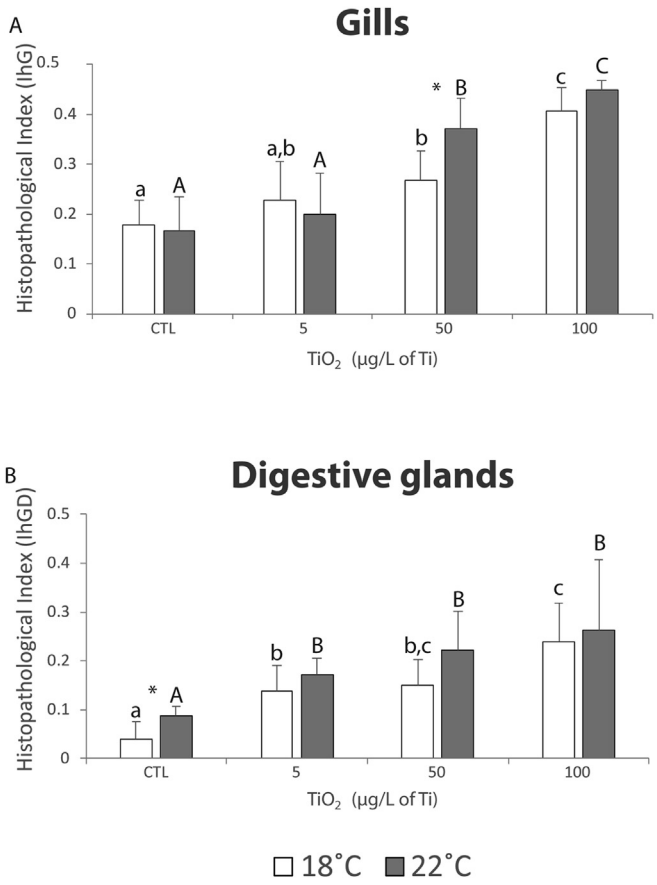

Fig. 2. A: Histopathological index in gills (I_hG); B: Histopathological index in digestive tubules (I_hDG), in *Mytilus galloprovincialis* exposed to each condition (CTL-0, 5, 50 and 100 µg/L) and both temperatures (18 and 22 °C). Values are mean + standard deviation. Significant differences ($p < 0.05$) among concentrations (CTL-0, 5, 50 and 100 µg/L) for each temperature (18 and 22 °C) are represented with different letters (lower case letters for 18 °C, upper case letters for 22 °C); while significant differences between temperatures for each Ti concentration are represented with an asterisk (n = 36 per organ).

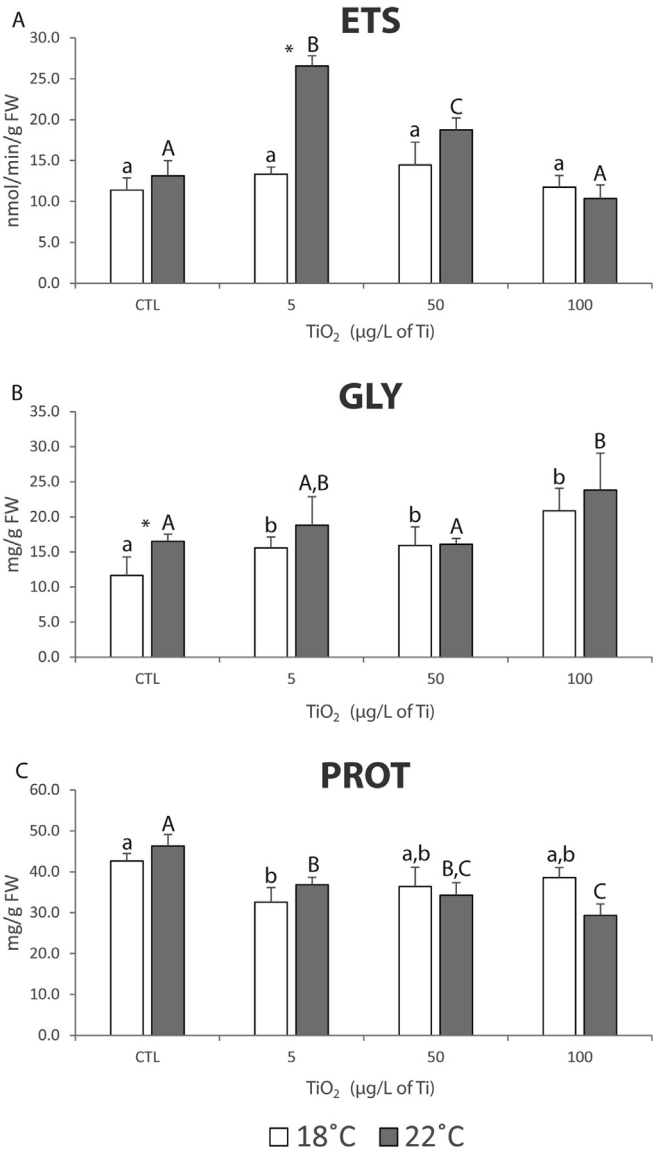

Fig. 3. A: Electron transport system activity (ETS); B: Glycogen content (GLY); C: Protein content (PROT), in *Mytilus galloprovincialis* exposed to each condition (CTL-0, 5, 50 and 100 µg/L) and both temperatures (18 and 22 °C). Values are mean + standard deviation. Significant differences ($p < 0.05$) among concentrations (CTL-0, 5, 50 and 100 µg/L) for each temperature (18 and 22 °C) are represented with different letters (lower case letters for 18 °C, upper case letters for 22 °C); while significant differences between temperatures for each tested concentration are represented with an asterisk.

sedimentation of the large *rutile* particles at the bottom of the aquaria, while the small ones, more stable, remain in suspension. Similar behaviour was also reported by Canesi et al. (2010) in artificial seawater. The authors reported the precipitation of large TiO_2 particles deep down the aquarium, making difficult the quantification of the amount of TiO_2 suspended in water. In addition, Zhu et al. (2011) related the agglomeration and the rapidly precipitation to the high ionic strength of the seawater due to the presence of chloride salts. The energy barrier to avoid agglomeration is inversely proportional to the ionic strength (Jiang et al., 2009). Thus, the observed decrease on the Z-average size in seawater can be explained by the highest tendency of the particles to aggregate as consequence of the ionic strength, leading to precipitation of the largest agglomerates.

4.1. Titanium concentrations in mussels' tissues

The present study revealed that differences in Ti concentrations found in mussels' tissues were only observed at the highest exposure concentration level, with higher values at the lowest temperature. The study conducted by Mikulášek et al. (1997) revealed that the interactive forces of TiO_2 are affected by temperature, showing that the increase of the temperature leads to a reduction of dispersion and shear stress of *rutile*. The authors explained this effect as a result of a decrease in the interactive forces between particles with temperature. For this reason, it was expected that at the highest temperature, the particles aggregate the most. Previous studies identified this possibility as a contributing factor to the highest accumulation and toxicity at superior temperatures. In particular, Ward and Kach (2009) demonstrated that bivalves could more efficiently capture and ingest particles that are incorporated into agglomerates than those freely dispersed. However, in the present study the highest accumulation was observed at the lowest temperature (18 °C) and not at the expected 22 °C. At higher temperature the lowest accumulation may be explained by higher precipitation of larger aggregates limiting the availability and accumulation of the particles.

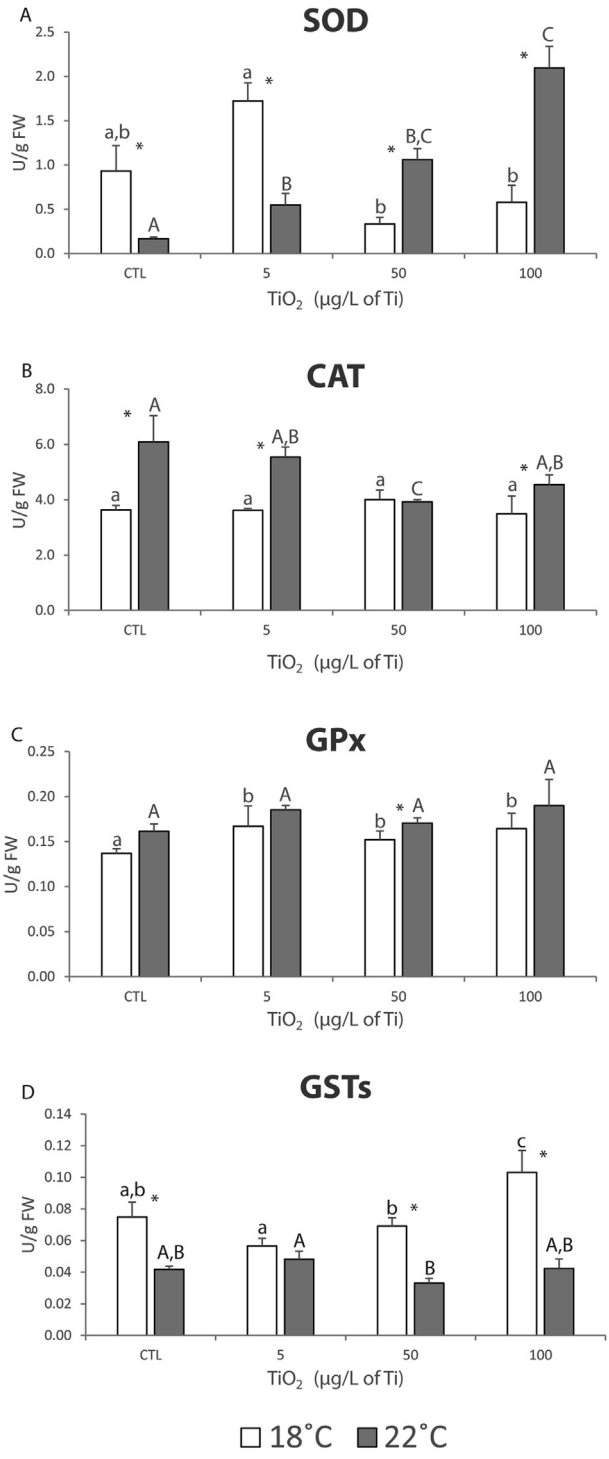

Fig. 4. A: Superoxide dismutase activity (SOD); B: Catalase activity (CAT); C: Glutathione peroxidase activity (GPx); D: Glutathione S-transferases activity (GSTs), in *Mytilus galloprovincialis* exposed to each condition (CTL-0, 5, 50 and 100 µg/L) and both temperatures (18 and 22 °C). Values are mean + standard deviation. Significant differences ($p < 0.05$) among concentrations (CTL-0, 5, 50 and 100 µg/L) for each temperature (18 and 22 °C) are represented with different letters (lower case letters for 18 °C, upper case letters for 22 °C); while significant differences between temperatures for each tested concentration are represented with an asterisk.

4.2. Histopathological alterations

The assessment of histopathological alterations is an important method to evaluate the impacts of pollutants in bivalves (Bignell et al., 2011; Cuevas et al., 2015). Gills are one of the major target organs for contaminants because they are in direct contact with the surrounding environment, playing an important role in respiration (Au, 2004; Rajalakshmi and Mohandas, 2005). The digestive glands of bivalves is the main organ for xenobiotic biotransformation, a mechanism of immune defence and homeostatic regulation (Livingstone et al., 2006), it has also been extensively used for toxicity assessments (Marigómez et al., 2013).

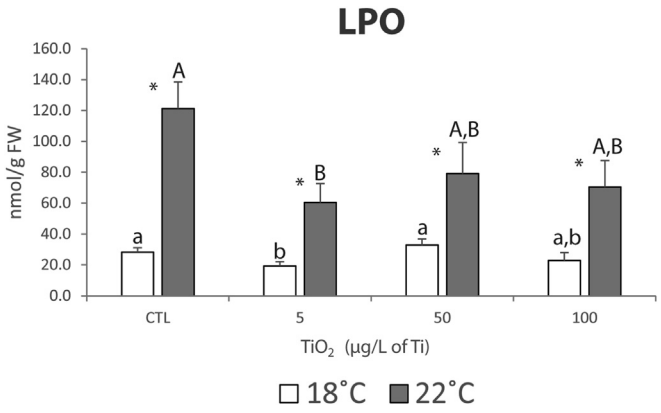

Fig. 5. Lipid peroxidation levels (LPO), in *Mytilus galloprovincialis* exposed to each condition (CTL-0, 5, 50 and 100 µg/L) and both temperatures (18 and 22 °C). Values are mean + standard deviation. Significant differences ($p < 0.05$) among concentrations (CTL-0, 5, 50 and 100 µg/L) for each temperature (18 and 22 °C) are represented with different letters (lower case letters for 18 °C, upper case letters for 22 °C); while significant differences between temperatures for each tested concentration are represented with an asterisk.

The present study revealed dose-dependent histopathological alterations in gills and digestive glands of mussels exposed to *rutile*. In particular, the results obtained demonstrated that both *rutile* and temperature induced histopathological alterations in gills and digestive glands of contaminated mussels. Regarding the mussels' gills, the presence of *rutile* mainly caused hemocyte infiltration which, according to different authors (Bignell et al., 2011; Costa et al., 2013; Cuevas et al., 2015; Rocha et al., 2016), is associated with inflammatory responses. Additionally, exposure to *rutile* caused the enlargement of mussels' central vessel and an abundance of lipofuscin aggregates. According to Höhn and Grune (2013), the presence of lipofuscin aggregates may indicate oxidative stress in the affected cells, which corroborates the LPO levels observed in mussels exposed to 22 °C. On the other hand, at 18 °C there was no evidence of LPO indicating low toxicity of *rutile* at control temperature, which was not corroborated by the histopathological results that showed alterations in gills due to the presence of *rutile*. At increased temperature contaminated organisms also evidenced loss of cilia which can lead to difficulties in filtering food and breathing problems (Pagano et al., 2016). Therefore, the present findings are in line with other studies that already demonstrated histopathological alterations in bivalve's gills when exposed to pollutants, namely lanthanum (Pinto et al., 2019), mercury (Amachree et al., 2014; Coppola et al., 2020), and 'bulk' TiO_2 and TiO_2 NPs (D'Agata et al., 2014). Regarding the mussels' digestive tubules, for both temperatures, the exposure to *rutile* mainly caused hemocytes infiltration, accumulation of lipofuscin and atrophy, which consists of a reduction in the thickness of epithelia accompanied by the enlargement of the digestive tubule lumen (Cuevas et al., 2015). At 22 °C mussels exposed to 50 and 100 µg/L showed signs of necrosis in digestive tubules, which is characterized by cellular rupture (do Amaral et al., 2019). Similarly, studies assessing impacts of contaminants, such as lanthanum

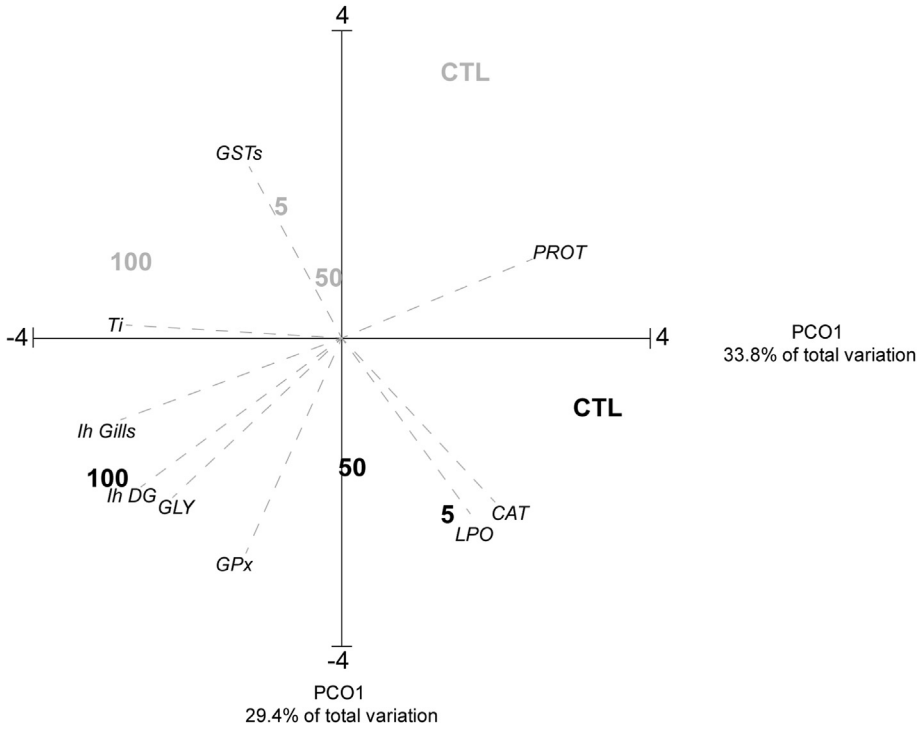

Fig. 6. Centroids ordination diagram (PCO) based on Ti concentrations, biochemical and histopathological parameters, measured in *Mytilus galloprovincialis* exposed to each condition (CTL-0, 5, 50 and 100 μg/L) and both temperatures (18 and 22 °C). Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data ($r > 0.75$): Ih Gills, Ih DG, Ti, GLY, CAT, PROT, GPx, GSTs, LPO. Gray letters: 18 °C conditions; Black letters: 22 °C conditions.

(Pinto et al., 2019) cadmium-based quantum dots (Rocha et al., 2016) and various metals (cadmium, chromium, copper, mercury, nickel, lead, zinc) (Coppola et al., 2020; Cuevas et al., 2015) demonstrated histopathologicalalterations in digestive glands in mussels.

4.3. Biochemical responses results

The results obtained evidenced that temperature greatly influenced mussels' biochemical performance, as identified by the PCO analysis, which separated conditions at 18 °C at the positive axis and conditions under 22 °C at the negative side of PCO2 (Fig. 6). However, the present study also demonstrated that *rutile* particles were responsible for biochemical alterations in mussels, with non-contaminated mussels and mussels exposed to the highest tested concentration in opposite sides of PCO1. This response may be related to greatest Ti bioaccumulation in mussels exposed to the highest exposure concentration. Furthermore, temperature influences the accumulation of Ti in mussels' tissues, with the highest accumulation levels found at 18 °C. Therefore, because at 22 °C lower accumulation was observed, differences in mussels' biochemical responses observed at different temperatures may also result from increased sensitivity of organisms to *rutile* due to temperature rise. Previous studies already demonstrated that bivalves increased the accumulation of pollutants along an increasing exposure gradient (Velez et al., 2015, 2016) while studies with Ti demonstrated significantly higher accumulation in mussels' tissues only at the highest exposure concentration (100 μg/L, Monteiro et al., 2019b). Studies assessing the impacts of pollutants under warming conditions evidenced contrasting results, with higher accumulation levels of arsenic in *M. galloprovincialis* mussels under increased temperatures (Coppola et al., 2018), while triclosan concentrations were higher in the same species exposed to control temperature and no temperature effects were noticed on lead bioaccumulation (Pirone et al., 2019). Thus, according to the present findings and results from previously published studies, it is possible to hypothesise that the effects of temperature on pollutants bioaccumulation will depend on the pollutant.

In general, the present results demonstrated that the effects caused by *rutile* were higher in mussels contaminated under warmer temperature (22 °C). In particular, the metabolic capacity of mussels was not altered in organisms exposed to *rutile* at 18 °C, revealing that the concentrations tested were not enough to impact their metabolism. However, when exposed to increased temperature the impacts of *rutile* on mussels' metabolism were noticeable, especially at 5 and 50 μg/L, evidencing that its toxicity may be enhanced under warmer conditions or, alternatively, the sensitivity of mussels to these particles may increase under higher temperatures. Because non-contaminated mussels exposed to 22 °C did not show any significant alteration on ETS activity compared to non-contaminated mussels exposed to 18 °C we may hypothesise that alterations on mussels' metabolism resulted from the increased toxicity of *rutile* under 22 °C, with mussels increasing their metabolic capacity to activate their defence mechanisms. These results can indicate that mussels exposed to the higher temperature and lower *rutile* concentrations were able to activate their metabolism probably to fight against high stress levels, but with increasing exposure concentrations mussels were no longer able to maintain this behaviour. Metabolic depression was already described in mussels as a response to pollutants exposure, normally associated with bivalves' capacity to maintain their valves closed, reduce the filtration rate and avoid accumulation of xenobiotics (Gosling, 2003). Thus, the present findings are in agreement with previous studies, also conducted with *M. galloprovincialis,* which demonstrated a metabolic depression as a response to the exposure to pollutants, namely titanium (Monteiro et al., 2019a), lanthanum (Pinto et al., 2019) and gadolinium (Henriques et al., 2019).

In terms of energy reserves, the present study found that

mussels were able to preserve the expenditure of GLY, especially at the most stressful conditions (100 μg/L at both temperatures). These results follow the decrease of ETS activity at 22 °C, indicating that higher stressful conditions mussels tried to prevent the negative impacts by limiting their metabolic activity and saving GLY. However, regarding PROT content, the results obtained showed a different response, with a tendency to decrease the total PROT content with the increase of *rutile* concentration, especially at the highest temperature. These findings pointed out that the stress induced was not enough to increase the production of proteins (namely enzymes), therefore this energy resource tended to decrease (especially at the highest exposure concentration). Previous studies already evidenced the bivalves' capacity to preserve their energy reserves when under stressful conditions, a behaviour normally associated with a decreasing metabolic activity. In particular, the work of Monteiro et al. (2019a,b) found an increase in GLY content in *M. galloprovincialis* with increasing Ti concentration. Duquesne et al. (2004) also demonstrated the same pattern in *Macoma balthica* exposed to cadmium. De Marchi et al., 2018 showed a decrease in PROT content in *Ruditapes philippinarum* exposed to carbon nanotubes.

When organisms, including mussels, are exposed to pollutants the production of reactive oxygen species (ROS) normally increases, leading to the activation of the antioxidant defences, including the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Regoli and Giuliani, 2014). The present results suggest that, at 18 °C, the activity of antioxidant defences was not activated even with increasing exposure concentrations, corroborating the hypothesis that the concentrations tested were not enough to activate the antioxidant defences, or other mechanisms of defence, such as detoxification mechanisms, were enough to prevent impacts especially at higher concentrations. In fact, the detoxification capacity was activated, here evidenced by increased glutathione-S-transferases (GSTs) activity, especially at higher *rutile* concentrations. When exposed to pollutants organisms develop mechanisms of defence that are responsible for lowering the stress induced. Such mechanisms involve the detoxification of xenobiotic substances, as is the case of GSTs, whose main function is to catalyse the conjugation of a diverse array of electrophilic compounds with glutathione (Regoli and Giuliani, 2014). Coppola et al. (2018) demonstrated that at control temperature and in the presence of arsenic, the activity of antioxidant defences (SOD and CAT) in *M. galloprovincialis* were not significantly increased which was associated with the capacity of bivalves to activate detoxification mechanisms (GSTs). Moreover, Ale et al. (2019) showed that the activity of CAT was not activated in *M. galloprovincialis* exposed to silver nanoparticles while the activity of GSTs was activated. A study conducted by Mezni et al. (2018) showed that the activity of SOD was not significantly increased in digestive glands of *M. galloprovincialis* at control temperature when exposed to a gradients of TiO_2 NPs. Also, Monteiro et al. (2019a) demonstrated an increase of GSTs activity with increasing exposure concentrations of Ti in *M. galloprovincialis*. Nevertheless, at the higher temperature (22 °C) an opposite behaviour was observed, with mussels increasing the activity of SOD with the increase of *rutile* concentration while CAT was inhibited in contaminated organisms. Such findings may result from the inefficient capacity of GSTs to detoxify *rutile* at the warmest conditions which, in turn, resulted in higher stress levels, which activate the antioxidant defences as SOD and inhibit CAT. It was already demonstrated that GSTs may be inhibited in bivalves exposed to warm conditions, resulting into high stress levels and inhibition of antioxidant enzymes at extreme conditions. Coppola et al. (2018) showed that *M. galloprovincialis* increases the activity of antioxidant defences when exposed to arsenic under warm conditions. Pirone et al. (2019) also demonstrated that the activity of antioxidant defences was not activated in *M. galloprovincialis* at control temperature exposed to lead but when temperature is raised to 22 °C, mussels increase their SOD activity. The inhibition of GSTs was demonstrated by Andrade et al. (2019) by exposing *M. galloprovincialis* to carbon nanotubes under warm conditions (21 °C).

Regarding cellular damage, the results here presented showed that no lipid peroxidation (LPO) occurred in contaminated mussels at 18 °C evidencing that no cellular damage was observed in mussels exposed to *rutile* under control temperature probably due to low toxicity of *rutile*. These results also highlight the efficiency of the biotransformation defence system to detoxify *rutile*. Such response may result from the mussels' increased capacity to activate their detoxification mechanisms, preventing organisms from cellular damage and oxidative stress. On the other hand, at increased temperature, cellular damages were observed despite the enhancement of antioxidant mechanisms in contaminated organisms. These damages were a result of general oxidative status in mussels exposed to higher temperature and *rutile*. This oxidative status may also result from the inefficient capacity of mussels to activate GSTs and eliminate *rutile*. Previous studies conducted by Coppola et al. (2017, 2018) have already showed that no LPO occurred in contaminated mussels (*M. galloprovincialis*) exposed mercury and arsenic at control temperature, while under higher temperature (22 °C) LPO levels increased. Additionally, Freitas et al. (2017) showed higher LPO levels in *M. galloprovincialis* exposed to mercury under warm conditions compared to control temperature.

5. Conclusions

Overall, the present study demonstrates that temperature rise may significantly increase the sensitivity of bivalves towards *rutile*, revealing higher toxic impacts in mussels exposed to this pollutant under warming conditions than in mussels maintained at control temperature. Such response can result from the mussels' inefficient capacity of biotransformation enzymes (GSTs) that at the highest temperature are inhibited (concentrations 50 and 100 μg/L). Nevertheless, regardless of temperature, mussels exposed to *rutile* demonstrated histopathological effects in a dose-dependent patterns, with gills and digestive glands showing impacts that can compromise its physiological performance, including filtration and respiration rates, thus impacting growth and reproductive capacities.

Author statement

Rosa Freitas and Eduarda Pereira are supervisors of Carla Leite (master student) and Francesca Coppola and Rui Monteiro (PhD students) that help Carla Leite in the lab, including help to perform biochemical analyses (Francesca Coppola) and chemicals preparation and Ti quantification (Rui Monteiro). Rosa Freitas and Eduarda Pereira gave the idea of this study to the students that accepted this challenge and performed all the analyses in the lab. Eduarda Pereira is the responsible for the laboratory where chemical quantifications were done. Mariana R. F. Silva, Mirtha A. O. Lourenço, Paula Ferreira were responsible for *rutile* characterization analyses. Gianluca Polese supervised the histopathological analyses performed by Carla Leite. Rosa Freitas and Amadeu Soares are the responsible persons for the labs where 1biomarkers were determined. Eduarda Pereira, Rosa Freitas, Paula Ferreira and Amadeu Soares funded this study.

Declaration of competing 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.

Acknowledgments

Carla Leite benefited from BSc grant under the project ASAR-ISAFE (NSFC/0001/2016) funded by the Portuguese Science Foundation (FCT). Francesca Coppola, and Rui Monteiro benefited from PhD grants (SFRH/BD/118582/2016 and SFRH/BD/108535/2015, respectively), given by the National Funds through the FCT, supported by FSE and Programa Operacional Capital Humano (POCH) e da União Europeia. Paula Ferreira acknowledge the grant IF/00300/2015. Rosa Freitas was funded by national funds (OE), through FCT, in the scope of the framework contract foreseen in the numbers 4, 5 and 6 of the article 23, of the Decree-Law 57/2016, of August 29, changed by Law 57/2017, of July 19. This work was also financially supported by the project BISPECIAl: BIvalveS under Polluted Environment and ClImate chAnge PTDC/CTA-AMB/28425/2017 (POCI-01-0145-FEDER-028425) funded by FEDER, through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI), and by national funds (OE), through FCT/MCTES. Thanks are due for the financial support to CESAM (UIDB/50017/2020+UIDP/50017/2020), CICECO (UIDB/50011/2020 & UIDP/50011/2020) and Smart Green Homes Project (POCI-01-0247-FEDER-007678), financed by national funds through the FCT/MEC and co-financed by FEDER under the PT2020 Partnership Agreement and Compete 2020.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.126563.

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