Chemosphere 271 (2021) 129775

Contents lists available at ScienceDirect

Chemosphere

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

# Effects of temperature on caffeine and carbon nanotubes co-exposure in *Ruditapes philippinarum*

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## HIGHLIGHTS

- Higher caffeine accumulation in the presence of f-MWCNTs, especially at 21 °C.
- Lower metabolic capacity at 18 °C, especially in the presence of caffeine.
- Increased antioxidant capacity at 18 °C, especially when caffeine was acting alone.
- Higher biotransformation capacity at 18 °C, namely when pollutants were combined.
- Cellular damage occurred in contaminated clams, regardless the temperature.

## ARTICLE INFO

*Article history:*
Received 5 December 2020
Received in revised form
20 January 2021
Accepted 21 January 2021
Available online 27 January 2021

Handling Editor: Michael Bank

*Keywords:*
Clams
Mixture of pollutants
Warming
Oxidative stress
Metabolism
Histopathological alterations

## ABSTRACT

In the marine environment, organisms are exposed to a high and increasing number of different contaminants that can interact among them. In addition, abiotic factors can change the dynamics between contaminants and organisms, thus increasing or even decreasing the toxic effect of a particular compound. In this study, the effects of caffeine (CAF) and functionalized multi-walled carbon nanotubes (f-MWCNTs) induced in the clam *Ruditapes philippinarum* were evaluated, acting alone and in combination (MIX), under two temperature levels (18 and 21 °C). To assess the impact of such compounds, their interaction and the possible influence of temperature, biochemical and histopathological markers were investigated. The effects of f-MWCNTs and caffeine appear to be clearly negative at the control temperature, with lower protein content in contaminated clams and a significant decrease in their metabolism when both pollutants were acting in combination. Also, at control temperature, clams exposed to pollutants showed increased antioxidant capacity, especially when caffeine was acting alone, although cellular damages were still observed at CAF and f-MWCNTs treatments. Increased biotransformation capacity at 18 °C and MIX treatment may explain lower caffeine concentration observed. At increased temperature differences among treatments were not so evident as at 18 °C, with a similar biological pattern among contaminated and control clams. Higher caffeine accumulation at MIX treatment under warming conditions may result from clams' inefficient biotransformation capacity when exposed to increased temperatures.



## 1. Introduction

As the majority of the human population lives near the coast, it is expected that the population growth and technological advances

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https://doi.org/10.1016/j.chemosphere.2021.129775
0045-6535/

will lead to an increase of pollutants discharges in the marine environment (Schiedek et al., 2007; Ostle et al., 2019; Daoji and Daler, 2004). Industries, agriculture, farms, aquaculture plants and domestic inputs are the main sources of pollution in coastal areas (Yuan et al., 2007). Among the various chemicals discharged (Beg et al., 2001; El Zrelli et al., 2015; Muniz et al., 2015), the common presence of caffeine in coastal areas (del Rey et al., 2012; Siegener and Chen., 2002) has led to its use as an indicator for human pollution (Hillebrand et al., 2012). Caffeine, or 1,3,7-trimethylxanthine is an alkaloid naturally present in some plants, such as *Coffea arabica*, *Camellia thea* and *Cola acuminate* and in beverages like tea, coffee and energy drinks due to its stimulant effect on the central nervous system (Barone and Roberts, 1996; Rogers and Smith, 2011). Most of caffeine is metabolized by the human body while a small amount (up to 10%) is excreted and, through wastewater treatment plants (WWTPs), may reach aquatic environments (Ferreira et al., 2005; Buerge et al., 2003). Studies revealed that caffeine concentration levels may range between 18 and 525 ng/L in coastal areas (as measured in Portugal coasts by Paíga and Delerue-Matos, 2017), but higher concentrations (3.0 μg/L) have been reported for other coastal systems (Ali et al., 2017). Caffeine has already been shown to have negative impacts in marine species, including bivalves (Cruz et al., 2016; Munari et al., 2020) and other invertebrates (Pires et al., 2016a and b), with changes in organism's oxidative status, metabolic capacity, regeneration ability and immunological parameters.

Besides caffeine, several other pollutants arrive to coastal areas, including newly developed chemicals and substances (e.g. nanoparticles, parabens, flame retardants and personal care products), currently identified as emerging contaminants (Sauvé and Desrosiers, 2014). Due to inefficient elimination processes in WWTPs, these compounds have been identified in several aquatic environments (Gogoi et al., 2018). Among the most prominent class of emerging contaminants are carbon-based nanoparticles and, in particular, carbon nanotubes (CNTs) (Esawi and Farag, 2007; Liu et al., 2013). Carbon nanotubes are entirely made by one layer of carbon atoms folded to form a hollow cylinder. They are considered to be one of the most widely used nanomaterials, employed in different applications including electronic devices, reinforced materials and biomedical applications (Trojanowicz, 2006). They can be categorized by their structure, in single-walled (SWCNTs) and multi-walled (MWCNTs), and by their functionalization through the addition of specific chemical functionalities (Hirsch and Vostrowsky, 2005). This modification can greatly change CNTs' behaviour in the ecosystem, namely improving their solubility and dispersion (Sun et al., 2002). In the aquatic environment, the predicted concentrations (PECs) for MWCNTs are projected to range between 0.001 and 1000 μg/L (Zhang et al., 2017). Nevertheless, previous studies already pointed out the negative impacts caused by CNTs in bivalves: Zha et al. (2019) showed an immunotoxic effect on *Terillarca granosa*; Freitas et al. (2018) and De Marchi et al. (2020) revealed metabolic impacts and oxidative stress on *Ruditapes philippinarum* and *Mytilus galloprovincialis*; Ward and Kach (2009) observed enhanced uptake and gut retention in *M. edulis* and *Crassostrea virginica* when exposed to nanoparticles' aggregates, which could be significant in biomagnification and trophic transfer processes.

Accompanying the major threat represented by pollution, another source of concern is global warming. Currently, climate change is considered one of the most important causes of ecosystems degradation (Malcolm et al., 2006; Prăvălie, 2018; Traill et al., 2010), with important ecological and economic consequences (Hughes et al., 2018; Micheli et al., 2013; Rogelj et al., 2013). Impacts are greatly derived from the continuous emission of greenhouse gases, among which carbon dioxide ($CO_2$) is the main contributor (Montzka et al., 2011), leading to temperature rise in the atmosphere and aquatic environments (IPCC, 2018). It is predicted that $CO_2$ in the atmosphere already caused a warming of 1.0 °C compared to pre-industrial levels, while it seems likely to reach 1.5 °C between 2030 and 2052 (IPCC, 2018). As for the atmosphere, the oceans are warming up (Gleckler et al., 2012) with sea surface temperature projections ranging between 0.64 (RCP2.6) and 0.95 (RCP8.5) for the period 2031-2050, and between 0.73 (RCP2.6) and 2.58 (RCP8.5) for the period 2081-2100, relative to the recent past (1986-2005) (IPCC, 2019). Such changes are already causing strong impacts on marine organisms. Focusing on bivalves, rising seawater temperature seems to affect *Limecola balthica* recruitment (Philippart et al., 2003); also, a relationship between climate change and the immune response has been reported in *Chamelea gallina* and *M. galloprovincialis* (e.g. Matozzo et al., 2012a and b); furthermore, it was demonstrated that mass mortality events in temperature sensitive species, such as *C. virginica* and *M. galloprovincialis* are caused by rising temperature (Ivanina et al., 2013; Gazeau et al., 2014); changes in *R. philippinarum* and *M. galloprovincialis* metabolic capacity, oxidative and neurotoxic status were also demonstrated after exposure to warming conditions (Andrade et al., 2019b; Freitas et al., 2019a; Velez et al., 2017).

*Ruditapes philippinarum* , is a bivalve species native of Japan, Korea and the Philippines, that was imported to the Mediterranean and Atlantic coasts of Europe for aquaculture purposes (Gosling, 2008). This species is considered a good indicator to assess pollutants effects (Shin et al., 2002; Moraga et al., 2002; Aguirre-Martínez et al., 2013; Liu et al., 2011; Matozzo et al., 2012a and b), reason why it was selected for the present study. Although in literature single effects of caffeine and MWCNTs on *R. philippinarum* were already described (as for example, Cruz et al., 2016; De Marchi et al., 2017), to the best of our knowledge, no studies evaluated the combined effects of these two pollutants under actual and predicted temperature scenarios. For this reason, the aims of this study were focused on the evaluation of the effects of caffeine (CAF) and -COOH functionalized form of MWCNTs (f-MWCNTs) acting alone and in combination (MIX) in *R. philippinarum*, considering two temperatures (18 and 21 °C). Biochemical and histopathological alterations were investigated after a twenty-eight days exposure period.

## 2. Methodology

### 2.1. Sampling and experiment conditions

*Ruditapes philippinarum* organisms were sampled in February from the Óbidos lagoon (39° 25′ 0″ N, 9° 13′ 0″ W), in central Portugal. Clams of similar size (mean length of 4.0 ± 0.3 cm; mean width of 3.0 ± 0.3 cm) were selected in order to avoid variability in biological responses. Before starting the exposure, clams were subjected to an acclimation period that lasted two weeks. During this period clams were maintained in tanks of 50 L with constant aeration and exposed to conditions resembling the sampling site: temperature 18 ± 1 °C, artificial seawater with salinity 30 ± 1, prepared with Red Sea Salt (Red Sea) and tap water purified by reverse osmosis (four stage unit, Aqua-win RO-6080, Thailand), pH 8.0 ± 0.1 and light/dark cycles of 12 h. After the first three days of acclimation, clams were fed every other day with 10 mL/L of algae solution (prepared with 1 g of Algamac Protein plus per 1 L distilled water) as described by Coppola et al. (2020b) and Freitas et al. (2020).

After acclimation clams were divided and submitted to the following treatments during twenty-eight days: control, 0 μg/L of

CAF and 0 μg/L of −COOH functionalized multi-walled carbon nanotubes, f-MWCNTs (CTL); caffeine, 0.5 μg/L (CAF); −COOH functionalized multi-walled carbon nanotubes, 10 μg/L (f-MWCNTs), and the combination of both contaminants with 0.5 μg/L of CAF and 10 μg/L f-MWCNTs (MIX). This experimental setup was performed under two different temperatures (18 and 21 °C). MWCNTs functionalized with -COOH are characterized by a better dispersibility in aqueous media, higher colloidal stability and are more prone to adsorb different molecules. As such they were selected for the present study.

For each treatment, three replica aquaria (5 L) were prepared, each aquarium with 6 clams. In addition, 4 aquaria *per* temperature (8 in total) without clams were used as blanks for caffeine quantification: 2 contaminated with 0.5 μg/L CAF, 2 contaminated with 0.5 μg/L CAF and 10 μg/L f-MWCNTs. Water conditions (temperature, salinity, pH, aeration), feeding and photoperiod were maintained constant, as described for the acclimation period. The concentration of caffeine was selected to resemble environmental conditions (Paíga and Delerue-Matos, 2017; Siegener and Chen, 2002). The concentration of f-MWCNTs was chosen to fall within predicted environmental values (Zhang et al., 2017) and values already shown to cause effects in marine invertebrates (De Marchi et al., 2017, 2018, 2020).

Mortality was checked daily and the water was completely renewed each week, after which conditions were re-established for each treatment (temperature and pollutants concentrations).

For quantification purposes water samples were taken immediately after recontamination (to compare nominal concentration of caffeine with the real ones − exposure medium, and assess concentration losses along the exposure period - blanks) and before water renewal (for f-MWCNTs characterization − exposure medium; and for caffeine quantification to assess concentration losses along the exposure period - blanks).

After the exposure period, five organisms *per* aquarium were frozen with liquid nitrogen and stored at −80 °C to be used for biomarker analyses and caffeine quantification. One clam *per* aquarium was fixed in Bouin solution for 24 h at room temperature for histopathological analysis. During the exposure period, no mortality was registered for all treatments.

### 2.2. Caffeine quantification in seawater and organisms

Caffeine concentration in the exposure medium, blanks and clams' tissues were determined by direct competitive ELISA, following the protocol of Silva et al. (2014). Initially 96-well high-binding microtiter plates were coated with a polyclonal antibody overnight. After this period, the plates were washed and the monoclonal antibody against caffeine was incubated for 90 min. Then another washing step was performed and the sample buffer, the standards, the samples and the tracer solution were incubated for 40 min. After another washing step, the substrate solution was added to each well and the plate incubated for 30 min. The enzyme reaction was stopped by the addition of sulfuric acid to each well and the optical density was read on a microplate spectrophotometer at 450 nm and referenced to 650 nm. The results were expressed in μg/L for caffeine in the exposure medium and blanks, and in ng/g fresh weight (FW) for caffeine in clams' tissues.

### 2.3. Functionalized multiwalled carbon nanotubes characterization

Dynamic Light Scattering analysis (DLS) was used to assess the presence of macro/micro/nano-sized particle aggregates of f-MWCNTs suspended in aqueous media containing organisms at salinity 30 and different temperatures (18 and 21 °C) either alone (f-MWCNTs) or in combination with caffeine (MIX). The characterization was also performed on the samples not exposed to contaminants (CTL). DLS measurements were carried out by using a Delsa Nano C from Beckman Coulter, Inc. (Fullerton, CA) equipped with a laser diode operating at 658 nm. Scattered light was detected at 165° angle and analysed by using a log correlator over 120 accumulations for a 1.0 mL of sample in a UV cuvette semi-micro. Each sample was reproducibly shaken before analysis and exposed to four DLS measurements. The calculation of the particle size distribution and distribution averages was performed by using CONTIN particle size distribution analysis routines through Delsa Nano 3.73 software. The hydrodynamic radius and polydispersity index of the analysed dispersions were calculated by using the cumulant method. Undetectable colloidal material at the end of each measurement is indicated as invalid data (i.d.). The analyses were repeated several times due to the inherent heterogeneity and colloidal instability of the analysed samples.

### 2.4. Biochemical parameters

To perform biochemical analyses, soft tissues were carefully removed from the shells of three animals *per* aquaria (nine *per* treatment) and were manually homogenized with a mortar and a pestle under liquid nitrogen. The samples were divided in aliquots of 0.5 g of FW. For each treatment, the markers selected were: electron transport system (ETS) activity, to assess metabolic capacity; glycogen (GLY) and total protein (PROT) contents, to estimate the amount of energy reserves; the activity of catalase (CAT) as a measure of antioxidant capacity; the activity of glutathione S-transferases (GSTs) as an indicator of biotransformation defense; lipid peroxidation (LPO) as an indicator of cellular damage. The extraction for each biomarker was performed with specific buffers, as already described by Andrade et al. (2019a and b) and Coppola et al. (2020a and b). Before performing each biochemical analysis, the tissues were homogenized for 30 s at 4 °C with Tissue Lyser II and centrifuged for 20 min at 10000 *g* (or 3000 *g* for ETS) at 4 °C. The collected supernatants were stored at −80 °C. All the biomarker evaluations were duplicated and performed using a microplate reader (Biotek).

#### 2.4.1. Metabolic capacity

The ETS activity was determined according to King and Packard (1975) with modifications by Coen et al., 1997. The absorbance was read at 490 nm every 25 s for 10 min ($\varepsilon = 15.900\ M^{-1}cm^{-1}$). Results were expressed in nmol/min *per* g of FW.

#### 2.4.2. Energy reserves

The GLY content was measured according Dubois et al. (1956), with a standard curve consisting of glucose standard concentrations (0−10 mg/mL). The absorbance was read at 492 nm. The results were expressed as mg *per* g of FW.

The total PROT content was determined by the biuret method according to Robinson and Hogden (1940). A single reading of absorbance was performed at 540 nm. The standard curve consisted of a series of bovine serum albumin solutions (0, 5, 10, 20, 40 mg/mL). Results were expressed as mg *per* g of FW.

#### 2.4.3. Antioxidant capacity

The activity of CAT was determined according Johansson and Borg (1988) with a curve made by standard solutions of formaldehyde (0−150 μM). The reading was performed at 540 nm and the results were expressed as U *per* g of FW where U indicates the formation of 1 nmol of formaldehyde per min at 25 °C.

#### 2.4.4. Biotransformation defense

The activity of GSTs was determined according to Habig et al.,

1974 with a reading wavelength at 340 nm ($\varepsilon = 9.6\ mM^{-1}cm^{-1}$) and the results were expressed as U *per* g of FW, where U stands for the formation of 1 μmol of dinitrophenyl thioether per min.

#### 2.4.5. Cellular damage

Levels of LPO were assessed according to Ohkawa et al. (1979), with the quantification of malondialdehyde (MDA, a by-product of lipid peroxidation) at 535 nm ($\varepsilon = 156\ mM^{-1}cm^{-1}$). The results were expressed in nmol of MDA *per* g of FW.

### 2.5. Histopathological analyses

The whole soft tissue from one clam per aquaria (three per treatment) was fixed in Bouin solution for 24 h. Afterwards, clam's tissue was placed in ethanol 70%, which was daily changed to remove the fixative solution. Gills and digestive tubules were separated, dehydrated and included in paraffin (see also Coppola et al., 2020a and b).

Hardened paraffin containing gills and digestive tubules was cut in sections of 7 μm with a microtome. The sections were placed on slides that were stained using hematoxylin. For each treatment, gills and digestive tubules were examined to point out histological alterations.

Following the procedure described by Costa et al. (2013), the histopathological index was calculated using the formula:

$$I_h = \frac{\sum_1^j w_j a_{jh}}{\sum_1^j M_j}$$

Where $w_j$ indicates the weight of the $j_{th}$ alteration, $a_{jh}$ stands for a score given to the $h_{th}$ specimen for the $j_{th}$ alteration and $M_j$ represents the maximum value that can be reached by the $j_{th}$ alteration.

The $I_h$ was determined following the concepts of the differential biological significance of each surveyed alteration (weight) and its degree of dissemination (score). The weights range between 1 (least severe) and 3 (most severe), while score ranges between 0 (not present) and 6 (diffuse).

### 2.6. Integrated biomarker response

The integrated biomarker response (IBR) index was calculated following Beliaeff and Burgeot (2002). Biomarkers were arranged in the following order: ETS, GLY, PROT, CAT, GSTs and LPO. Higher IBR value corresponds to higher organism response.

### 2.7. Statistical analysis

Caffeine quantification, biomarker results and histopathological analysis results were all submitted to hypothesis testing with permutational multivariate analysis of variance using PERMANOVA + for PRIMER v6 statistical software (see Anderson et al., 2008).

The null hypothesis were: i) for caffeine in water and clams, no significant differences exist between treatments (CAF *vs* MIX) at 18 °C (uppercase letters) and 21 °C (lowercase letters) (Table 1); ii) for histopathological analysis no significant differences exist among treatments at 18 °C (uppercase letters in Table 3) and 21 °C (lowercase letters in Table 3); iii) for biomarkers analysis no significant differences exist among treatments at 18 °C (uppercase letters in Table 3) and 21 °C (lowercase letters in Table 3); iv) for each response and for each treatment no significant differences exist between temperatures (18 and 21 °C), represented in Tables 1 and 3 by an asterisk. Only differences with a $p < 0.05$ were considered significant.

The matrix expressing biochemical markers, histopathological indexes and caffeine quantification in water and tissues under 18 and 21 °C was normalized and the Euclidean distances among centroids were calculated. Such distances were visualized in principal coordinates ordination (PCoA) analysis.

## 3. Results

### 3.1. Caffeine quantification

Caffeine concentrations in the exposure medium, both when acting alone (CAF) and as a mixture with f-MWCNTs, did not differ significantly between treatments (CAF *vs* MIX) at each temperature, as well as between temperatures for each treatment (Table 1). These results showed that after spiking, caffeine concentrations found in the exposure medium were similar to the nominal concentrations, thus demonstrating the effective clam exposure.

In blanks no significant differences were observed along the exposure period (beginning and end of each week), for both temperatures and caffeine treatments (acting alone or combined with f-MWCNTs), demonstrating the stability of caffeine in the medium along one-week exposure period, regardless the temperature (Table 1).

When analyzing clams' tissues, although clams tended to accumulate higher caffeine concentration at 21 °C (both at CAF and MIX treatments), significant differences between temperatures were only found at the MIX treatment (Table 1). At each temperature no significant differences were found between CAF and MIX treatments, although higher caffeine values were found at MIX treatment (Table 1).

### 3.2. Functionalized multi-walled carbon nanotubes characterization

The results from Dynamic Light Scattering (DLS) analysis of aqueous media containing *Ruditapes philippinarum* as tested organisms exposed to functionalized multi-walled carbon nanotubes either alone (f-MWCNTs) or in combination with caffeine (MIX) at two different temperatures (18 and 21 °C) are reported in the Table 2. CNT-based aggregates were unambiguously detected in seawater samples containing *R. philippinarum* exposed to f-MWCNTs and CAF as contaminants at 18 °C since no aggregates were found in the controls at the same experimental conditions

**Table 1**
Caffeine concentration in CAF and MIX treatments for the two temperature levels (18 and 21 °C) obtained for the exposure medium and blanks (collected at the beginning and end of the exposure weeks) and in *Ruditapes philippinarum* clams' tissues (collected at the end of the experiment). Significant differences between treatments (CAF and MIX) at each sampling moment and temperature level were represented with different letters (uppercase letters for 18 °C samples; lowercase letters for 21 °C samples). An asterisk (*) was used to identify differences comparing temperature levels for each treatment at each sampling moment.

| Conditions | 18 °C | | 21 °C | |
|---|---|---|---|---|
| | Beginning | End | Beginning | End |
| **Exposure medium (μg/L)** | | | | |
| CAF | $0.5 \pm 0.1^{A}$ | | $0.53 \pm 0.07^{a}$ | |
| MIX | $0.52 \pm 0.07^{A}$ | | $0.5 \pm 0.1^{a}$ | |
| **Blanks (μg/L)** | | | | |
| CAF | $0.5 \pm 0.1^{A}$ | $0.55 \pm 0.06^{A}$ | $0.5 \pm 0.1^{a}$ | $0.59 \pm 0.08^{a}$ |
| MIX | $0.5 \pm 0.1^{A}$ | $0.59 \pm 0.05^{A}$ | $0.55 \pm 0.08^{a}$ | $0.58 \pm 0.09^{a}$ |
| ***R. philippinarum* tissues (ng/g FW)** | | | | |
| CAF | – | $0.8 \pm 0.2^{A}$ | – | $1.0 \pm 0.2^{a}$ |
| MIX | – | $0.93 \pm 0.02^{A*}$ | – | $1.4 \pm 0.3^{a}$ |

(Table 2). Conversely, DLS analysis of seawater samples exposed to the selected contaminants at 21 °C did not evidence a significant discrimination among control and CNT-based aggregates. However, aggregates dimensions were found higher in the samples exposed to f-MWCNT and caffeine (MIX) with respect to those incubated with f-MWCNTs alone either at 18 or 21 °C (Table 2).

### 3.3. Biochemical parameters

#### 3.3.1. Metabolic capacity

At 18 °C the ETS activity was significantly lower in clams exposed to both pollutants (MIX treatment) in comparison to the remaining treatments, while at 21 °C significantly higher ETS activity was found in organisms when exposed to both f-MWCNTs and CAF treatments compared to CTL and MIX treatments. Significant differences between temperatures were found at f-MWCNTs, CAF and MIX treatments, with higher values at 21 °C (Table 3).

#### 3.3.2. Energy reserves

At 18 °C GLY content showed a significant increase in clams when exposed to CAF compared to CTL, while no significant differences were observed among treatments at 21 °C. Also, no significant differences were found when comparing clams exposed to the same treatment but at different temperatures (Table 3).

At 18 °C significantly higher PROT content was found at CTL in comparison to the remaining treatments, while no significant differences were observed among treatments at 21 °C. Comparing both temperatures, significantly higher values were found at 18 °C in CTL clams (Table 3).

#### 3.3.3. Antioxidant capacity

The activity of CAT at 18 °C differed significantly among all treatments, with the lowest values at CTL and the highest values at CAF. At 21 °C the lowest CAT activity was observed at CAF treatment, with significant differences to CTL and MIX treatments, while the highest activity was observed in clams exposed to both pollutants (MIX) with significant differences to f-MWCNTs and CAF treatments. Comparing temperatures, significantly higher CAT activity was observed at 18 °C for CAF and MIX treatments, while higher values were observed at 21 °C for CTL clams (Table 3).

#### 3.3.4. Biotransformation enzymes

Both at 18 and at 21 °C, GSTs activity levels showed no significant differences among treatments. Comparing temperatures, significantly higher GSTs activity was found at 18 °C for the following treatments: CTL, f-MWCNTs and MIX (Table 3).

#### 3.3.5. Cellular damage

At 18 °C LPO levels were significantly higher at f-MWCNTs and CAF treatments in comparison to CTL and MIX treatments, while no significant differences among treatments were found at 21 °C. When comparing temperatures, significantly higher values were found at 21 °C in CTL, CAF and MIX treatments (Table 3).

### 3.4. Histopathological analyses

At 18 °C significantly higher histopathological index was recorded in organisms' gills exposed to caffeine compared to the remaining treatments. At 21 °C significantly higher values were observed at CTL in comparison to f-MWCNTs and CAF treatments. When comparing different temperatures for each treatment a significant increase in $I_h$ was found at CTL clams at 21 °C, while a significant decrease was found at this temperature in clams exposed to CAF (Table 3).

The histopathological index calculated for digestive tubules at 18 °C showed significantly higher values at CTL and f-MWCNTs treatments in comparison to CAF and MIX ones, while at 21 °C significant differences were noticed between MIX treatments and f-MWCNTs and CAF treatments. No significant differences were observed between temperatures regardless the treatment tested (Table 3).

Most of the alterations found in gills were lipofuscin aggregates and haemocytes infiltration. In digestive tubules the most common histopathological alterations were lipofuscin aggregates and atrophy. No necrotic tissue has been observed (Fig. 1).

### 3.5. Integrated biomarker response

The highest IBR value (2.83) was found in organisms exposed to MIX at 18 °C, while the lowest value (0.12) was found in clams exposed to f-MWCNTs at 18 °C (Table 3).

### 3.6. Principal coordinates analysis

The principal coordinates analysis (PCoA) revealed that PCO1 explained 33.9% of the total variation, while PCO2 explained 27.5% of the total variation (Fig. 2). The PCO1 axis clearly separated CAF and MIX treatments at 18 °C on the positive side from all the remaining treatments on the negative side. The PCO2 axis separated on the positive side all treatments at 21 °C as well as CAF at 18 °C, with CTL, f-MWCNTs and MIX at 18 °C at the negative side. High correlation between CAT and GLY with PCO1 positive side was observed ($p > 0.85$). Treatments at 21 °C were closely correlated to LPO and ETS at PCO2 positive side ($p > 0.76$).

## 4. Discussion

In the marine environment, a multitude of pollutants coexist in

**Table 2**
Dynamic Light Scattering (DLS) data of Size (nm) and Polydispersity Index (PDI) of control (CTL), f- MWCNTs and MIX suspensions (salinity 30) in contact with *Ruditapes philippinarum* clams, collected along time (days 7, 14, 21 and 28), at both tested temperatures (18 and 21 °C). Undetectable colloidal material at the end of each measurement is indicated as invalid data (i.d.).

| Temperature | Collection period | CTL | | f-MWCNTs | | MIX | |
|---|---|---|---|---|---|---|---|
| | | *Size (nm)* | *PDI* | *Size (nm)* | *PDI* | *Size (nm)* | *PDI* |
| 18 °C | *T7* | i.d. | – | i.d. | – | i.d. | – |
| | *T14* | 81.7 | 0.87 | i.d. | – | 962.85 | 0.41 |
| | *T21* | 75.0 | 0.10 | 2042.8 | 0.90 | 8635.5 | 3.65 |
| | *T28* | i.d. | – | i.d. | – | 6072.0 | – |
| 21 °C | *T7* | 2797.2 | 1.10 | i.d. | – | 1299.5 | 0.61 |
| | *T14* | i.d. | – | i.d. | – | 4530.4 | 2.04 |
| | *T21* | 3049.3 | 1.42 | i.d. | – | 4359.05 | 1.84 |
| | *T28* | 26.7 | 0.64 | 1484.9 | 0.68 | 2977 | 1.67 |

**Table 3**
Biochemical markers in *Ruditapes philippinarum* after 28 days of exposure: electron transport system (ETS) activity expressed in nmol/min/g fresh weight (FW); glycogen (GLY) content in mg/g FW; total protein content (PROT) in mg/g FW; catalase activity (CAT) in U/g FW; Glutathione S-transferases (GSTs) activity in U/g FW; lipid peroxidation (LPO) levels in nmol/g FW. Histopathological indexes ($I_h$) calculated for gills and digestive tubules. IBR: Integrated biomarker response. Results are mean ± standard deviation. For each biomarker, uppercase letters are used for statistical differences among the treatments at 18 °C, while lowercase letters are used for statistical differences among treatments at 21 °C. Statistical differences in the same treatment between temperatures (18 and 21 °C) are identified with an asterisk (*). The highest value for a given biomarker, histopathological index and IBR it is highlighted in bold while the lowest value it is underlined.

| | | CTL | | f-MWCNTs | | CAF | | MIX | |
|---|---|---|---|---|---|---|---|---|---|
| | | 18 °C | 21 °C | 18 °C | 21 °C | 18 °C | 21°C | 18 °C | 21 °C C |
| **Biochemical markers** | *ETS* | $22.7 \pm 2.7^{A}$ | $22.5 \pm 8.5^{a}$ | $24.2 \pm 3.0^{A}$ | $40.4 \pm 8.3^{b}$ | $24.3 \pm 6.5^{A*}$ | $\mathbf{42.5 \pm 8.3^{b}}$ | $11.7 \pm 2.6^{B*}$ | $29.1 \pm 7.2^{a}$ |
| | *GLY* | $7.68 \pm 0.75^{A}$ | $8.95 \pm 1.78^{a}$ | $8.46 \pm 1.5^{A,B}$ | $8.31 \pm 1.5^{a}$ | $\mathbf{11.60 \pm 1.65^{B}}$ | $9.59 \pm 1.71^{a}$ | $10.55 \pm 1.52^{A,B}$ | $7.66 \pm 1.43^{a}$ |
| | *PROT* | $\mathbf{20.09 \pm 3.80^{A}*}$ | $9.55 \pm 2.21^{a}$ | $11.23 \pm 1.24$ | $10.59 \pm 1.73^{a}$ | $9.69 \pm 1.06^{B}$ | $11.37 \pm 1.92^{a}$ | $9.35 \pm 1.95^{B}$ | $11.73 \pm 2.43^{a}$ |
| | *CAT* | $4.71 \pm 0.76^{A}*$ | $8.72 \pm 1.03^{a,c}$ | $9.00 \pm 1.12^{B}$ | $7.58 \pm 0.81^{a,b}$ | $\mathbf{32.35 \pm 2.82^{C}*}$ | $6.86 \pm 0.50^{b}$ | $15.54 \pm 1.26^{D}*$ | $9.32 \pm 1.15^{c}$ |
| | *GSTs* | $0.33 \pm 0.09^{A}*$ | $0.22 \pm 0.07^{a}$ | $.31 \pm 0.09^{A}*$ | $0.21 \pm 0.07^{a}$ | $0.30 \pm 0.02^{A}$ | $0.28 \pm 0.10^{a}$ | $\mathbf{0.35 \pm 0.11^{A}*}$ | $0.18 \pm 0.01^{a}$ |
| | *LPO* | $5.32 \pm 0.29^{A}*$ | $7.32 \pm 1.21^{a}$ | $6.90 \pm 1.05^{B}$ | $\mathbf{9.07 \pm 1.77^{a}}$ | $6.94 \pm 0.88^{B}*$ | $8.73 \pm 1.22^{a}$ | $4.73 \pm 0.23^{A}*$ | $9.01 \pm 1.52^{a}$ |
| $I_h$ | *Gills* | $0.083 \pm 0.024^{A}*$ | $0.20 \pm 0.047^{a}$ | $0.12 \pm 0.12^{A}$ | $0.13 \pm 0^{b}$ | $\mathbf{0.28 \pm 0.024^{B}*}$ | $0.13 \pm 0.047^{b}$ | $0.10 \pm 0.047^{A}$ | $0.12 \pm 0.071^{a,b}$ |
| | *Digestive tubules* | $0.36 \pm 0.034^{A}$ | $0.31 \pm 0.101^{a,b}$ | $\mathbf{0.37 \pm 0.084^{A}}$ | $0.26 \pm 0.101^{a}$ | $0.30 \pm 0.051^{B}$ | $0.29 \pm 0^{a}$ | $0.29 \pm 0.034^{B}$ | $0.32 \pm 0.017^{b}$ |
| **IBR** | | | 0.65 | 0.12 | 0.47 | 0.18 | 0.32 | **2.83** | 0.86 |

the same area, with an increasing number of studies pointing out effects of mixtures of substances (Coppola et al., 2020a and b; Ferreira et al., 2014; Pires et al., 2016a and b; Rodea-Palomares et al., 2012). Alongside with the presence of pollutants, aquatic systems are also experiencing alterations related with climate changes, including temperature rise, with different studies demonstrating the impacts of temperature on the sensitivity of marine species and/or changes on pollutants toxicty (among others: Beukema et al., 2009; Chıcharo and Chıcharo, 2001; Freitas et al., 2007; Rodrigues et al., 2015; Verdelhos et al., 2015).

Carbon nanotubes already demonstrated the capacity to adsorb contaminants and metals (Pan and Xing, 2008), a mechanism known as trojan horse effect (Naasz et al., 2018). Nevertheless, in the present study, although higher caffeine concentration was found in the presence of f-MWCNTs (MIX treatment), significant differences were only observed when comparing temperature

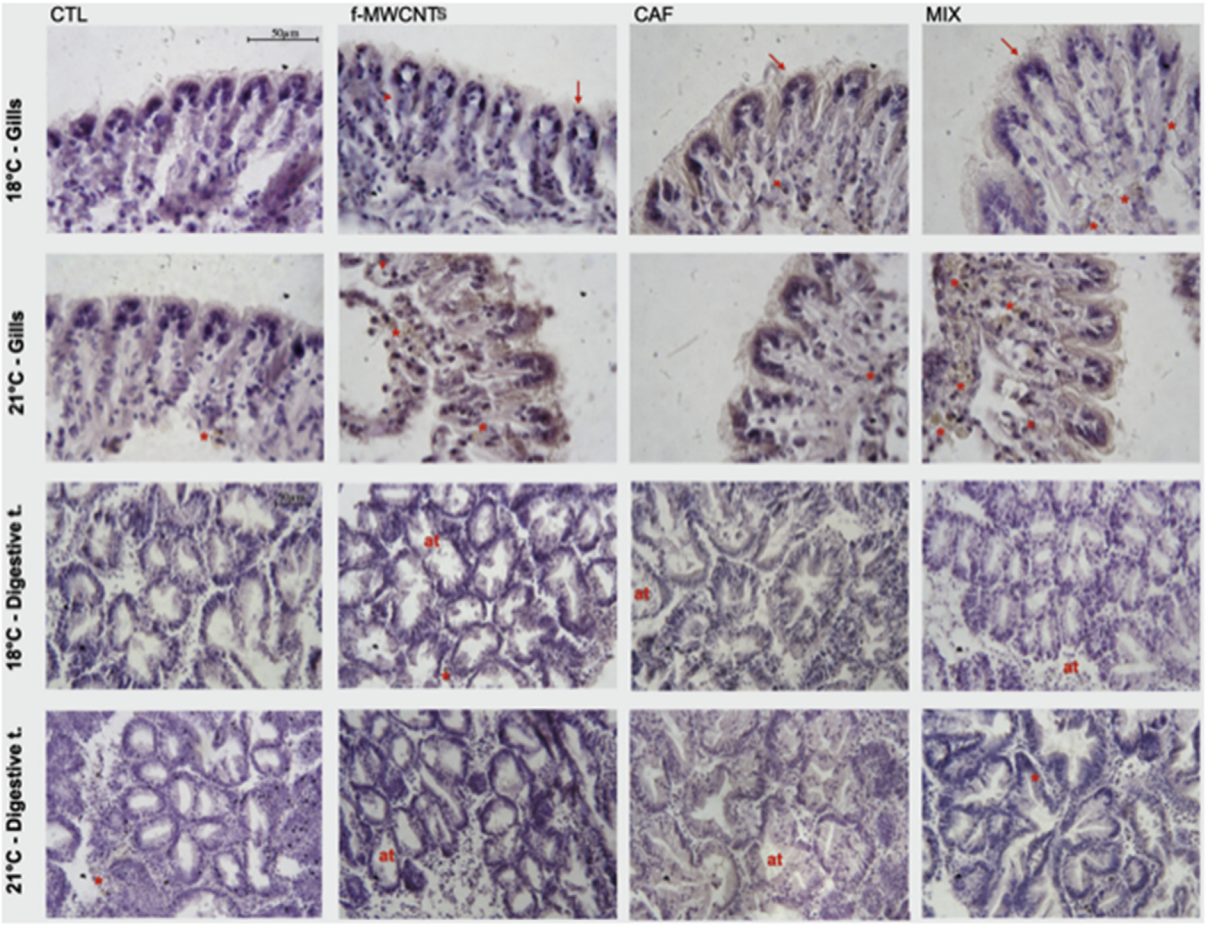


**Fig. 1.** Micrographs of tissues in *Ruditapes philippinarum* exposed to different treatments (CTL, f-MWCNTs, CAF, MIX and two temperature levels) stained with hematoxylin. i) Gills: haemocytes infiltration (short arrow), abundance of lipofuscin aggregates (asterisks) and cilia loss (long arrow). (ii) Digestive tubules: lipofuscin aggregates (asterisks) and atrophied digestive tubules ("at"). Scale bar = 50 μm.

levels, with higher values at 21 °C for the MIX treatment. This result may indicate that f-MWCNTs are able to bind caffeine, as already revealed by Lee et al. (2013), with higher capacity at warming conditions. As already demonstrated, high temperatures facilitate the aggregation of nanoparticles (Chen et al., 2015), increasing their surface area which in turn, makes them ideal vehicles for some drugs/contaminants (Pérez-Luna et al., 2018). The obtained results could also be explained by decreased detoxification capacity in clams exposed to CAF and f-MWCNTs (MIX treatment) at increased temperature. In particular, the lowest GSTs activity was found in clams exposed to MIX treatment at 21 °C. The present results are in accordance with previous studies that also demonstrated lower biotransformation capacity of GSTs enzymes at increased temperature. Andrade et al. (2019a) observed lower GSTs activity in mussels exposed to f-MWCNTs (0.01 mg/L) at increased temperature (21 °C) compared with values recorded at control temperature, indicating the inhibition of these enzymes due to temperature. Also, Balbi et al. (2017) confirmed this evidence, demonstrating a seasonal variation in GSTs activity in mussels, with the lowest activity found during the warmer months.

Overall, the present study demonstrated that contaminated clams under control temperature maintained their metabolic capacity, except when exposed to CAF and f-MWCNTs at the same time (MIX treatment) where ETS activity decreased compared to control values. This result may indicate that, at control temperature, concentrations of both pollutants were not high enough to change the ETS activity, while higher stress was generated by the combination of both pollutants, which resulted in clams' metabolic depression. It was already demonstrated that up to certain contamination levels bivalves can maintain or even reduce their metabolism, most probably associated with the filtration and respiration rates reduction, in an attempt to avoid accumulation of pollutants. Coppola et al. (2020c) revealed that the mussels *M. galloprovincialis,* decreased their metabolic capacity in comparison to CTL when in the presence of contaminants (lead, manganese spinel ferrite nanoparticles and the combination of both). On the other hand, the present study further revealed that at warming conditions, contaminated clams showed increased metabolic capacity, especially in the presence of CAF and f-MWCNTs acting alone. These results evidence that under higher stress levels (contamination and increased temperature acting together), metabolic maintenance was no longer the strategy followed by clams and in this case an increased metabolic capacity was observed, which may be associated with activation of defence mechanisms. A previous work by Coppola et al. (2020b) found, in *Ruditapes philippinarum*, an increase in ETS in specimens exposed to mercury (Hg) at higher temperature (22 °C) compared to clams exposed to Hg at control temperature (17 °C).

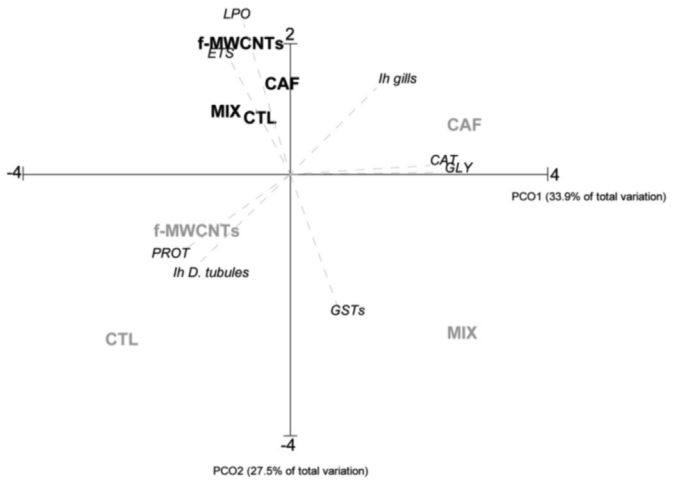


**Fig. 2.** Principal coordinates analysis (PCoA) based on biochemical markers and histological alterations in *Ruditapes philippinarum* after 28 days exposure. Tested treatments were CTL, CAF, f-MWCNTs, MIX at 18 °C (grey letters) and 21 °C (black letters). Pearson correlation vectors are superimposed as supplementary variables (r > 0.75): ETS (eletron transport system activity), GLY (glycogen content), PROT (protein content), CAT (catalase activity), GSTs (glutathione S-transferases activity), LPO (lipid peroxidation levels), histopathological index ($I_h$) for gills and digestive tubules (D. tubules).

To better understand the energy budget of an organism and, thus, how they will respond to unfavourable conditions, energy reserve biomarkers, namely GLY and PROT contents, can also be taken into account (Ansaldo et al., 2006; Freitas et al., 2019b). The obtained results clearly demonstrated that although the metabolic capacity was affected by contaminants and temperature, the GLY content was, in general, maintained regardless the tested treatment. These findings may indicate that GLY was not the first energy source of *R. philippinarum* when exposed to these contaminants. The maintenance of GLY content under stressful conditions is a strategy already suggested by other studies that observed stable GLY content in *Limecola balthica* after Cd exposure (Duquesne et al., 2004). Furthermore, a study by Freitas et al. (2017) assessing the effect of Hg and temperature, demonstrated that even an increase in energy reserves can be a possible strategy in *M. galloprovincialis*. Regarding the PROT content, the present study showed a significant decrease across all conditions compared to control clams (non-contaminated clams at 18 °C), highlighting that clams were probably using proteins as a source of energy to fuel defence mechanisms and/or the amount of enzymes, decreased under stress conditions as a result of lower protein synthesis. Total PROT content decrease was already observed in the bivalves *R. philippinarum*, *Scrobicularia plana* and the polychaete species *Diopatra neapolitana*. In their study on the effect of carbamazepine exposure in *R. philippinarum*, Almeida et al. (2015) observed a significant decrease of PROT in clams under the highest contaminant concentration (9.00 μg/L). Furthermore, Freitas et al. (2015) demonstrated a decrease on the PROT content in *S. plana* specimens submitted to 0.30 and 6.00 μg/L of carbamazepine exposure, while for *D. neapolitana* significant reduction in PROT was found in organisms exposed to 0.30, 3.00 and 6.00 μg/L of the same contaminant.

The exposure to contaminants can enhance the production of Reactive Oxygen Species (ROS) which, if not eliminated, can cause oxidative damage to cell structures such as lipid membranes and DNA (Regoli and Giuliani, 2014). A common response to oxidative stress is the overexpression of antioxidant enzymes such as catalase (CAT). The results obtained in the present study demonstrated that the antioxidant enzyme CAT was activated in contaminated clams under control temperature (18 °C), especially at CAF treatment. However, under warming conditions this enzyme maintained the activity regardless the treatment, with lower values at 21 °C compared with 18 °C, except in non-contaminated clams compared to non-contaminated clams at control temperature. The results observed could be explained by an increase in ROS production induced by the presence of contaminants (in clams maintained at 18 °C) but also by increased temperature (non-contaminated organisms at 21 °C), that caused the activation of the cells' defence mechanisms against oxidative damage. Although higher ETS activity was observed at 21 °C, the increased ROS production resulted from the respiration process was not associated to increased CAT activity in contaminated clams. Such findings may point out that, at the highest temperature, other antioxidant defence mechanisms were activated in an attempt to eliminate the increased ROS production resulting from higher ETS activity. At control temperature,

Cruz et al. (2016) also showed an increase in CAT activity in *R. philippinarum* at higher caffeine exposure concentration and Freitas et al. (2018) revealed an increase in antioxidant enzymes (including catalase) in *R. philippinarum* exposed to f-MWCNTs. A correlation between increased temperature and CAT activity was found also by Khessiba et al. (2005) after the exposure of *M. galloprovincialis* to a temperature increase regime (10 °C, 15 °C, 25 °C). Furthermore, Verlecar et al. (2007) also demonstrated that *Perna viridis* specimens under heat stress, increased antioxidant enzymes activities, namely CAT.

Under stressful conditions, including the presence of contaminants, organisms are able to increase the activity of biotransformation enzymes, such as the GSTs, in an attempt to substances detoxification (Strange et al., 2001). In the present study GSTs activity was lower at 21 °C compared to 18 °C, regardless the treatment, revealing the negative influence of temperature on these enzyme activities. Similarly, Andrade et al., 2019a observed that *M. galloprovincialis* specimens exposed to increased temperature (21 °C) presented lower GSTs than organisms under control temperature (18 °C). Furthermore, the results obtained showed a significant increase on GSTs activity in clams exposed to both contaminants (MIX treatment) at 18 °C compared to clams exposed to the same treatment but at 21 °C. These findings may explain limited caffeine accumulation under this treatment (MIX treatment at 18 °C) compared to clams exposed to both contaminants but under warming conditions.

Generally, an increased LPO is found in cells when the antioxidant defences fail to remove the excess production of ROS (Regoli and Giuliani, 2014). Results here presented, highlight that under control temperature cellular damage occurred in clams exposed to caffeine and to f-MWCNTs acting along. Cellular damages (measured by LPO levels) were also found in *R. philippinarum* exposed to caffeine with a concentration of 0.5 μg/L (Cruz et al., 2016). Regarding the effect of f-MWCNTs alone, Andrade et al. (2019b) observed a raise in LPO in *M. galloprovincialis* after exposure to 0.01 mg/L of f-MWCNTs. Also, De Marchi et al. (2017) demonstrated increased LPO at different concentrations (0.01, 0.1 and 1 mg/L) of functionalized and non-functionalized MWCNTs in the same species. On the other hand, under control temperature, clams exposed to both contaminants at the same time (MIX treatment), showed lower LPO levels than contaminated and control organisms. These results may be explained by lower ETS at this treatment, leading to lower ROS generation, but also due to increased GSTs activity (with the highest values at this treatment) that contributed to contaminants elimination. Under warming conditions LPO was in general higher than at 18 °C, regardless the treatment, indicating the negative impact of temperature on membrane lipids. The impacts of temperature on LPO levels was previously demonstrated by Coppola et al. (2017) as well as by Freitas et al. (2020) that observed increased LPO levels in *M. galloprovincialis* exposed to 21 °C after 28 days compared to control temperature (18 °C).

To better understand the effects of pollutants on aquatic species, histopathological analyses have become more and more prominent in the last years (Cuevas et al., 2015). The use of Histopathological indexes ($I_h$) has been a common approach when evaluating impacts of pollutants on fish species (Agamy, 2012; Marchand et al., 2012; Nimet et al., 2020), but more recently it has also been extended to bivalves (Coppola et al., 2020a and b; Costa et al., 2013; Larguinho et al., 2014). In the present study, an increased number of histopathological alterations were detected in the gills of clams subjected to CAF: the histopathological index was significantly higher in this group compared to CTL at 18 °C. These findings indicate that caffeine had a negative impact on *R. philippinarum* at the gills level as the respiratory organs are believed to be one of the first target for contaminants damage (Au, 2004). The most common alteration found in gills was the presence of lipofuscin aggregates that were already linked to oxidative stress (Livingstone et al., 1990). The absence of histopathological damages in gill of clams exposed to CAF and f-MWCNTs (MIX treatment) may indicate a protective role of the f-MWCNTs towards these organs, which could also explain the lowest LPO levels observed at this treatment. At 21 °C non-contaminated clams showed higher $I_h$ index than clams maintained under control temperature, while clams exposed to caffeine showed an opposite response. Coppola et al. (2020b) also demonstrated the negative impacts of temperature on clams due to increased temperature and the presence of Hg in *R. philippinarum*. In addition to the presence of lipofuscin aggregates, as well as loss of cilia and lumen enlargement was detected. Haemocytes infiltration was rarely noticed, but it seemed more prominent in clams exposed to f-MWCNTs. Similar histopathological alterations were also found by Leite et al. (2020) in *M. galloprovincialis* exposed to Rutile and Anatase.

The present results failed to find significative effects on digestive tubules, regardless the treatment tested and the temperature scenario, although higher $I_h$ values was obtained at f-MWCNTs treatment (18 °C). Such findings can reveal low impact of caffeine and f-MWCNTs on clams' digestive tubules. The results presented in this study are in opposition to findings by Anisimova et al. (2015) that investigated the effects of MWCNTs on tissues of *Modiolus modiolus*. These authors observed histopathological changes in the intestinal epithelium after an acute exposure (24–48h) to 100 mg/L of MWCNTs. The differences in our findings could be explained by the difference in contaminant concentrations, indicating that these nanoparticles do not have a strong impact on digestive tubules at low environmental concentrations.

Trying to identify the most stressful condition, the Integrated Biomarker Response (IBR) was applied, and the results showed the highest impact in clams exposed to both contaminants (MIX treatment) at 18 °C. This result may indicate an interactive effect between both contaminants, generating a higher stress condition to clams, here evidenced by significant metabolic decrease, lower protein content and higher detoxification capacity. The lowest IBR value was found for clams exposed to f-MWCNTs at 18 °C, indicating low stress induced by this contaminant. For example, Xia et al. (2017) found an increased IBR value in the scallop *Chlamys farreri* exposed to $TiO_2$, together with histopathological alterations, oxidative stress and neurotoxicity at predicted relevant environmental concentrations. Also, Coppola et al. (2019) found an increased IBR in *M. galloprovincialis* exposed to arsenic.

Overall, the results obtained revealed a clear separation between clams exposed to different temperatures (see PCoA graph). Also, clams exposed to increased temperature tended to group together, indicating similar responses regardless the treatment; while at 18 °C a clear distinction between clams exposed to caffeine (both acting alone and in combination with f-MWCNTs) and clams exposed to CTL and f-MWCNTs was noticed, with clams under control showing similar responses to clams exposed to f-MWCNTs (as demonstrated by IBR results).

## 5. Conclusions

Our findings confirmed the negative effects of caffeine and f-MWCNTs in *Ruditapes philippinarum*, revealing metabolic alterations, oxidative stress and some histological changes associated with exposure to these two pollutants in gills. Furthermore, it seems that temperature played an important role in modulating these responses, but the highest impacts were observed at 18 °C, when both pollutants were acting together. Overall, this study demonstrates an interplay between the two contaminants and

major impact of their mixture in *R. philippinarum* and proves that temperature may influence the sensitivity of this species, with consequences at the distributional and survival levels.

## Author statement

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgment

Ângela Almeida and Francesca Coppola benefited from a PhD grants (SFRH/BD/110218/2015 and SFRH/BD/118582/2016, respectively) given by the National Funds through the Portuguese Science Foundation (FCT), supported by FSE and Programa Operacional Capital Humano (POCH) and União Europeia. This work was also financially supported by the project BISPECIAl: BIvalveS under Polluted Environment and CIImate chAnge (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), to FCT/MEC through national funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020.

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