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Mytilus galloprovincialis: A valuable bioindicator species for understanding the effects of diclofenac under warming conditions

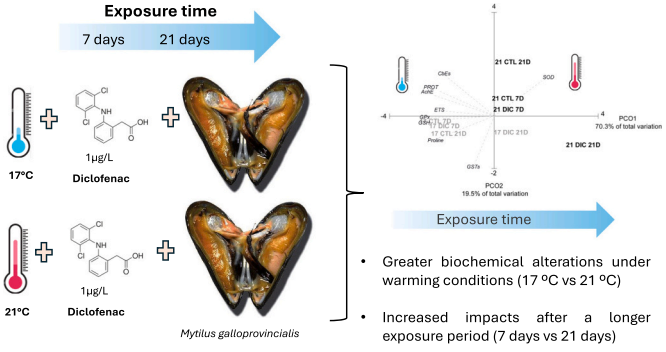
Federica Arrigo [d], Lucia De Marchi [b], Valentina Meucci [b], Giuseppe Piccione [a], Amadeu M.V.M. Soares [c], Caterina Faggio [d,e], Rosa Freitas [c,*]

[a] Department of Veterinary Sciences, University of Messina, Viale Giovanni Palatucci snc, 98168 Messina, Italy
[b] Veterinary Teaching Hospital, Department of Veterinary Sciences, University of Pisa, 56122 Pisa, Italy
[c] Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal
[d] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, 98166 S. Agata-Messina, Italy
[e] Department of Ecosustainable Marine Biotechnology, Stazione Zoologica Anton Dohrn, Naples, Italy

HIGHLIGHTS

- Diclofenac (DIC) negatively influenced the biochemical performance of mussels.
- Warming conditions caused a worsening of the biochemical responses.
- At 21 °C the metabolic capacity decreased, especially in the presence of DIC.
- Carboxylesterases activity was inhibited at 21 °C after 21 days of exposure to DI
- DIC caused neurotoxicity after 21 days of exposure, regardless of the temperature.
- Drugs aquatic contamination interconnects the health of humans and ecosystems.

GRAPHICAL ABSTRACT



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ABSTRACT

Drugs are chemical compounds used to treat and improve organic dysfunctions caused by diseases. These include analgesics, antibiotics, antidepressants, and antineoplastics. They can enter aquatic environments through wastewater streams, where their physico-chemical properties allow metabolites to distribute and accumulate. Current climate change and associated extreme weather events may significantly impact these substances' toxicity and aquatic organisms' sensitivity. Among the chemicals present in aquatic environments is the non-steroidal anti-inflammatory drug diclofenac (DIC), which the EU monitors due to its concentration levels. This study investigated the influence of temperature (control at 17 °C vs. 21 °C) on the effects of DIC (0 µg/L vs. 1 µg/L) in the mussel species *Mytilus galloprovincialis*. Significant results were observed between 17 and 21 °C. Organisms exposed to the higher temperature showed a decrease in several parameters, including metabolic capacity and detoxification, particularly with prolonged exposure. However, in some parameters, after 21 days, the *M. galloprovincialis* showed no differences from the control, indicating adaptation to the stress. The results of this study confirm that DIC concentrations in the environment, particularly when combined with increased temperatures, can produce oxidative stress and adversely affect *M. galloprovincialis* biochemical and physiological

* Corresponding author.
E-mail address: rosafreitas@ua.pt (R. Freitas).

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performance. This study also validates this species as a bioindicator for assessing environmental contamination with DIC. Beyond its direct impact on aquatic organisms, the presence of pharmaceuticals like DIC in the environment highlights the interconnectedness of human, animal, and ecosystem health, underscoring the *One Health* approach to understanding and mitigating environmental pollution.

1. Introduction

Aquatic ecosystems are continuously subjected to pollutants, including pharmaceutical and personal care products, with concentrations tending to increase in the environment (Banaee et al., 2023; Costa et al., 2020a; Impellitteri et al., 2023a; Martyniuk et al., 2023a; Multisanti et al., 2023; Sehonova et al., 2017; Shiry et al., 2023a). Anti-inflammatories are pharmaceuticals with antipyretic and analgesic functions (Montinari et al., 2019) that, given their therapeutic function, are among the most frequently prescribed in the world. The actual amount consumed, however, is difficult to determine due to their wide availability under different trade names (Panchal and Prince Sabina, 2023). Their therapeutic uses and benefits are numerous: the most common is the treatment of acute pain after surgery or chronic pain, but they are also used to treat osteoarthritis and rheumatoid arthritis (Wojcieszyńska et al., 2023). In addition, recently, they have been used to treat COVID-19 (Wojcieszyńska et al., 2022). Diclofenac, 2-[2-(2,6-dichloroanilino)phenyl]acetic acid (DIC) is a non-steroidal anti-inflammatory drug, commonly used for pain relief. It is frequently offered for sale as topical gels or oral pills. Currently, on the market, the most popular trade names are Algosenac, Almiral, Diclomex, Dicuno, Voltaren. Diclofenac is also used to treat epilepsy, autoimmune conditions, and acute pancreatitis (Amanullah et al., 2022). Due to its excessive use in human and veterinary fields, it has been among the most detected substances in the environment since 2000. Approximately 75 % of DIC active ingredient used reaches aquatic environments and adversely affects organisms living in these habitats (He et al., 2017). Therefore, DIC has been placed on the European Union's first watch list so that its concentration in the environment and its effects are monitored periodically. Nevertheless, DIC has been identified at different concentrations worldwide in different environmental compartments such as groundwater, soil, sediment, and even freshwater at ecotoxicological risk (Sathishkumar et al., 2020). In the marine environment, concentrations can vary from a few ng/L to several hundreds of ng/L. For example, on the Atlantic coast of Portugal, a concentration of 241 ng/L was found in seawater (Lolić et al., 2015), while on the west coast of Ireland, 550 ng/L was detected in seawater (McEneff et al., 2014). Higher concentrations (10 μg /L) were found in seawater of the Al-Arbaeen Lagoon (Red Sea coast) (Ali et al., 2017). Among the effects on bivalves, deterioration in osmoregulation and a decrease in gamete release was reported in the mussel species *Mytilus galloprovincialis,* while in clams *(Ruditapes philippinarum)* antioxidant defences were enhanced and a decrease in respiration rate was observed (Costa et al., 2020a; Freitas et al., 2019; Trombini et al., 2019).

Besides pollutants, several studies already demonstrated that climate change and in particular warming can also affect aquatic environments. According to the most recent reports of the Intergovernmental Panel on Climate Change (IPCC, 2023), the steady increase in greenhouse gas emissions has caused the global surface temperature to rise by approximately 1.1 °C in recent years, particularly between 2011 and 2020, and it is predicted that temperatures could rise 1.5 °C by 2040 and 4 °C by 2100 (IPCC, 2021; IPCC, 2023). The current rise in global temperatures is fuelling a surge of extreme weather events in aquatic environments. This includes extended marine heatwaves, which are becoming both increasingly frequent and more intense (Pastor and Khodayar, 2023). This alarming warming trend, coupled with the growing frequency of extreme weather events, poses a significant threat to the health of aquatic ecosystems and all the species that inhabit them. For example, an increase in water temperature can affect organisms' sensitivity to pollutants. Attig et al. (2014) studied the effects of warming on the impacts caused by nickel *M. galloprovincialis* and showed a negative effect of temperature increase on mussel's antioxidant and detoxification response to Ni exposure. It was already reported by Lopes et al. (2022) that warming can enhance the impacts of 17 α-ethinylestradiol (EE2) by lowering the defence capacity of bivalves like *Mytilus galloprovincialis* when subjected to increased temperatures compared to the ones maintained under control temperature. Also, Coppola et al. (2017), showed that heating can worsen impacts of mercury (Hg) on *M. galloprovincialis*. However, warming can also change pollutants' behaviour and bioavailability, enhancing their toxicity. For example, Hg methylation (i.e., the transformation of inorganic Hg, less toxic, to the organic form, more toxic) is favoured by warming conditions (Pack et al., 2014). Dinh et al. (2022) confirmed that the toxicity of contaminants is influenced by high temperatures that cause their degradation and accumulation. It is also known that the transformation of organic to inorganic arsenic form (the most toxic one) is potentiated by increased temperature (Neff, 1997). Furthermore, at the same time, pollution may increase the sensitivity of organisms to temperature, limiting their defence mechanisms (Lannig et al., 2006). These authors investigated the combined effects of temperature and cadmium (Cd), on the energy metabolism of the oyster *Crassostrea virginica*, revealing that both increasing temperature and Cd exposure led to a rise in standard metabolic rates. Additionally, the combination of these stressors appeared to overwhelm the oyster's capacity for aerobic energy production, ultimately compromising its tolerance to stress. According to Sokolova and Lannig (2008), aquatic pollution, including metal pollution, can change the sensitivity of aquatic organisms to global warming, negatively impacting physiological mechanisms such as growth and reproduction rate.

Considering the above-mentioned, and the lack of information regarding the combined effects of pharmaceuticals and predicted temperature increase, the present study aimed to evaluate the influence of warming on the effects caused by DIC on the mussel *M. galloprovincialis.* According to the literature available, no studies are known regarding the effects of DIC on *M. galloprovincialis* subjected to predicted warming conditions. Biochemical parameters were analysed to understand the effects of DIC on metabolic, oxidative stress, and neurotoxic status after exposure to present and predicted temperature scenarios. These findings are crucial given predicted climate change scenarios, where more frequent and intense extreme weather events associated with higher temperatures are expected. Predicting drug impacts in these scenarios will raise awareness of threats to aquatic environments and their social and economic consequences. The presence of pharmaceutical pollutants like non-steroidal anti-inflammatory drugs (NSAIDs) in aquatic environments not only affects aquatic organisms and biodiversity but also raises concerns about human exposure through the consumption of contaminated water or seafood. By studying the effects of these pollutants on aquatic organisms, researchers will gain insights into broader ecological and public health implications, emphasizing the importance of a One Health approach to address environmental challenges.

2. Materials and methods

2.1. Experimental conditions

Mytilus galloprovincialis specimens (length: 58.1 ± 5.2 mm; width: 32.82 ± 3.1) were collected from the coastal lagoon of Ria de Aveiro (Portugal) in February. After collection, organisms were transported to the laboratory where they were maintained for 15 days under the same

conditions as the sampling site (temperature 17.0 ± 1.0 °C; salinity 30 ± 1; pH 8.0 ± 0.1). Artificial seawater was obtained from commercial salt and deionised water. During this period mussels were fed with Algamac protein plus (150,000 cells/animal/day) three times a week. After the first week mussels were divided into two different climatic rooms: 17.0 ± 1.0 °C and 21 ± 1.0 °C. The highest temperature (21 ± 1.0 °C) was obtained gradually, with an increase of 1–2 °C per day. At the end of the acclimation period, animals were maintained for 7 and 21 days in the presence or absence of diclofenac (DIC) at both temperatures, making a total of 4 treatments *per* exposure period: CTL (temperature 17 ± 1 °C without DIC); temperature 17 ± 1 °C with DIC (1 μg/L); temperature 21 ± 1 °C without DIC; temperature 21 ± 1 °C with DIC (1 μg/L). Six glass containers (1 L) with two mussels in each were used per treatment and sampling period. The temperature (17 or 21 °C) was checked daily and, if necessary, adjusted. Water samples were renewed every week and the conditions were re-established, including the restoration of DIC concentrations. Spiking was done once a week considering previous studies that demonstrated the stability of DIC over a week (e.g., Costa et al., 2020b).

Test conditions (temperature 21 °C and DIC concentration of 1 μg/L) were selected based on: i) measured water temperature at the sampling site and predicted warming scenarios (IPCC, 2021); ii) previous studies testing the same DIC concentration and/or warming conditions in different bivalves species (Costa et al., 2020a, 2020b; Freitas et al., 2020); iii) and DIC levels in seawater from contaminated coastal areas (Ali et al., 2017). Exposure periods of 7 and 21 days were identified as being used in some studies to evaluate the effects of xenobiotics on bivalve organisms such as *Unio tumidus*, *Perna perna* or *Mytilus galloprovincialis* (Charissou et al., 2004; Trevisan et al., 2014).

At the end of each exposure period (7 and 21 days) animals were frozen for DIC quantification and biochemical analyses.

2.2. Diclofenac concentration in mussels' soft tissues

Diclofenac concentrations were measured in mussels' tissue samples by high-performance ultraviolet liquid chromatography (HPLC-UV), following the methodology described by Gatidou et al. (2007), with minor adjustments as previously applied by Costa et al. (2020a, 2020b). In summary, approximately 1.5 g fresh weight (FW) of whole tissue from two individuals were dehydrated and subjected to sonication at 50 °C for 30 min using a solvent mixture comprising 62.5 % methanol and 37.5 % Milli-Q water. Following centrifugation, the resulting supernatant was collected and diluted to a final volume of 100 mL using Milli-Q grade water, then subjected to purification via solid-phase extraction. The chromatographic setup involved a PerkinElmer Series 200 gradient pump connected to a PerkinElmer Series 200 variable UV detector set to 280 nm. The mobile phase consisted of a mixture of acetonitrile and water containing 0.2 % formic acid in a ratio of 60:40 (*v*/v). The analytical column employed was a Haisil LC column (5 μm, 150 × 4.60 mm, Higgins) maintained at room temperature. Recovery efficiency exceeded 78 % for soft tissue samples, with a limit of detection (LOD) of 5 ng/g dry weight (DW) for soft tissue.

2.3. Biochemical parameters

For each treatment, the biomarkers measured included indicators of metabolic capacity (electron transport system activity, ETS); energy reserves (total protein content, PROT); antioxidant (activity of the enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx)) and biotransformation (activity of the enzymes carboxylesterase (CbEs) (with substrate *p*-nitrophenyl acetate) and glutathione S-transferases (GSTs)) capacity; cellular redox homeostasis (proline (PROL) and reduced glutathione (GSH) contents); neurotoxicity (acetylcholinesterase activity (AChE)).

To conduct the biochemical analyses, frozen organisms' soft tissues were homogenised under liquid nitrogen and then divided into 0.5 g FW aliquots. The samples were then extracted using specific buffers using the TissueLyzer II, at a rate of 20 1/s for 90 s, centrifuged at 4 °C for 20 min at 10,000 *g* (or 3,000 *g* for ETS) and the supernatant was collected for analysis. The samples obtained were kept at −80 °C or used immediately to determine biochemical responses.

The methods used for each biochemical parameter are described in detail by other authors (Andrade et al., 2021; Cunha et al., 2023). Briefly, mussel's metabolism was assessed by measuring ETS activity based on the methods by King and Packard (1975). The total PROT content was quantified following the Biuret method described by Robinson and Hogden (1940). The antioxidant capacity of mussels was assessed by quantifying the activity of the enzymes SOD and GPx. SOD activity was measured following the Magnani et al. (2000) method and expressed by U per g of FW where U represents the amount of enzyme required to induce a 50 % inhibition of pyrogallol autoxidation. GPx activity was measured following the Paglia and Valentine (1967) method and expressed in U per g FW where U represents the number of enzymes which catalyse the conversion of 1 μmol nicotinamide adenine dinucleotide phosphate (NADPH) per min. Mussels' detoxification capacity was evaluated by measuring the activity of CbEs and GSTs enzymes. The phase I enzyme (CbEs) was quantified according to Hosokawa and Satoh (2001) with adaptations made by Solé et al. (2018) while phase II enzymes (GSTs) were quantified using the method adopted by Habig et al. (1974). PROL was quantified according to the method developed by Bates et al. (1973). The redox balance was measured by the GSH which was quantified following Rahman et al. (2006). Neurotoxicity was determined according to the method of Ellman et al. (1961) with modification by Mennillo et al. (2017).

2.4. Data analysis

2.4.1. Diclofenac concentrations in mussels

The statistical analysis was conducted using GraphPad Prism® software (v.7, La Jolla, CA, USA). All data underwent distribution testing using the Kolmogorov-Smirnov test. The data were presented as mean ± standard deviation. A significance level of $p < 0.001$ was applied. Sidak's multiple comparisons test (two-way ANOVA) was utilized to compare diclofenac concentrations in samples collected under different temperature conditions (17 °C vs 21 °C) for each time point of collection (7 and 21 days).

2.4.2. Biochemical responses

All biochemical parameters were subjected separately to a non-parametric permutational analysis of variance (PERMANOVA add-on in Primer v7). Significant values were assessed as those <0.05 ($p < 0.05$). The null hypothesis was: DIC has no toxic effects on mussels regardless of the temperature and exposure period. Significant differences among treatments at 17 °C were identified with different lowercase letters while significant differences among treatments at 21 °C were identified with uppercase letters. Differences between temperatures for each treatment and sampling period are identified with *p-values* in Table 2.

The matrix showing the biomarkers per treatment was normalised and the calculated Euclidean distance between the centroids (mean value per treatment) was used to perform the Principal Coordinate Ordering (PCO) analysis. In the PCO plot, variables with a correlation higher than 75 % with the spatial distribution of the tested treatments were represented as overlapping vectors.

3. Results

3.1. Diclofenac concentrations in mussels' tissues

In terms of DIC concentrations in mussel tissues, no significant differences were detected among temperatures (Table 1). However, significant differences were observed among exposure times, with the

Table 1
Diclofenac concentrations (means±ds) in mussel tissues (ng/g dry weight) at 7 and 21 experimental days under two different temperatures (17 and 21 °C).

	Conditions	
	17 °C	21 °C
7 days	32.1 ± 3.5****	28.0 ± 4.8****
21 days	61.6 ± 3.2****	56.6 ± 2.7****

**** $p < 0.0001$.

highest DIC concentrations observed after 21 days compared to 7 days ($p < 0.0001$) (Table 1).

3.2. Mussels' biochemical responses

3.2.1. Metabolic capacity and protein content

At both temperatures, no significant differences were found in ETS activity among treatments (Fig. 1A). Comparing the two temperatures (17 and 21 °C), except for mussels exposed to DIC for 21 days, significant differences were observed in all the remaining treatments, with higher values at 17 °C (Table 2).

The PROT content (Fig. 1B) showed no significant differences among treatments regardless of the temperature tested. Comparing both temperatures, a statistical significance was represented by a decrease in PROT content in mussels exposed to DIC after 7 days at the temperature of 21 °C compared to 17 °C (Table 2).

Table 2
p-values compare the two temperatures (17 and 21 °C) for each treatment (0 μg/L - CTL, 1 μg/L - DIC) and for each exposure period (7 and 21 days). The various biochemical parameters were tested: electron transport system activity, ETS; total protein content, PROT; superoxide dismutase activity, SOD; glutathione peroxidase activity, GPx; Carboxylesterase activity, CbEs; Glutathione S-transferase activity, GSTs; Proline content, PROL; Reduced glutathione content, GSH; Acetylcholinesterase activity, AChE.

	17 CTL 7D vs 21 CTL 7D	17 DIC 7D vs 21 DIC 7D	17 CTL 21D vs 21 CTL 21D	17 DIC 21D vs 21 DIC 21D
ETS	**0.0013**	**0.0485**	**0.0292**	0.0503
PROT	0.7409	**0.0418**	0.1045	0.3875
SOD	0.6061	0.0809	**0.0392**	**0.0018**
GPx	**0.0104**	**0.0344**	0.1068	0.1257
CbEs	0.3853	0.8967	0.1918	**0.002**
GSTs	**0.0223**	0.9321	**0.017**	0.7682
PROL	**0.0003**	**0.0426**	0.0832	0.3381
GSH	**0.0392**	0.0042	**0.0258**	0.2354
AChE	0.0679	0.1344	**0.015**	0.1485

3.2.2. Antioxidant and biotransformation defences

The activity of SOD at each tested temperature showed no significant differences among treatments (Fig. 2A). Comparing the two temperatures, significantly higher activity was observed at 21 °C both at the control and after exposure to DIC regardless of the exposure period (Table 2).

At 17 °C, GPx activity (Fig. 2B) showed a significant decrease in mussels exposed to DIC compared to the control regardless of the

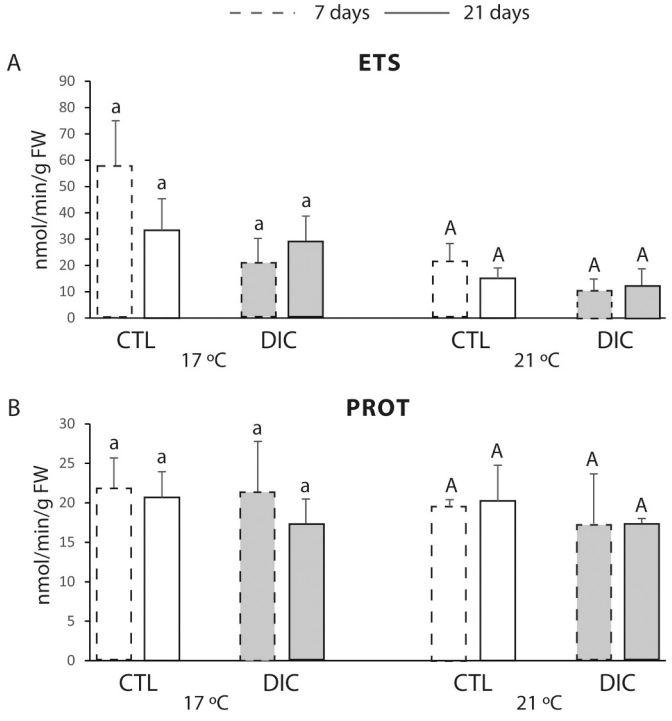

Fig. 1. A: Electron transport system (ETS) activity and B: Protein (PROT) content, in *M. galloprovincialis* exposed to two different treatments (0 μg/L CTL, 1 μg/L DIC) for 7 and 21 days at both temperatures (17 °C -control, 21 °C). Results are the means + standard deviation. Significant differences ($p < 0.05$) among treatments at 17 °C are identified with lowercase letters and uppercase letters for organisms exposed to 21 °C.

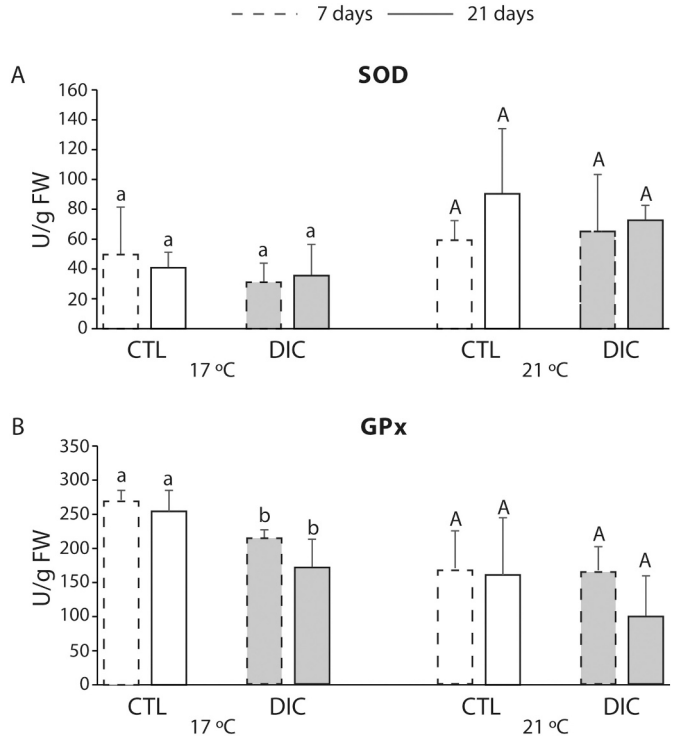

Fig. 2. A: Superoxide dismutase (SOD) and B: Glutathione peroxidase (GPx) activities, in *M. galloprovincialis* exposed to two different treatments (0 μg/L CTL, 1 μg/L DIC) for 7 and 21 days at both temperatures (17 °C - control, 21 °C). Results are the means + standard deviation. Significant differences ($p < 0.05$) among treatments at 17 °C are identified with lowercase letters and uppercase letters for organisms exposed to 21 °C.

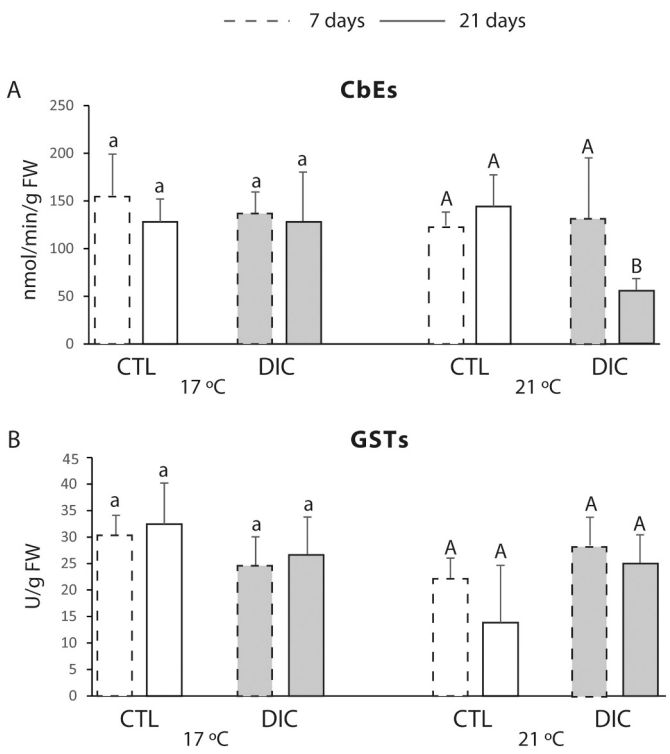

Fig. 3. A: Carboxylesterase (CbEs) and B: Glutathione S-transferases (GSTs) activities, in *M. galloprovincialis* exposed to two different treatments (0 μg/L CTL, 1 μg/L DIC) for 7 and 21 days at both temperatures (17 °C - control, 21 °C). Results are the means + standard deviation. Significant differences ($p < 0.05$) among treatments at 17 °C are identified with lowercase letters and uppercase letters for organisms exposed to 21 °C.

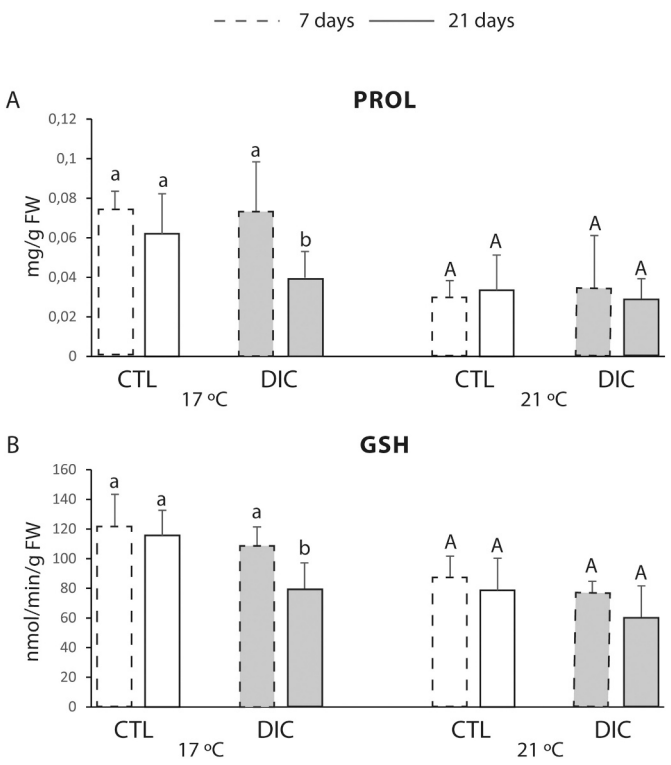

Fig. 4. A: Proline (PROL) and B: Reduced glutathione (GSH) contents, in *M. galloprovincialis* exposed to two different treatments (0 μg/L CTL, 1 μg/L DIC) for 7 and 21 days at both temperatures (17 °C - control, 21 °C). Results are the means + standard deviation. Significant differences ($p < 0.05$) among treatments at 17 °C are identified with lowercase letters and uppercase letters for organisms exposed to 21 °C.

exposure period. At 21 °C, no significant differences were observed among treatments. Comparing 17 and 21 °C, significant differences were observed in CTL and DIC-exposed mussels after 7 days, with lower activity at the highest temperature (Table 2).

The carboxylesterase (CbEs) activity (Fig. 3A) showed no significant differences among treatments at 17 °C while at 21 °C mussels exposed to DIC for 21 days presented significantly lower activity in comparison to the remaining treatments. Comparing the two temperatures, significant differences were only observed in contaminated mussels after 21 days of exposure, with lower values at 21 °C (Table 2).

The activity of GSTs showed no significant differences among treatments regardless of the temperature (Fig. 3B). Between temperatures, a significant decrease was observed at 21 °C for CTL mussels at both exposure periods (Table 2).

3.2.3. Proline content and redox balance

At a temperature of 17 °C, a significant decrease in PROL levels was evidenced after 21 days of exposure to DIC while at 21 °C no significant differences were observed among treatments (Fig. 4A). Comparing both temperatures, significant differences were observed after 7 days of exposure in CTL and DIC-exposed mussels, with lower values at 21 °C (Table 2).

At 17 °C, levels of GSH decreased significantly in DIC-exposed mussels after 21 days of exposure in comparison to the remaining treatments. At 21 °C no significant differences were observed among treatments (Fig. 4B). Comparing temperatures, significant differences were observed with lower values at 21 °C in mussels after 7 days at CTL and after exposure to DIC (Table 2).

3.2.4. Neurotoxicity

The activity of AChE was significantly lower in mussels exposed to DIC after 21 days, regardless of the temperature tested (Fig. 5). Comparing both temperatures significant differences were found in CTL mussels after 21 days of exposure, with lower values at 21 °C (Table 2).

3.3. Ordination analysis

The results of the PCO analysis (Fig. 6) revealed that the first main

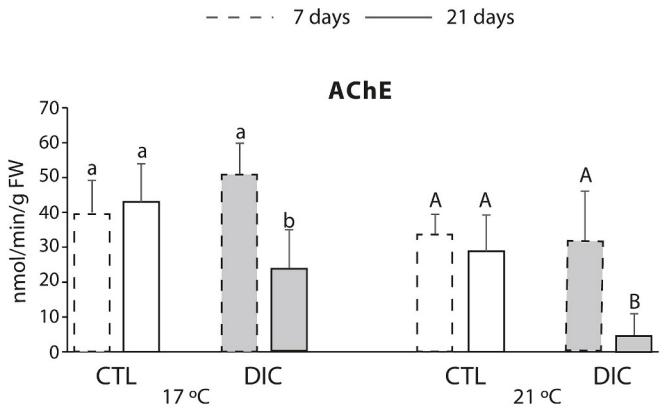

Fig. 5. A: Acetylcholinesterase (AChE) activity, in *M. galloprovincialis* exposed to two different treatments (0 μg/L CTL, 1 μg/L DIC) for 7 and 21 days at both temperatures (17 °C - control, 21 °C). Results are the means + standard deviation. Significant differences ($p < 0.05$) among treatments at 17 °C are identified with lowercase letters and uppercase letters for organisms exposed to 21 °C.

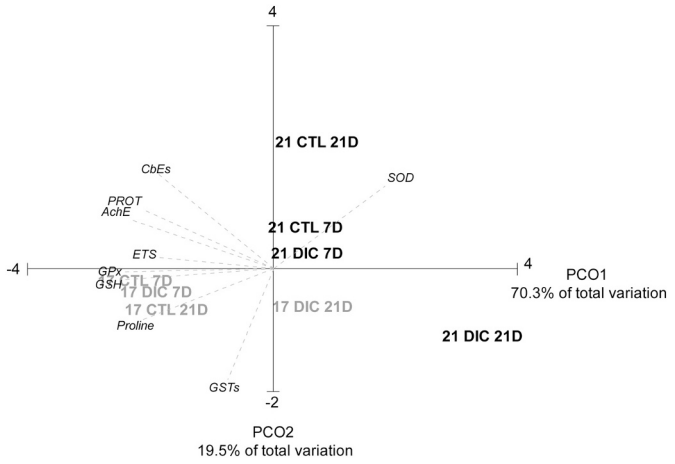

Fig. 6. Principal coordinated analyses (PCO) based on biochemical parameters, measured in *M. galloprovincialis* exposed for 7 and 21 days to different temperatures (17 °C - control, 21 °C) in the absence and presence of diclofenac (DIC). Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data ($r > 0.75$).

component (PCO1) explained 70.3 % of the total variation among treatments, separating mussels exposed to a temperature of 21 °C (CTL 7-21D, DIC 7-21D) at the positive side from mussels exposed to the remaining treatments in the negative side. Biochemical descriptors superimposed on PCO showed that GPx, GSTs, AChE and ETS activities, as well as PROL, GSH and PROT contents were the variables that best explained the variation in PCO1. PCO2 explained 19.5 % of the total variation. The overall PCO2 separates the individuals exposed to 21 °C (CTL 7-21D, DIC 7D) on the positive side from the remaining treatments on the negative side.

4. Discussion

In recent years, the effects of pollutants, including drugs, in the aquatic environment have been particularly studied, investigating not only the concentrations in the environment but also the effects on inhabiting organisms (Impellitteri et al., 2023c; Pagano et al., 2022; Schmidt et al., 2011a, 2011b; Yuan et al., 2023). Among drugs, non-steroidal anti-inflammatory drugs are frequently detected in the aquatic environment. Numerous studies have already documented their ability to induce toxic effects in various organisms, particularly aquatic species. Studies have consistently reported biochemical and physiological alterations in aquatic invertebrates exposed to NSAIDs such as salicylic acid, ketoprofen, and ibuprofen. These alterations include changes in respiration rate, oxidative stress induction, metabolic disturbances, and neurotoxicity. Examples of affected species include *Daphnia magna*, the Manila clam (*Ruditapes philippinarum*), and the Mediterranean mussel (*Mytilus galloprovincialis*) (Almeida et al., 2015; Bownik et al., 2020; Freitas et al., 2019; Matozzo et al., 2012a, 2012b; Nunes, 2019). Effects have been also detected in the gills and digestive gland of *M. galloprovincialis* together with neurotoxicity and endocrine disruption after exposure to DIC (Gonzalez-Rey and Bebianno, 2014). According to the literature, DIC can exert adverse effects on mussels, including histopathological changes (Świacka et al., 2020), oxidative stress, and DNA damage (Mirzaee et al., 2021; Freitas et al., 2019). Physiological impairment, decreased metabolism, and oxidative stress in bivalves exposed to DIC were also reported (Costa et al., 2020a; Freitas et al., 2020). Although few studies report the effects of increased temperature and non-steroidal anti-inflammatory drugs, according to the literature available this combination can cause neurotoxicity and negatively affect organisms' metabolic capacity and antioxidant capacity, namely in marine invertebrates (Bethke et al., 2023; Costa et al., 2020a; Freitas et al., 2019; Freitas et al., 2020).

To our knowledge, there are no studies investigating how biochemical parameters change after exposure to DIC at increased temperatures in mussels. *M. galloprovincialis*. Due to its efficient filtering capabilities, high abundance, and widespread presence in aquatic ecosystems, this species has emerged as a valuable bioindicator organism. Also, because it can tolerate and accumulate contaminants (xenobiotics), it serves as a reliable indicator of potential environmental effects (Impellitteri et al., 2022; Impellitteri et al., 2023d; Impellitteri et al., 2024; Martyniuk et al., 2023b; Porretti et al., 2023; Tresnakova et al., 2023a, 2023b; Torre et al., 2013). In the present study, this species was used as a bioindicator, assessing the metabolic, oxidative stress, and neurotoxic responses after acute and chronic exposure to DIC, investigating the influence of a 4 °C increase on the impacts observed at 17 °C (control temperature).

To investigate organisms' metabolic capacity, the activity of the electron transport system (ETS) can be used as a proxy, and it proved to be a useful measure of the impacts induced by drugs and warming (Freitas et al., 2019; Freitas et al., 2020). The results obtained showed that in the presence of DIC mussels tended to decrease their metabolic capacity in comparison to non-contaminated ones, which was more evident at 17 °C, with contaminated mussels under warming conditions showing a smaller difference to non-contaminated mussels at the same temperature in comparison to the difference observed at 17 °C, where the decrease in the metabolism was higher between non-contaminated and contaminated mussels. Such results indicated that under warming conditions the decreased metabolic capacity in contaminated mussels has reached mussels' metabolic limit, probably indicating that the reduction in mussels' aerobic respiration was no longer a viable defence strategy. Metabolic depression was already demonstrated by Freitas et al. (2020) when subjecting *M. galloprovincialis* to another nonsteroidal anti-inflammatory drug (salicylic acid) at a control temperature (17 °C) and warming conditions (21 °C). Lower metabolism in contaminated mussels could be related to valve closure, a strategy to avoid the accumulation of the drug. Gosling (2003) reported the closing of valves as a protective effect against contaminants. Anestis et al. (2010), further demonstrated that lowered metabolism was associated with valve closure due to an increase in temperature (24 °C). The present findings further demonstrated that after a shorter exposure period (7 days) at 21 °C DIC-exposed mussels decreased their ETS activity in comparison to 17 °C, while after 21 days of exposure, no differences were observed between contaminated mussels maintained at different temperatures. Such results might explain similar DIC concentration levels in mussels exposed to different temperatures. A similar response pattern at two distinct temperatures after a longer exposure period may indicate that organisms, after undergoing the stress caused by DIC, adopted an adaptive response due to a phenomenon known as hormesis, enhancing their defence responses (Sun et al., 2021).

Protein content can be used to assess environmental stress because organisms can use proteins as energy reserves for stress management (Smolders et al., 2004). At the same time, it is known that under stressful conditions organisms can increase the production of defence enzymes, which can be associated with an increase in protein production (Freitas et al., 2019). The results of the present study showed that at 17 and 21 °C uncontaminated and DIC-exposed mussels presented similar PROT content regardless of the exposure period, indicating that associated with lower metabolic capacity contaminated mussels decreased the expenditure of PROT and/or the use of proteins was compensated by their production. Recent studies assessing the impacts of drugs demonstrated a similar response pattern, as reported by Silva et al. (2022) after exposing the species *R. philippinarum* for 28 days to 17 α-ethinylestradiol at 21 °C. In what concerns to temperature effect, as it occurred with mussels' metabolic capacity, after 7 days DIC-exposed mussels at 21 °C presented lower PROT content in comparison to contaminated mussels at 17 °C, but this difference disappeared after 21 days with contaminated mussels presenting similar PROT content regardless of the

temperature tested. Coppola et al. (2018) report that no significant differences in PROT content are noted after exposure of *Mytilus galloprovincialis* to increased temperature from 17 to 21 °C and for up to 28 days of exposure to arsenic. The obtained results may indicate that the influence of temperature on DIC impacts could be attenuated after a longer exposure period, leading to organisms' adaptation. Nevertheless, the present findings might also indicate that after more prolonged stress mussels may exhaust their ability to further reduce protein content. This should be investigated in future studies, with longer exposure periods, trying to understand the limited capacity of mussels to produce and use their protein content against stress.

Besides changes in organisms' metabolic capacity, exposure to stressful conditions often results in an overproduction of reactive oxygen species (ROS) which, if not eliminated efficiently by antioxidant defence mechanisms, can lead to cell damage (Huchzermeyer et al., 2022). The results showed that at each temperature (17 and 21 °C) contaminated and uncontaminated mussels presented similar SOD activity, regardless of the exposure period. Similarly, Trombini et al. (2019) showed after 7 and 14 days of exposure a decrease in SOD activity in the gills and digestive gland in the organism *R. philippinarum* after exposure to ibuprofen. Freitas et al. (2016), on the other hand, showed maintenance of the activity of antioxidant enzymes such as SOD after 28 days of exposure of *Scrobicularia plana* to carbamazepine. Furthermore, the obtained results demonstrated that warming led to SOD activation, regardless of the presence or absence of DIC and exposure time. Such results can indicate that this enzyme is more affected by warming than by DIC. However, this behaviour could depend on the contaminant tested since studies conducted by Almeida et al. (2021) showed higher SOD activity in *M. galloprovincialis* organisms exposed to heating conditions (21 °C), but the combined effect of higher temperature and carbamazepine or cetirizine resulted in enhanced SOD activity. Similarly, Freitas et al. (2020) showed that the highest SOD activity was observed when *M. galloprovincialis* specimens were exposed to the combined effect of warming and salycilic acid. Nevertheless, in this study, the activity of this enzyme increased in comparison to the control when organisms were only exposed to warming conditions. Therefore, regarding the antioxidant SOD, the present results can indicate that this enzyme activity is more affected by warming than by DIC or that under the combined scenario, mussels reached their capacity to activate this antioxidant enzyme. This hypothesis can be related to the limited capacity of mussels to increase protein production when in the presence of DIC.

Regarding the activity of GPx, at 17 °C there was a decrease in DIC-exposed mussels regardless of the exposure period, while at 21 °C the activity of this enzyme was similar both in contaminated and non-contaminated mussels. Comparing temperatures, differences in terms of GPx activity were only significant after 7 days of exposure with lower values at 21 °C, which can be explained by the fact that after the shorter exposure, the organisms became accustomed to it and therefore did not show an effect in the longer exposure. Similarly, the inhibition of GPx was observed by Freitas et al. (2020) when studying the effects of temperature rise on the exposure of *M. galloprovincialis* to salicylic acid after a 28-day exposure period. The same was shown by Costa et al. (2020a) after 7 days of exposing *R. decussatus* clams to Triclosan at a temperature of 21 °C. Therefore, these findings suggest that GPx activity may require a longer exposure time to respond to certain pollutants, like salicylic acid or triclosan; in contrast, for other pollutants such as carbamazepine (Trombini et al., 2019), GPx activity can be altered even after short-term exposure, as observed in this study.

In terms of detoxification capacity, phase I enzymes (CbEs) showed no activation in the presence of DIC at 17 °C indicating that at control temperature the presence of DIC was not enough to alter this enzyme activity, probably due to the low concentration tested. However, at 21 °C the activity of CbEs decreased in the presence of DIC after a longer exposure period, which can indicate that a prolonged exposure to the combined effect of DIC and warming led to the inhibition of this enzyme. Comparing temperatures, CbEs showed lower activity at 21 °C after 21 days of exposure while 7 days after the onset of the experiment no differences were observed between temperatures. Such results may corroborate the hypothesis that inhibition may need longer exposure to the stressors. The same inhibition was shown by Queirós et al. (2021) after exposure of cyclophosphamide (100 ng/L) to the *M. galloprovincialis* organism at control temperature after 28 days. This decrease was also noted by Andrade et al. (2022) in which CbEs activity decreased after exposure to another type of pollutant, Lanthanum to *M. galloprovincialis*, for 28 days.

In terms of phase II biotransformation enzymes, at each temperature tested the presence of DIC did not change the activity of GSTs, regardless of the exposure time. Also, between temperatures, the activity of GSTs was similar in contaminated mussels. These findings evidence the limited role of GSTs in DIC detoxification. Almeida et al. (2021) observed that, at 17 °C, GSTs activity increased in *R. philippinarum* clams contaminated with carbamazepine. By contrast, no increase or decrease was shown at 21 °C. Overall, the present findings demonstrated that after 7 and 21 days of exposure to DIC, mussels presented a limited role in the drug biotransformation, which can even be negatively affected by warming. An exposure period of 28 days to DIC and warming showed the activation of GSTs (Freitas et al., 2019) which corroborates the hypothesis that this enzyme activity is time-dependent. However, the DIC concentration in mussel tissues significantly differed between exposure times, with the highest levels observed after 21 days compared to 7 days. These results might be attributed to the limited activity of CbEs, which showed the lowest levels in mussels exposed to DIC at 21 °C after 21 days compared to those recorded in mussels under the same treatment for 7 days.

Proline is important in osmotic regulation and is also crucial in stress tolerance (Ábrahám et al., 2010). In the present study, at 17 °C a decrease in PROL content was observed in contaminated mussels after 21 days of exposure but at 21 °C the presence of DIC did not change the content of PROL. Proline is commonly used to assess stress conditions on marine invertebrates. Elevated PROL levels were detected in *Theodoxus fluviatilis* (Wiesenthal et al., 2019) and *Crassostrea virginica* (Li et al., 2022) subjected to hyperosmotic stress for 3 and 7 days, respectively. Since our results showed a decrease, this indicates that organisms such as *M. galloprovincialis* fail to maintain their PROL levels under certain stress conditions, namely pollutants such as DIC. A similar response was observed regarding reduced glutathione, with lower GSH content in mussels maintained at 21 °C, especially in the presence of DIC. Mezzelani et al. (2016) also reported a decrease in GSH after *M. galloprovincialis* exposure to DIC for 14 days. The same was shown by Costa et al. (2020a), with a decrease in GSH levels at a temperature of 17 °C in the two species of *R. philippinarum* and *R. decussatus* after 7 days of exposure to DIC.

Acetylcholinesterase (AChE) is an enzyme belonging to the cholinesterase class. It plays a critical role in neurotransmission at cholinergic synapses and its activity has been established as a valuable tool for evaluating the impact of xenobiotics. In bivalves, numerous studies have documented the inhibition of this enzyme when organisms were exposed to contaminants, such as pesticides (Haque et al., 2019; Petrovici et al., 2020; Plhalova et al., 2018), metals (Parra et al., 2021) and pharmaceuticals (Almeida and Nunes, 2019). Furthermore, the combined effect of warming and copper (Cu) and oxyfluorfen led to AChE inhibition in the bivalve *Cerastoderma edule* (Mesquita et al., 2023). In the present study, AChE was inhibited in the presence of DIC both at 17 and 21 °C only after a 21-day exposure period, indicating that inhibition may need a longer exposure period than 7 days. In this regard, Mezzelani et al. (2016) and Freitas et al. (2019) report an inhibition of AChE activity in the organism *M. galloprovincialis* after exposure to other NSAIDs such as paracetamol and salicylic acid for 14 and 28 days, respectively. Comparing temperatures, AChE activity tended to be lower at 21 °C (at both exposure days) corroborating the examples given before that showed the negative impacts of warming and a contaminant on mussels'

neurotoxic status.

The concept of One Health emphasizes the interconnectedness of human health, animal health, and the environment. In this context, the study of DIC effects on *M. galloprovincialis* reflects the interplay between environmental pollution, ecosystem health, and potentially human health. The presence of pharmaceuticals like DIC in aquatic environments not only impacts aquatic organisms but also raises concerns about human exposure through the consumption of contaminated seafood or water. Furthermore, the study underscores the importance of understanding how environmental stressors, such as pollutants like DIC and temperature changes, can affect organisms' biochemical processes. By studying the responses of *M. galloprovincialis*, which serves as a bioindicator species, researchers gain insights into broader ecological and public health implications. In summary, this research aligns with the One Health approach by recognizing the interconnectedness of environmental, animal, and human health. Studying the effects of pollutants like DIC on aquatic organisms contributes to our understanding of ecosystem health and underscores the need for holistic approaches to address environmental challenges.

5. Conclusions

In conclusion, DIC, which is commonly found in aquatic systems, negatively affected the biochemical performance of *M. galloprovincialis*, and these effects were aggravated by warming, with a lack of increasing response capacity over time. Considering the tested conditions, *M. galloprovincialis* proves to be an excellent bioindicator species for assessing the effects of drugs under different environmental conditions. Sub-cellular impacts might induce alterations at higher biological levels, leading to a potential decrease in bivalves' growth and reproduction capacity, with social and economic impacts.

CRediT authorship contribution statement

Federica Arrigo: Writing – original draft, Methodology, Formal analysis. **Lucia De Marchi:** Writing – review & editing, Methodology, Formal analysis. **Valentina Meucci:** Resources. **Giuseppe Piccione:** Resources. **Amadeu M.V.M. Soares:** Resources, Funding acquisition. **Caterina Faggio:** Writing – review & editing, Supervision, Resources. **Rosa Freitas:** Writing – review & editing, Supervision, Resources, Formal analysis, Conceptualization.

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.

Data availability

Data will be made available on request.

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