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SILVANA TEIXEIRA COSTA

Marine heatwaves: a new insight of climate change. The combined effect with Carbamazepine upon ***Mytilus galloprovincialis*****.**

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Marine heatwaves: uma nova abordagem das alterações climáticas. O efeito combinado com Carbamazepina em *Mytilus galloprovincialis.*

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Doutor Alessandro Nardi, Investigador Post-Doc no Dipartimento di Scienze della Vita e dell'Ambiente da Università Politecnica delle Marche (Itália) e da Doutora Rosa Freitas, Investigadora auxiliar (Nível 1) do Departamento de Biologia da Universidade de Aveiro (Portugal).

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palavras-chave

Toxicidade, fármacos, mexilhões, stress térmico, parâmetros bioquímicos, resposta oxidativa.

resumo

Os sistemas costeiros marinhos estão entre os ecossistemas mais afetados pelas alterações climáticas. Neste âmbito, uma preocupação crescente tem surgido em torno dos impactos causados por eventos meteorológicos, nomeadamente, ondas de calor (MHWs) sobre o biota. As ondas de calor são períodos nos quais a água do mar fica extremamente quente e que podem durar dias ou meses e estenderem-se por milhares de quilómetros. Os mais recentes eventos têm revelado a elevada vulnerabilidade dos ecossistemas marinhos para com as ondas de calor que, além disso, estão sujeitos também a variados poluentes que alcançam o compartimento aquático e podem constituir perigo. Foi já documentado na literatura que os efeitos da temperatura podem alterar a biodisponibilidade, propriedades dos poluentes presentes na água, alterando a toxicidade e a sensibilidade dos organismos para estes. No entanto, a informação é ainda escassa no que diz respeito aos impactos das MHWs no biota e quais são os efeitos causados pela exposição combinada com poluentes, nomeadamente Carbamazepina (CBZ). Carbamazepina é um fármaco emergente que pode ser encontrado no ambiente aquático na ordem dos ng/L até aos µg/L. Dado isto, mexilhões *Mytilus galloprovincialis* foram escolhidos como organismos modelo para desenvolver a investigação. Os organismos foram expostos a dois cenários climáticos distintos: condições térmicas consideradas ótimas, onde a temperatura foi constante (18 ºC) durante 20 dias; ação de uma onda de calor compreendida entre os 18 ºC e os 22.5 ºC que durou 11 dias (pico de temperatura ao dia 6) seguida por um período de recuperação de 10 dias (a 18 ºC). Os impactos das MHWs foram avaliados de forma isolada e em combinação com 1 µg/L de CBZ, resultando num total de quatro tratamentos diversos: controlo (CTL), contaminação por carbamazepina (CBZ), recriação de uma onde de calor (MHW) e a combinação dos dois (CBZ + MHW). A bioacumulação de CBZ foi determinada e os impactos biológicos avaliados considerando uma larga bateria de biomarcadores toxicológicos. As análises mostraram um claro sinergismo entre MHW e acumulação de CBZ nos tecidos dos organismos. Apesar das interações entre a temperatura e CBZ mostrarem ser dependentes do tempo de exposição, notou-se uma clara coordenação entre a resposta oxidativa total, parâmetros dependentes de glutationa, metabolismo de ácidos gordos e concentração de malondealdeído. Foram observadas variações significativas do sistema imune, causadas pela exposição múltipla CBZ + MHW (especialmente no teste de Micronúcleo) enquanto a condição MHW gerou mais danos genéticos a nível dos hemócitos. Concluindo, os resultados confirmaram que as MHWs podem influenciar os efeitos ecotoxicológicos dos contaminantes, realçando a importância de desenvolver mais investigação de forma a compreender os mecanismos celulares e prever respostas biológicas de organismos de elevada relevância em termos económicos e de saúde pública.

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keywords

Toxicity, pharmaceuticals, mussels, thermal stress, biochemical parameters, oxidative response.

abstract

Marine coastal systems are among the main climate change targets. In particular, an increasing concern has raised on the impacts caused by weather events, namely marine heatwaves (MHWs), towards organisms from these areas. MHWs are periods of extreme warm sea surface temperature that persist for days to months and can extend up to thousands of kilometres. Some of the recently observed MHWs revealed the high vulnerability of marine ecosystems and fisheries to such extreme climate events. Beside thermal aggression, inhabiting organisms are also exposed to several pollutants that reach the aquatic compartment and constitute potential hazardousness. Furthermore, it is already reported in literature that warming may affect chemicals' bioavailability and speciation, changing pollutants toxicity, but temperature rise may also change organism's sensitivity to pollutants. Nevertheless, limited information is reported regarding MHWs impacts on wildlife and how they cope with pollutants, namely pharmaceuticals such as the case of Carbamazepine (CBZ). Carbamazepine is a pharmaceutical of emerging concern, found in aquatic systems in concentrations ranging from ng/L to µg/L. For this, mussels *Mytilus galloprovincialis* were chosen as model organisms to develop the present research study. Organisms were exposed to two different climatic scenarios: an optimal scenario where organisms were subjected to 18 ºC water temperature for 20 days and a heatwave scenario ranging between 18 ºC to 22.5 ºC that lasted 11 days (temperature peak at day 6), followed by a recovery period of 10 days (constant temperature 18 ºC). The impacts caused by MHW were evaluated alone and in combination with CBZ contamination of 1 µg/L corresponding to four different treatments: controls (CTL), Carbamazepine exposure (CBZ), marine heatwave scenario (MHW) and the combination of both stressor (CBZ + MHW). Carbamazepine bioaccumulation was assessed and biological impacts were evaluated considering a wide battery of toxicological biomarkers. The results obtained revealed a clear synergism of MHW treatment on CBZ uptake. However, interactions between temperature and CBZ exposure had effects on induction of antioxidant responses, data showed a time dependent impact. Nevertheless, a general impairment was observed between total oxidative scavenging capacity, glutathione dependent parameters, fatty acids metabolism and malondialdehyde content. Significant variations of the immune system were mainly caused by multiple exposure (CBZ + MHW), specially for Micronuclei test. Moreover, MHW showed to be much more effective in what concerns DNA damage in haemocytes. The overall results confirmed that MHWs could influence ecotoxicological effects of environmental contaminants, praising the concern of developing more and better researches in order to understand cellular mechanisms and predict toxicological responses of marine organisms of economic relevance and public health concern.

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1. INTRODUCTION

1.1. SCIENTIFIC CONTEXTUALISATION

Marine ecosystems play a key role by providing mankind with important services such as primary protein provision for a seventh of world's population (FAO, 2012) and climate regulation through uptake and storage of atmospheric carbon dioxide (Parekh et al., 2006; Kwon et al., 2009). The structure and the functioning of these ecosystems are based on a balance of abiotic and biotic factors, constantly challenged by alterations caused by natural and anthropogenic stressors affecting several aspects of this network, namely oxygen concentration, temperature, pH, food availability and input of chemical substances (Kappel et al., 2005; Doney et al., 2011; Gattuso et al., 2015).

The occurrence of anthropogenic CO_2-driven climate changes gained the scientific attention, in particular due to the negative consequences of water's parameters shifts (oxygen concentration, temperature, pH, food availability and input of chemical substances) towards aquatic biota (Sokolova et al., 2004; Mubiana and Blust et al., 2007; Coppola et al., 2018; Maulvault et al., 2018a; Serra-Compte et al., 2018; Costa et al., 2020a; 2020b). For instance, the continuous release of CO_2 into the atmosphere led to changes in ocean carbon system, lowering seawater pH: this process, known as ocean acidification (OA), may cause several biological adverse effects such as reduced calcification rates and, consequently, lowered viability of marine calcifying organisms (Doney et al., 2011). Moreover, the increase of CO_2 in the atmosphere is enhancing greenhouse effect, causing the increase of air and sea mean temperatures: warming scenarios are frequently linked with ocean stratification which is more likely to originate nutritional restrictions for water surface's photosynthetic organisms (Steinacher et al., 2010). Higher temperatures of surface water are also likely to reduce oxygen solubility (Vaquer-Sunyer et al., 2008). Obviously, it is foreseen that the combined effect of these changes will lead to biodiversity and habitat reduction, shifts of species distributional patterns, and ecosystem loss (Brierley and Kingsford, 2009; Cheung et al., 2009; Doney et al., 2011; Sarà et al., 2014).

Human-induced climate change is even closely related with daily temperature and precipitation extremes which in recent years have been more frequent, intense and of wider occurrence (Stott, 2016; Bindoff et al., 2013; Zwiers et al., 2013). Despite the rising concern regarding the impacts of extreme weather events (EWE), this topic is still poorly documented considering their irregular and difficult to predict occurrence and limitations for their determination and quantification (Ummenhofer and Meehl, 2017). Despite EWEs are defined as rare occurrences (Alexander, 2016), a huge increase has been registered. The American Meteorological Society's annual report of extreme events was first published in 2012 with six

extreme event studies for the year 2011 (Peterson et al., 2012). The number of studies rose sharply to 32 EWEs in 2016 worldwide (Herring et al., 2014; Stott, 2016). According to Intergovernmental Panel on Climate Change Special Report on Extreme events (IPCC, 2012), a certain occurrence should be considered EWE if climate variable is above or below a threshold value near upper or lower (respectively) end of the previously obtained records for the studied variable and area. Usually, thresholds are considered variations of 1 %, 5 % or 10% in comparison to reference periods (Ummenhofer and Meehl, 2017). The uncertainty and unpredictability associated with EWEs, regarding their intensity, frequency, duration, extension, seasonality or preconditioning, are on top of the research interests (Seneviratne et al., 2012). Nevertheless, to define an EWE, it is required not only a shift in the mean of probability distribution but also in the shape of the distribution (Ummenhofer and Meehl, 2017). It was already demonstrated that changes in the frequency of extremes are consistent with overall shifts in the distribution (Ballester et al., 2010; Hartmann et al., 2013; Rhines and Huybers, 2013). Moreover, both space and time can influence impact-related thresholds and, due to this, EWEs can be defined in two ways: i) in relation to a given impact, i.e., related to a specific threshold; ii) by determining the probability of occurrence of a specific EWE (Seneviratne et al., 2012). The complex interaction stablished between all the variables praise the need of reliable detection and attribution of changes of a given climate event (Ummenhofer and Meehl, 2017), leading to fulfilment of information gaps in terms of EWEs' impacts and respective adaptation and mitigation (Westra et al., 2014; Easterling et al., 2016; Stott et al., 2016).

For what concerns the ocean, it has been observed an increasing frequency, intensity and duration of extreme warming events, called marine heatwaves (MHWs) (Perkins et al., 2012; Frölicher et al., 2018; Oliver et al., 2018): based on the previous definition of extreme events, these are defined as periods during which seawater temperature exceeds a seasonally-varying threshold, usually the 90th or 99th percentile, for at least 5 consecutive days (Hobday et al., 2018). Globally, the number of anomalous warm days per year has increased by 50 % since the early 20th century and average intensity of these showed a linear trend of almost +0.1 °C per decade since 1982 (Oliver et al., 2017; 2018). These trends, attributed to the anthropic influence on climate stability, are projected to worsen on a global scale by the end of the century, with many areas of the ocean experiencing a nearly permanent state of MHW (Oliver et al., 2017; 2018).

The mounting evidence around heatwaves and their potential irreversible effects become more overwhelming regarding the lack of knowledge related with ocean impacts (IPCC, 2014). Although a growing number of studies had been performed in order to register

individual events occurrence (Marbà and Duarte, 2010; Mills et al., 2013; Di Lorenzo and Mantua, 2016; Wernberg et al., 2016; Oliver et al., 2017), scarce information is yet available regarding MHWs drivers, occurrence and above all biological impacts (Frölicher et al., 2018). This knowledge gap is of considerable concern regarding the high vulnerability of marine ecosystems, fisheries and human societies, to these events (Frölicher and Laufkötter, 2018). This susceptibility was well observed in previous events worldwide, like the year 2003 marked by extremely high temperature values in central and southern Europe (Schär et al., 2004). Even considering within seasonal fluctuations, that was the hottest summer since the early 16th century (Luterbacher et al., 2004). With no background of such event, scientific community faced the challenge to predict responses of ecological systems that caused mass mortalities in the north-western Mediterranean (Garrabou et al., 2009). Posterior occurrences were registered in the Western Australian coast in 2011 (Pearce and Feng, 2013), Northwest Atlantic in 2012 (Mills et al., 2013) or even more severe events, namely the 2015-2016 abnormal warming recorded across most of the tropical and extratropical oceans (Banzon et al., 2016). All these events showed to cause changes in biological production, toxic algal blooms (Cavole et al., 2016), regime shifts in reef communities (Wernberg et al., 2013; Wernberg et al., 2016), mass coral bleaching (Hughes et al., 2017) and mortalities of commercially important fish species (Caputi et al., 2016) with socio-economic negative consequences (Mills et al., 2013). The demand for deeper knowledge of the impacts of these events gains even more relevance when considering that, according with Frölicher et al. (2018), it is estimated that nowadays a 3.5 °C warming is 41 times more likely to occur when comparing with the pre-industrial times. This means that by the end of the 21st century, it is foreseen that ecosystems will be exposed to 3.5 °C MHWs every 3 days. The probability of MHWs occurrence is expected to increase almost everywhere, especially for the tropics and Arctic Ocean given the fact that these areas have limited sea surface temperatures' variations. It is less probable to occur in Southern Ocean given that surface waters are projected to stay relatively cool, and therefore the probability ratio does not increase much under all warming levels. Nonetheless, the Mediterranean Sea stands among the most endangered basins, both in terms of projected increase of intensity of MHWs events of + 2-4 °C and impacts on marine ecosystems structure, functioning and services (Galli et al., 2017; Oliver et al., 2018; Darmaraki et al., 2019). In particular, Galli et al. (2017) showed that the increase of heatwaves occurrence will impact many species that live in shallow waters: suggesting a decrease of the survival horizon for red coral (*Corallium rubrum*, a commercially exploited benthic species already subjected to heat-related mass mortality events) and coralligenous reefs. Noticeably, these events are documented worldwide such as the investigation reported by Sanford et al. (2019) on which

the authors analysed the effects on coastal biota of heatwaves reported within 2014-2016, in California. Sanford et al. (2019) reported substantial changes in geographic distributions and/or abundances across a diverse suite of 67 southern species, including an unprecedented number of poleward range extensions (37) and striking increases in the recruitment of owl limpets (*Lottia gigantea*) and volcano barnacles (*Tetraclita rubescens*). Considering these scenarios, deeper knowledge regarding organisms' biological responses to MHWs is essential.

Marine ecosystems are also threatened by continuous release of pollutants throughout a plethora of sources and modes of input among which emerging pollutants (EPs) like pharmaceuticals are of primary concern. Pharmaceuticals are a large and diverse group of compounds used in huge amounts all around the globe that can reach the marine ecosystem through inefficient wastewater treatment plants (WWTPs), industrial and hospital discharges, aquaculture facilities, animal farming and runoff of soils (Huerta et al., 2012; Almeida et al., 2014; Mezzelani et al., 2018). The issue becomes even more overwhelming when considering that some pharmaceuticals are not completely metabolized and parent compounds, metabolites and conjugates are excreted (Heberer et al., 2002). Pharmaceuticals are now ubiquitously detected in aquatic ecosystems at concentrations ranging from ng/L to µg/L (Fent et al., 2006), and since they can be persistent in the environment a widespread bioaccumulation in marine organisms has been demonstrated (Mezzelani et al., 2018; Mezzelani, personal communication).

Within the most relevant pharmaceuticals is Carbamazepine (CBZ), an antiepileptic drug. Bahlmann et al. (2012) observed a significant increase in CBZ concentrations in surface waters in Berlin (Germany) in 2009, where environmental concentrations were monitored for 10 months. This subject become of high concern due to low CBZ removal in WWTPs (below 10 %) (Zhang et al., 2008). This substance is currently found in WWTP influents and effluents, surface waters, groundwater and even in treated drinking water in concentrations ranging from 0.03 to 6.3 µg/L (Bahlmann et al., 2009; Bahlmann et al., 2012; Aguirre-Martínez et al., 2013b). Some authors even classified CBZ as "R52/53 Harmful to aquatic organisms and may cause long term adverse effects in the aquatic environment" (Tsiaka et al., 2013). Taking the previous information under consideration, CBZ is highly tested in ecotoxicological studies to evaluate its hazard toward non-target organism (Almeida et al., 2014; 2015; Freitas et al., 2016; Pires et al., 2016; Qiang et al., 2016; Oliveira et al., 2017; Serra-Compte et al., 2018). In particular, Almeida et al. (2014; 2015) demonstrated that CBZ (0.00-9.00 µg/L and 0.03-0.30 µg/L, respectively) is responsible for alterations in clams (*Ruditapes decussatus* and *Ruditapes philippinarum*) oxidative status and metabolic capacity. Studying the effects of CBZ in polychaetes, Pires et al. (2016) evaluated the impacts of this drug on the regenerative capacity

of *Diopatra neapolitana*. Polychaetes were exposed, during 28 days, to CBZ (0.0 to 9.0 µg/L) and the obtained results revealed that with the increase of drug concentrations, organisms regenerated less new segments and took longer to completely regenerate. Regarding toxicological tests on fishes, Qiang et al. (2016) examined the effects of CBZ on zebrafish embryos in terms of phenotype, behaviour and molecular responses. The results showed that 1 µg/L CBZ conducted to increased hatching rate, body length, swim bladder appearance, higher sensitivity to touch and light stimulation and disturbed the expression pattern of neural-related genes. Moreover, Oliveira et al. (2017) also assessed CBZ (0.0-9.0 µg/L) effects on mussels *Mytilus galloprovincialis.* Results demonstrated that after a chronic exposure, gonadosomatic indices were negatively affected.

In order to understand organisms' responses towards EWEs and CBZ, an ideal biological indicator must be chosen. Given the previously mentioned wide ecotoxicological application, mussels were chosen to carry the experiment. Mussels have been extensively used in literature to monitor anthropogenic pollution trends in coastal waters (Farrington et al., 2016; Beyer et al., 2017). This species was one of the first animals used to evaluate environmental quality of seawater by Goldberg in 1975. Mussels are worldwide distributed, easy to access and are highly tolerant but responsive to environmental shifts such as salinity, temperature, oxygen levels and food availability (Li et al., 2019). Moreover, mussels are benthic filter feeder organisms that can efficiently accumulate pollutants from water column providing, therefore, an accurate environmental quality measurement tool (Beyer et al., 2017). In addition, beside all the environmental importance, another reason contributing to the huge usage of this species is related to the fact that mussels may constitute a contamination vector and, therefore, represent a risk for human health through dietary intake (UNEP, 2016; Van Cauwenberghe and Janssen, 2014; Beyer et al., 2017). For this, mussels have been widely used in many environmental monitoring programs worldwide (Beyer et al., 2017) and *M. galloprovincialis* (Fig. 1) chosen for present research.

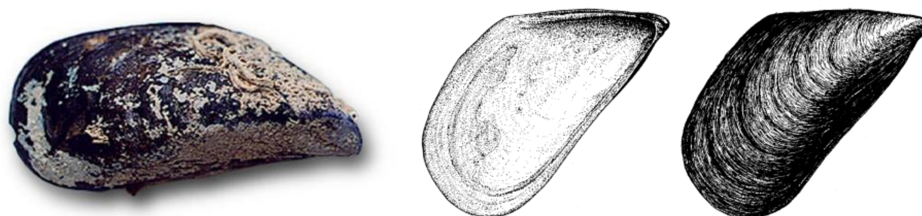

Fig. 1- *Mytilus galloprovincialis* (Food and Agricultural Organisation (FAO), 2012)

Scientific literature is rich of studies regarding the biochemical, cellular and biological impacts of chemical pollution under actual and more rarely predicted warming scenarios, towards mussels. Increased water temperature might negatively impact organism's thermal tolerance (Hofmann and Todgham, 2010), affecting physiological processes like growth and reproduction (Boukadida et al., 2016) and inducing biochemical alterations (Andrade et al., 2019; Freitas et al., 2017; Nardi et al., 2017; Velez et al., 2017). There are several evidences that thermal stress, by affecting metabolism, could increase the production of intracellular reactive oxygen species (ROS) and consequently affect the antioxidant system, with damage to proteins, lipids and DNA (Verlecar et al., 2007; Coppola et al., 2018; Freitas et al., 2017; 2019; 2020). Temperature elevation can also induce higher rates of bioaccumulation of certain compounds, as already showed in literature (Sokolova et al., 2004; Mubiana and Blust et al., 2007; Coppola et al., 2018; Maulvault et al., 2018a; Serra-Compte et al., 2018), and this effect could be influenced by seasonal organisms biology (Nardi et al., 2018; Giuliani et al., 2020). Despite this, to date there is no information available on the interactive effects of environmental realistic scenarios of MHWs on the bioaccumulation and biological effects of contaminants in marine organisms. For this, the present work will add significant value to the present-day ecotoxicological research for climate change and emerging pollutants, evaluating immune function, neurotoxic alterations, antioxidant and metabolic capacity, oxidative damages and DNA integrity, widely used biomarkers to assess impacts of pollutants and climate change related factors in bivalves, including mussels.

It is well known that the accumulation of xenobiotics and their related metabolic products inside the lysosomes weakens the lysosomal membrane stability and may induce diffusion of hydrolytic lysosome enzymes into the cytosol. The assessment of mussels' haemocytes lysosomal membrane stability has been proposed as a rational biomarker of general stress to both pollutants and climate change related factors (Gorbi et al., 2008; Mezzelani et al., 2016b; Nardi et al., 2017; 2018). Mussels haemocytes can be classified in two subpopulations, granulocytes and hyalinocytes, whose quantification constitute a widely used ecotoxicological biomarker that may highlight important alterations on the immune capacity of mussels in case of insult (Carballal et al., 1997); among the two subpopulations, granulocytes have phagocytic role, and alterations of both granulocytes vs hyalinocytes ratio and phagocytosis have been demonstrated in *M. galloprovincialis* exposed to environmental contaminants (metals, pharmaceuticals, microplastics), alone or in combination with climate change scenarios (Avio et al., 2015; Mezzelani et al., 2018; Nardi et al., 2018). In what regards to neurotoxicity, Acetylcholinesterase (AChE) activity has been widely used as an indicator of potential neurotoxicity (Gorbi et al., 2008; Mezzelani et al., 2016b), being sensitive to organic

contaminants that can permanently block the active site. Acetylcholinesterase breaks acetylcholine in acetic acid and choline, and thus terminate neurotransmission: acetylcholine is a neurotransmitter used in efferent systems and also in some central circuits (Woolf, 1991). It is synthesized in the cytoplasm of cholinergic neurons by the enzyme choline acetyltransferase and degraded at the synaptic cleft by the enzyme AChE. Organic compounds, but even environmental stressors, can alter key pathways of lipid metabolism, such as β-oxidation of very long-chain fatty acids affecting the activity of Acyl-CoA oxidase (Small et al., 1985; Mezzelani et al., 2016b). Alterations on lipid metabolism can be reflected on accumulation or usage of energy reserves, like neural lipids (Mezzelani et al., 2016b; Nardi et al., 2017).

Among the most investigated cellular processes in ecotoxicology, stands the antioxidant system, responsible of limiting damages caused by reactive oxygen species (ROS). Several environmental contaminants, both inorganic and organic, can alter the oxidative status of cells, by either directly increasing the production of ROS or altering the functioning of the complex network of scavenging molecules and enzymes that characterize the antioxidant system (Regoli and Giuliani et al., 2014). Mostly present within peroxisomes, catalase (CAT) is an extremely active catalyst for reduction of H_2O_2 to H_2O (Halliwell, 2007). Therefore, the antioxidant role of this enzyme is essential in promoting the degradation of this ROS. Hydrogen peroxide is substrate also for glutathione peroxidases (GPx), using reduced glutathione (GSH) as electron donor to catalyse the reduction of H_2O_2 to H_2O, while GSH is contemporaneously oxidized to GSSG. The opposite reaction, i.e., the reduction of GSSG to GSH is catalysed by glutathione reductase (GR) which, despite not a real antioxidant enzyme, is nonetheless essential to maintain the correct GSH/GSSG ratio and the intracellular redox status in marine organisms. Reduced glutathione is considered one of the most important antioxidant agents involved in protection of cell membranes from lipid peroxidation by scavenging oxygen radicals (Meister, 1989). Moreover, glutathione is the cofactor of many enzymes catalysing the detoxification and excretion of several toxic compounds. Among these enzymes, the GPx (including Se-dependent as well as Se-independent enzymes), through reduction of both hydrogen peroxide and organic hydroperoxides, provide an efficient protection against oxidative damage and free radicals. Another group of enzymes, glutathione S-transferases (GSTs), acts as the catalyst of a very wide variety of conjugation reactions of glutathione with xenobiotic compounds and lipid hydroperoxides containing electrophilic centres. As anticipated, the cellular balance between prooxidant challenge and antioxidant defences can be altered by a variety of chemical compounds and measuring variation of single antioxidant defences may sometimes under- or over-estimate the oxidative challenge

experienced by the cell. It is therefore useful to have an integrated picture of the antioxidant status, achieved through the analysis of the Total Oxidative Scavenging Capacity assay (TOSCA) which provides quantitative information on the capability to counteract hydroxyl (HO•) and peroxyl (ROO•) radicals (Regoli and Winston, 1998; Regoli et al., 2000).

When oxidative pressure overwhelms antioxidant defences, oxidative damages to lipids, proteins and DNA can occur. The oxidative damage of membrane polyunsaturated lipids is characterized by free-radical mediated chain oxidations, which lead to the formation of lipid free radicals. The lipid peroxidation of polyunsaturated fatty acids will lead to malondialdehyde formation, which concentration is often measured in ecotoxicological studies (Gorbi et al., 2008). Nonetheless, enhanced lipid and protein oxidation can lead to accumulation within tertiary lysosomes of lipofuscin, end product not further degradable (Broeg and Gorbi, 2011); accumulation of lipofuscin was demonstrated to occur in organisms exposed to pollutants, warming and ocean acidification (Mezzelani et al., 2016b; Nardi et al., 2017; 2018).

Similarly, oxidative insult and genotoxicity of chemicals can cause damage to DNA and nuclear abnormalities through several processes. Alterations on cellular DNA can represent one of the first exposure impacts (Bolognesi et al., 2004) while long-term expositions can lead to chromosomal damage after cell replication (Jha, 2008; D'Agata et al., 2014). The haemocytes are often used for genotoxic investigations in terms of DNA damage (Comet assay) or onset of nuclear abnormalities (micronucleus test), that reflect different typology, severity of damages that could be repaired or not. These biomarkers have been successfully used to assess damages to DNA and nuclear alterations in mussels exposed to emerging contaminants (Avio et al., 2015; Mezzelani et al., 2016a: 2016b; 2018) and multiple environmental stressors (Nardi et al., 2017; 2018).

1.2. OBJECTIVES

Though several studies were made within the scope of understanding the interactions of predicted ocean changes and chemical pollution (among others, Sokolova et al., 2004; Mubiana and Blust et al., 2007; Coppola et al., 2018; Maulvault et al., 2018a; Serra-Compte et al., 2018; Costa et al., 2020a; 2020b), scarce information assessing the biological effect of already occurring changes, as marine heat waves intensification, upon mussels *M. galloprovincialis* is yet available. To the author's knowledge no previous study combined MHWs with pollutants (CBZ, in this case). It is considered of upmost relevance to understand

how both stressors interact and how these interactions are reflected in marine biota physiological and biochemical performance. Therefore, the objectives of the present study were: i) to assess the single and combined impact of MHW scenario and CBZ exposure in terms of antioxidant system efficiency, lipid metabolism, immune system alterations, oxidative damage to lipids and DNA and neurotransmission activity; ii) to investigate the effect of MHW scenario on CBZ bioaccumulation and effects in *M. galloprovincialis*; iii) to evaluate the recovery capacity of mussels after experiencing a dynamic temperature stress, both alone or in combination with CBZ. The research conducted intended to contribute to the growing knowledge regarding climate change and marine pollution impacts towards conservation of marine ecosystems, by producing the first valuable scientific study of a yet unexplored topic.

2. METHODOLOGY

2.1. ANIMAL COLLECTION AND EXPERIMENAL DESIGN

Mussels, *M. galloprovincialis* (5.3 ± 0.5 cm length), were obtained from a shellfish farm in the central area of Central Adriatic Sea from an unpolluted area (Regoli et al., 2014). The organisms collected in October 2019 were posteriorly acclimated for 7 days in aquaria with aerated artificial seawater (ASW; Instant Ocean) under seasonal climatological environmental conditions (18 ºC and salinity 35).

After acclimation, 60 mussels were assigned to each of four different treatments CTL (18 ºC, 0.0 µg/L of CBZ), CBZ (18 ºC, 1 µg/L), MHW (temperature ranged within 18 ºC – 22.5 ºC, 0 µg/L of CBZ) and CBZ + MHW (temperature ranged within 18 ºC – 22.5 ºC, 1.0 µg/L of CBZ), each in duplicate tanks, which reflected a full factorial experimental design 2x2 based on two CBZ doses (0 and 1 µg/L) and two temperature regimes (18 °C constant and MHW scenario), as represented in Fig. 2. Carbamazepine dose reflected environmental concentrations found in coastal areas (Gaw et al., 2014; Birch et al., 2015; Freitas et al., 2016; Mezzelani et al., 2018b). MHW scenario design was based on data available for central Adriatic sea in accordance with events previously recorded classified as strong (Moderate, Strong Severe, Extreme), with slight modifications due to experiment management (http://www.marineheatwaves.org/tracker, coordinates= 43.625 Lat., 14.125 Lon., event number 72, October 2014): temperature was increased daily by 0.9 ºC until the peak (22.5 ºC) was reached (day 5); this temperature was maintained for two days (day 5 and day 6) after which the opposite temperature variation was performed (daily decrease of 0.9 ºC) until control temperature (18 ºC) was restored at day 11. The temperature reestablishment was followed by a recovery period where the temperature was constant at 18 ºC until day 20 (end of the exposure).

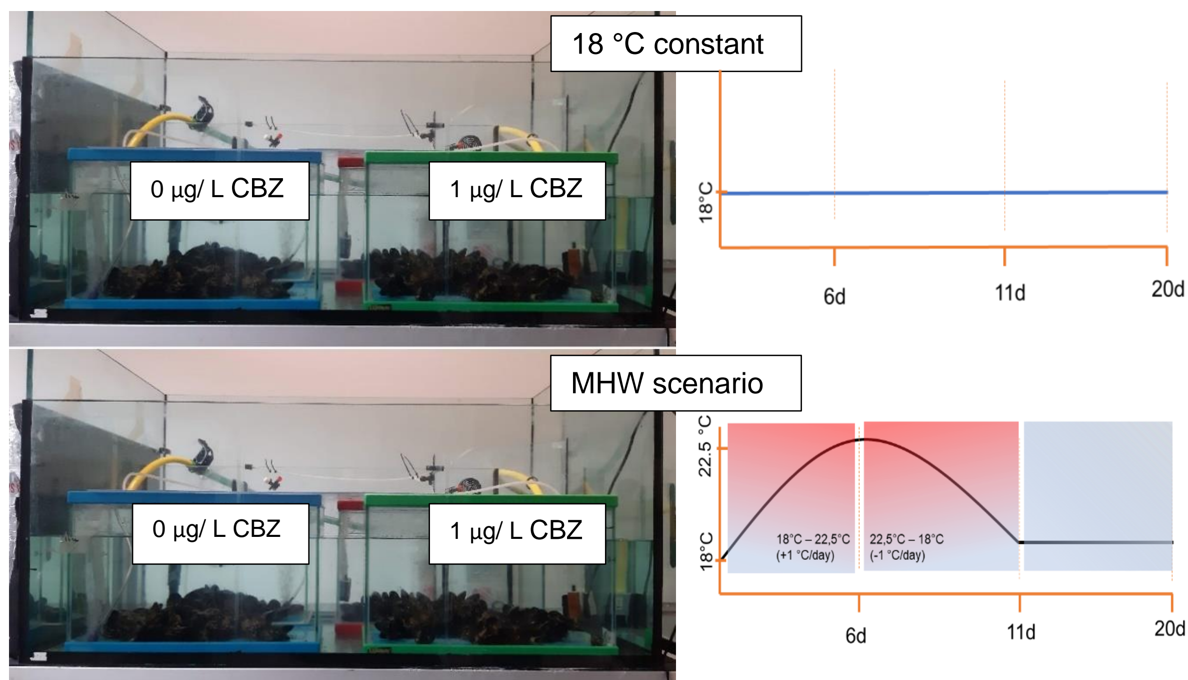

Fig. 2- Experimental design. CBZ= Carbamazepine and MHW= Marine heatwave.

Analysis were performed with organisms collected in three different sampling periods: days 6 (peak of the heat wave), day 11 (end of the heat wave) and 20 (end of recovery), using this identification for each sampling from here on. For each sampling 40 organisms per treatment were collected (20 per each duplicate tank) and immediately dissected. For a given treatment (n=40), digestive gland and haemolymph were collected from 21 organisms and divided in 7 pools of 3 organisms each, for biochemical analysis; 4 more organisms were sampled and digestive glands and haemolymph (for *in vivo,* histological and micronuclei analysis) were collected; whole tissue from the remaining 15 organisms was used for CBZ determination, by dividing organisms in 5 pools with the whole tissues of 3 animals each.

Samples for *in vivo* analysis were immediately used, while the others were flash frozen in liquid nitrogen and then stored at -80 °C or -20 °C (for biochemical analyses and CBZ determination, respectively).

2.2. CARBAMAZEPINE QUANTIFICATION

Analytical methods for measurements of CBZ in mussel's tissues (5 replicates with 3 organisms each).

High Performance Liquid Chromatography (HPLC) grade methanol, acetonitrile and analytical standard of CBZ (purity of ≥97 %) were purchased from Sigma Aldrich (Milan, Italy). Stock solution (1 mg/mL) was prepared in methanol and stored in amber vials (10 mL). Working solution was prepared daily in ultra-pure water, as required. All solutions were stored at +4 °C and in the dark to reduce possible degradation.

Acetic acid 0.1 % pH = 3.26 was used as homogenization and extraction buffer. About 3 g of wet tissues were homogenized in 5 mL of buffer at room temperature for 20 min. After centrifugation at 4500 *g* for 30 min, samples were purified by Solid Phase Extraction (SPE) with reversed-phase tubes (Discovery DSC-18, 1g x 6 mL, Supelco, Bellefonte, Pennsylvania, USA). SPE tubes were conditioned with 6 mL of methanol, followed by 18 mL of ultra-pure water. Samples were diluted (1:1) with ultra-pure water and loaded onto the SPE cartridges; after washing with 12 mL of potassium bicarbonate KHCO3 and 6 mL of ultra-pure water, analytes were eluted and recovered using 2 mL of methanol and acetic acid (0.1 %) (HPLC, gradient grade, Carlo Erba). Obtained samples were filtered using PhenexTM-RC membrane (Regenerated Cellulose/Polypropylene 0.45 µm, 15 mm syringe filters, Phenomenex, US) and then centrifuged again at 12000 *g* for 20 min.

Analytical detection of extracted pharmaceutical-active compounds (PhACs) was performed by HPLC, with fluorimetric and Diode Array Detectors, DAD (Agilent Infinity 1260 series). Chromatographic separations of CBZ were performed on a Kinetex column (C18,5 µm, 150 mm length, 4.6 mmID, Phenomenex, US), equipped with a security guard column (C18, 5 µm, 4 mm length, 2.0 mmID, Phenomenex, US). A mobile phase composed by ultra-pure water (26 %), acetonitrile (42 %) and Acetic acid 0.1 % pH = 3.26 (32 %) was used under isocratic condition. DAD was used for monitoring the spectra from 190 nm to 350 nm, and the signal for CBZ was obtained at 286 nm. This wavelength was selected among the spectra ranges assuring at least the 85% of the maximum absorbance, verifying the absence of other spectral interferences; additional qualifying signals (with about 50 %-75 % of the maximum absorbance) were also recorded for each compound for the quality control assurance. When necessary, the full spectra were used to verify the purity of the obtained peaks, comparing them with those obtained from pure standard solutions. Additional qualifying signals were obtained for quality control and pure standard solutions were used to further verify the purity of the obtained peaks.

Carbamazepine concentrations were quantified by comparison with signals of pure standard solutions. Due to the lack of appropriate Certified Standard Reference Materials (SRMs), CBZ recovery was estimated on samples of control mussels (n=10) spiked with various concentrations of investigated molecules. The minimum value of the optimal working

range corresponded to the analytical limit of measurement which guarantees an acceptable variability (CV<20 %) on 10 replicates and a good linearity (R2≥ 0.99), while the maximum value assured at least 95 % of recovery (n=10). Considering these analytical conditions and the described preparation procedures, the minimum measurable amounts (Limit of Detection, LOD) of CBZ in mussel's tissues were 1.03 ng/g dry weight (DW). All those values always ensure an appropriate analytical accuracy. During the protocols validation, samples spiked with levels of CBZ in the range of our experimental design, always provided significant reproducibility of results with low variability (CV<5 %, n=10) and elevated recovery yield (≥98%).

2.3. BIOMARKERS DETERMINATION

2.3.1. Immune system and neurotoxic alterations

Immunological parameters and neurotoxic alterations were evaluated in terms of haemocytes lysosomal membrane stability (LMS), granulocytes/hyalinocytes ratio, phagocytosis capacity and acetylcholinesterase activity (AChE).

The lysosomal membrane stability was assessed by using the cationic probe Neutral Red (NR) (Koukouzika et al., 2005). Neutral Red working solution (2 mL/mL filtered seawater) was freshly prepared from a stock solution of 20 mg neutral red dye dissolved in 1 mL of dimethyl sulfoxide. Samples were incubated on a glass slide with the previously prepared NR solution, and microscopically examined at 15 min intervals. Results are provided concerning the time at which 50 % of cells had lost to the cytosol the dye previously taken up by lysosomes.

The analysis of Granulocytes-Hyalinocytes ratio was based on the procedure described in Gorbi et al. (2013). Briefly, for each sample aliquots of haemolymph were firstly dispersed on glass slides, left in adhesion for 15 min and fixed in Beker's fixative (þ2.5 % NaCl). After washing in deionized water, slides were stained following Hematoxylin Eosin staining procedure and posteriorly mounted in Eukitt. Slides were observed using a light microscope and data obtained in percentage of granulocytes within a total of 200 counted cells per sample.

Carballal et al. (1997) protocol was followed to perform Phagocytosis assay. A volume of 100 mL of haemolymph was dispersed on glass slides and left for 15 min in the dark (at

room temperature) in order to adhere. Fluorescein-labelled Zymosan A bioparticles (Invitrogen) were added (10:1) to samples, which were posteriorly incubated for 2 h, under the former conditions. Uninternalized particles were removed by washing the slides with physiological solution and then treated with Beker's fixative (þ2.5 % NaCl). Finally, samples were mounted in glycerol gelatin and analysed under a fluorescence microscope. 200 cells per sample were counted and results were expressed as percentage of cells that internalized at least 3 fluorescent particles (positive cells).

Activity of AChE was measured according to Ellman method (Ellman et al., 1961). Samples were homogenized in Tris buffer 0.1 M, pH 7.5 and centrifuged at 9000 *g* for 20 min at 4 °C. Supernatants were removed and used for enzymatic quantification that used 1 mM acetylthiocholine as substrate and 0.1 mM 5,5′-dithiobis-2-dinitrobenzoic acid (DTNB). Enzyme activity was measured at 412 nm and expressed as nmol/min/mg of protein (PROT).

2.3.2. Single antioxidants and Total Oxyradical Scavenging Capacity

In order to evaluate alterations in single cellular antioxidants, enzymatic activity of catalase (CAT), glutathione peroxidase in both organic and inorganic (GPx total and GPx Se-Dep), glutathione reductase (GR), and glutathione S-transferases (GSTs) along to total glutathione content (TGSH) were assessed in organisms' digestive glands.

The activity of CAT (Johansson and Borg, 1988) was measured through absorbance decay as a consequence of hydrogen peroxide consumption. For sample extraction, a K-phosphate buffer pH 7.0 was used and an absorbance at 240 nm (extinction coefficient, ε = 0.04mM^{-1} cm^{-1}) to determine the enzyme kinetics. Results were expressed in µmol/min/mg PROT.

To perform GPx biomarker, procedure developed by Paglia and Valentine (1967) was followed in order to assess the activity of the coupled enzyme. Both parameters use the GR catalysed reaction that converts oxidized glutathione form (GSSG) into its reduced form (GSH) throughout the quantification of NADPH consumption. Samples were extracted in 100 mM K-phosphate buffer pH 7.5, 1 mM, Ethylenediaminetetraacetic acid (EDTA),1 mM dithiothreitol, 2 mM GSH, 1 unit GR, 0.24 mM NADPH, and 0.8 mM cumene hydroperoxide as substrates and the decay of NADPH content was monitored at 340 nm (ε = 6.22 mM^{-1} cm^{-1}). Results were expressed in µmol/min/mg PROT.

Nichoalds et al. (1974) procedure was carried to quantify GR. Activity was measured through the monitorization of oxidized NADPH considering as assay conditions 100 mM Na-

phosphate buffer pH 7.0, 1 mM GSSG and 60 µM NADPH. Reaction was measured at 340 nm (ε=6.22/mM/cm) and results expressed in µmol/min/mg PROT. Data obtained for TGSH parameter was analysed in accordance with Akerboom and Sies (1981) and samples' homogenization (1:5 w/v ratio) made in a solution of 5% sulfosalicilic acid with4 mM EDTA. The sum of the reduced and oxidized forms of glutathione can be determined using a kinetic assay in which catalytic amounts of GSH or GSSG and glutathione reductase bring about the continuous reduction of 5,5'-dithiobis (DTNB) by NADPH. Results were expressed in µmol/g fresh weight (FW).

The activity of GST enzyme was determined according to Habig (1981) and assay was carried out in 100mM K-phosphate buffer pH 6.5, 1.5mM 1-chloro-2,4-dinitrobenzen (CDNB), 1mM GSH. Reaction occurs by usage of CDNB as substrate at 340 nm (ε = 9.6mM^{-1} cm^{-1}). Results were expressed in µmol/min/mg PROT.

To assess the overall capability to counteract oxidative stress, the total oxyradical scavenging capacity (TOSC) assay was performed (Regoli, 2000). This method tests how efficiently the overall cellular antioxidant machinery counteract the dosed oxyradicals (peroxyl and hydroxyl radicals). Moreover, the process measures directly the inhibition of the oxidation of 0.2mMa-keto-g-methiolbutyric acid (KMBA) to ethylene gas.

In order to generate peroxyl radicals ($ROO^{\cdot}$), thermal homolyses reaction is triggered by 2,2'-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP) in 100 mM K-phosphate buffer, pH 7.4. Hydroxyl radicals ($OH^{\cdot}$) were produced by the Fenton reaction of iron-EDTA (1.8 mM Fe^{3+}, 3.6 mM EDTA) plus ascorbate (180 mM) in 100 mM K-phosphate buffer. Under these conditions, generated radicals react with α-Keto-γ-(methylthio)butyric acid (KMBA) and ethylene is produced; yields of ethylene is compared between samples reaction (with antioxidants) and control reaction (without antioxidants), thus relative efficiencies of cellular antioxidants toward a similar radical flux is quantified. Ethylene formation for each sample is quantified at regular intervals for a total of 90 min by a gas chromatograph, results are then plotted and TOSC value calculated through the formula

$$TOSC = 100 - (\int SA / \int CA * 100)$$

Where SA and CA are the integrated areas calculated under the least square kinetic curve produced during the reaction (Gorbi and Regoli, 2011) of each sample or control. The TOSC value is the normalized to the protein concentration and expressed in U TOSC/ mg PROT. Protein concentrations were measured according to Lowry (1951) method, using bovine serum albumin (BSA) as standard. PROT contents were expressed in mg.

2.3.3. Metabolic alterations and oxidative damage

Acyl-CoA (AOX) activity was assessed in organism's digestive gland; samples were firstly homogenized in 1 mM sodium bicarbonate buffer (pH 7.6) containing 1 mM EDTA, 0.1% ethanol, 0.01% Triton X-100 and centrifuged at 500 × g for 15 min at 4 °C. Reaction was followed by the oxidation of dichlorofluorescein-diacetate (DCF-DA) catalysed by an exogenous horseradish peroxidase (HRP).The reaction medium was 0.5 M potassium phosphate buffer (pH 7.4), 2.2 mM DCF-DA, 40 M sodium azide, 0.01% Triton X-100, 1.2 U/ mL HRP in a final volume of 1 mL. After a pre-incubation at 25 °C for 5 min in the dark with an appropriate volume of sample, reactions were started adding the substrates Palmitoyl-CoA, at final concentrations of 30 M at 502 nm. Results were expressed in nmol/min/mg PROT.

Oxidative damage was measured through levels of malondialdehyde (MDA), lipofuscin (LIPO) content, neutral lipids content (NL). Levels of MDA were measured according to Gorbi et al. (2008) through derivatization in 1-methyl-2-phenylindole (dissolved in acetonitrile/methanol 3:1) with HCl 32 % and calibrated against a malondialdehyde standard curve. Results were expressed in nmol/g FW.

Lipofuscin content was quantified on cryostat sections (8 μm thick) of digestive glands previously prepared at cryotome and stored at -80 °C. Procedure is in accordance with Belchier et al. (1998). Firstly, samples were fixed in Beker's fixative stained throughout Schmrol reaction and mounted in Eukitt. For analyses of NL, also cryostat sections (8 μm thick) were used. Samples' fixation into slides was made using Beker's fixative (þ2.5 % NaCl), stained with the Oil Red O (ORO) method and mounted in glycerol gelatin (Mehlem et al., 2013). For both biomarkers (LIPO and NL), 4 areas were selected to be representative of the overall histological section condition and for each quantification of staining intensity was measured using Image-Pro Plus Analysis Software and posteriorly normalized according to digestive tubules' area.

2.3.4. DNA integrity

Nuclear abnormalities and DNA fragmentation were respectively assessed in terms of micronucleus test (MN) and Comet assay (CometA). Micronuclei were evaluated in haemocytes according to Scarpato et al. (1990). Cells were fixed in Carnoy Solution (3:1, acetic acid : methanol). Posteriorly, the samples were spread onto slides (in replicates), dried and stained with DAPI. Two thousand cells with well-preserved cytoplasm were scored, for

each slide in order to determine the micronuclei frequency. To define which structures are referent to micronuclei, the following criteria were taken into account: round shape, smaller than 1/3 to a maximum of 1/13 of the main nucleus diameter, standing on the same optical plan, isn't refractile as the main nucleus and the former possess distinguishable boundaries from the micronuclei (Fenech, 2007).

Comet assay evaluated DNA integrity through single strand breaks. According to Gorbi et al. (2008), the procedure was developed on collected haemocytes from the organisms' adductor muscle. Cells were embedded in 0.6% low-melting-point agarose, spread onto slides and coated with 1 % normal-melting-point agarose. An extra layer of low-melting-agarose was added to the slides. Slides were embedded, at 4 °C, in a lysing solution (2.5 M NaCl, 100 mM EDTA, 1 % Triton X-100, and 10% DMSO, pH 10), for 90 min in the dark. Posteriorly, slides were treated with alkali (NaOH 300 mM, Na2EDTA 1 mM, pH> 13) for 10 min and placed in a horizontal electrophoresis apparatus. Electrophoresis was performed for 5 min at 25 V and 300 mA. After run, slides were neutralized with Tris–HCl (0.4 M, pH 7.5), stained with ethidium bromide and observed under a fluorescence microscope (400×). The amount of DNA fragmentation was quantified as the percentage of DNA migrated into the comet tail (tail DNA), using an image analyser (Kinetic Imaging, Ltd., Komet, Version 5). At least 50 nuclei for each sample (with replicates) were scored and the mean, of the three analysed organisms, per treatment, calculated.

2.4. DATA ANALYSIS

After checking normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test), two-way analysis of variance (ANOVA) was used to test the null hypotheses described below, followed by Fisher's Least Significant Differences (LSD) post-hoc tests for planned comparisons of the means of interest when significant statistical differences were found.

For the present experiment, the following null hypotheses were tested: i) for a given sampling period (day 6, day 11, day 20), no significant differences exist between treatments. For this hypothesis (i) significant differences ($p<0.05$) are represented in figures with different lower case letters; ii) for a given treatment (CTL, CBZ, MHW and CBZ + MHW), no significant differences exist between the sampling periods. For this hypothesis (ii) significant differences

($p<0.05$) are represented in figures with different upper case letters. When no significant differences were observed, no letters were added to the figures.

All the analyses were performed using the software RStudio (version 0.99.491).

3. RESULTS

3.1. MORTALITY

In this experiment, no mortality was recorded for any of the different treatments.

3.2. CARBAMAZEPINE CONCENTRATIONS

Carbamazepine bioaccumulation analyses revealed marked accumulation of the drug in all CBZ-exposed treatments compared to non-exposed ones (Fig. 3). Organisms showed a time-dependent kinetic of CBZ accumulation, with significantly lower CBZ burdens at day 6 compared with day 11 and 20 at control temperature and at day 6 and 11 compared with day 20 under MHW scenario (Fig. 3). The highest CBZ level was measured in organisms under the MHW scenario at day 20.

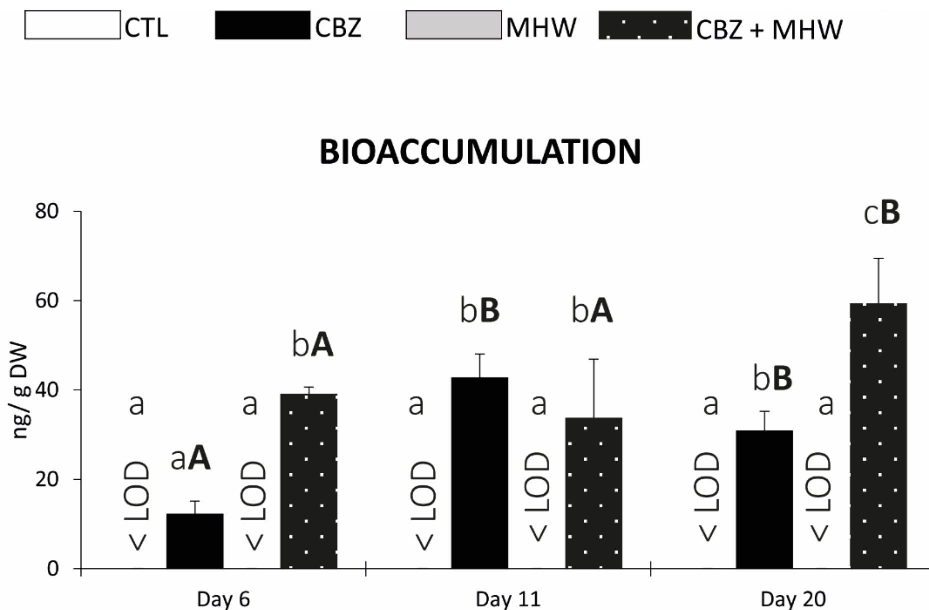

Fig. 3- Carbamazepine concentration on mussels' whole soft tissues. Data are given as mean values (n=3) ± standard deviations. Significant differences between group of means (ANOVA and SNK post-hoc) within the same sampling (Day 6, Day 11, Day 20) are identified with different lower case letters. Significant differences within the same treatment (ANOVA and SNK post-hoc) along time (Day 6, Day 11, Day 20) are identified with different upper case letters. Only significant differences are represented in the figures. CTL= Control; CBZ= Carbamazepine; MHW= Marine heatwave scenario; CBZ + MHW= Combination of Carbamazepine and Marine heatwave treatments.

3.3. BIOMARKERS RESPONSES

3.3.1. Immune system and neurotoxic alterations

Lysosomal membrane stability (LMS, Fig. 4A) was significantly lower for CBZ exposure, with major effects when the molecule was dosed in combination with the MHW scenario. No significant differences along time were reported.

Immunological responses highlighted a significant synergistic mechanism of effect of CBZ and MHW: at day 6 and 20, organisms exposed to CBZ under MHW showed significantly lower granulocytes vs hyalinocytes type cells ratio compared to organisms exposed to CBZ at control temperature. At day 11, when reaching the end of the MHW, the co-exposure to these stressors led to an increase of the ratio G/H (Fig. 4B). Time-dependent significant differences were observed only in organisms co-exposed, highlighting that synergistic effects of CBZ and MHW variables over time.

Similarly, phagocytosis (PHAGO, Fig. 4C) was significantly affected only in organisms co-exposed after 20 days. Organisms in the CBZ+MHW treatment showed a lower phagocytosis efficiency compared to those exposed to CBZ alone. Nonetheless, differences occurred in this treatment over time, and phagocytosis significantly decreased from day 11 to day 20.

Regarding neurotoxic effects, MHW exposure led to a significant inhibition of acetylcholinesterase enzyme (AChE, Fig. 4D) compared to CTL treatment after 6 days, which was no longer evident at day 11 and 20. Opposite effects of CBZ were observed after 11 and 20 days of exposure. The significant decrement measured at day 11 was followed by an increment at day 20, supported by the significant differences observed over time in this treatment (CBZ).

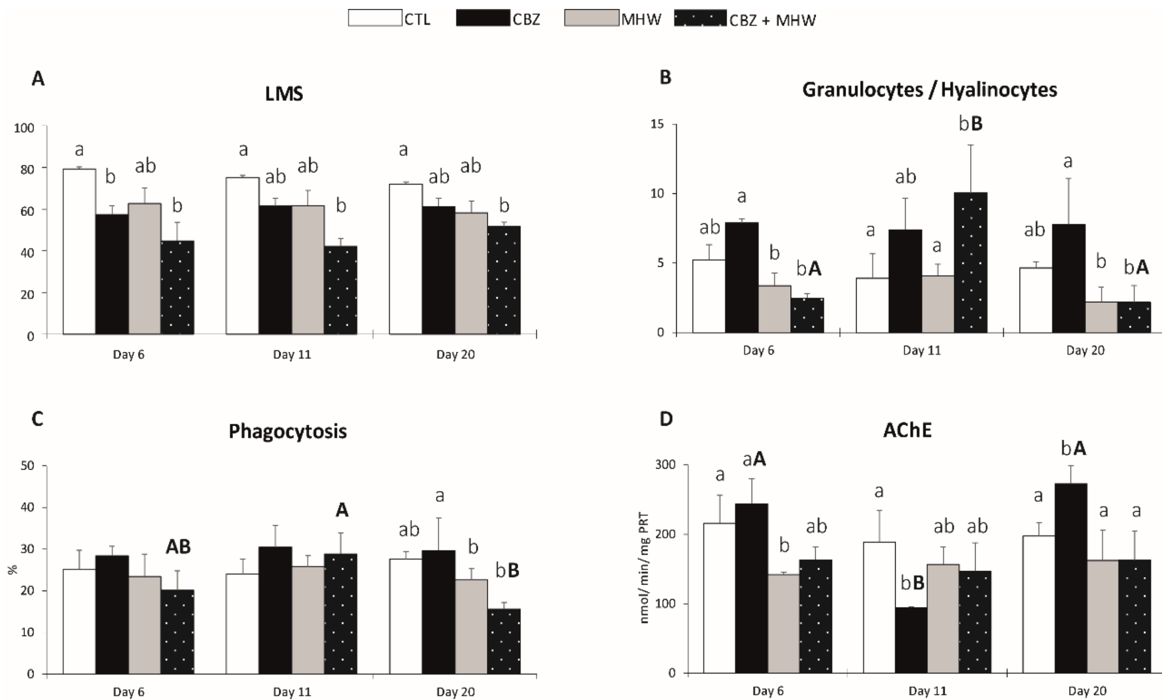

Fig. 4- **A**: Lysosomal membrane stability (LMS), **B**: Granulocytes *vs* Hyalinocytes ratio, **C**: Phagocytosis rate and **D**: Acetylcholinesterase activity (AChE), in haemocytes of mussels exposed to various treatments. Data are given as mean values (n=4 or n=5 for AChE) ± standard deviations. Significant differences between group of means (ANOVA and SNK post-hoc) within the same sampling (Day 6, Day 11, Day 20) are identified with different lower case letters. Significant differences within the same treatment (ANOVA and SNK post-hoc) along time (Day 6, Day 11, Day 20) are identified with different upper case letters. Only significant differences are represented in the figures. CTL= Control; CBZ= Carbamazepine; MHW= Marine heatwave scenario; CBZ + MHW= Combination of Carbamazepine and Marine heatwave treatments

3.3.2. Single antioxidants and Total Oxyradical Scavenging Capacity

Despite no statistical difference was registered among different treatments within each sampling for catalase activity (CAT, Fig. 5A), clear trends of effect of CBZ were found at both temperature regimes, with a significant increase of the activity of this enzyme over time in organisms exposed to the molecule at control temperature.

Total glutathione peroxidase (GPx TOTAL, Fig. 5B) did not show any statistical differences neither within samplings, nor among sampling periods. However, organisms exposed to carbamazepine (CBZ and CBZ + MHW treatments) showed a decreasing trend of enzymatic activity along time.

Se-dependent glutathione peroxidase activity (GPx Se-Dep., Fig. 5C) showed a significant increase over time from day 6 to 11 in CBZ treated organisms, followed by a

decrease at day 20, despite not statistically significant. Nonetheless, though not significant, results presented a general higher activity for MHW treatment alone.

Despite not significant, glutathione reductase (GR, Fig. 5D) activity was induced in all the treatments compared to each sampling CTL treatment. Moreover, statistically significant differences over time were observed between organisms exposed to the drug at control temperature from day 11. Nonetheless, co-exposure to CBZ and MHW seem to have a slight inhibitory effect, given that GR activity was always lower in CBZ + MHW treatment compared to single stressors.

Total glutathione (TGSH, Fig. 5E) showed generalized decrements in MHW exposed organisms, and significant differences were observed along time when comparing results of day 6 with the other sampling times in organisms exposed to MHW alone. The co-exposure (CBZ + MHW) led to a significant decrease of TGSH level at the end of the heatwave (day 11) compared to the peak (day 6) and the end of recovery (day 20).

Glutathione S-Transferases (GSTs, Fig. 5F) did not show any statistical difference neither within samplings, nor over time. Nevertheless, a generalized increasing trend was observed firstly due to MHW independently of CBZ exposure (day 11) and secondly due to CBZ exposure independently of temperature regime (day 20).

Single antioxidant defences analyses were integrated with overall capacity to counteract oxidative insult, total oxyradical scavenging capacity (TOSC) deriving from peroxyl (ROO•) and hydroxyl (HO•) radicals. TOSC ROO• (Fig. 5G) did not show any significant variation, though an increase in CBZ exposed organisms was observed after 11 days of exposure.

Results of TOSC HO• (Fig. 5H) showed a general sensitivity of this parameter to MHW during the heat wave: an initial increase was observed in organisms exposed to MHW and CBZ+MHW (day 6), followed by a marked decrement in this organisms at the end of the heatwave (day 11); 10 days after the heatwave, increased TOSC HO• values were caused by CBZ exposure (both CBZ and CBZ+MHW). Significantly different values occurred in CBZ+MHW exposed organisms at day 6, 11 and 20, compared to CTL, CBZ and MHW respectively. Regarding alterations along time, MHW exposed organisms showed a significantly decreased TOSC HO• along time, occurring at the end of the heatwave (day 11) and 10 days after the experiment (day 20) compared to day 6 (peak of the heatwave); on the other hand in the CBZ + MHW treatment the decrease observed at day 11 in comparison to day 6, was not paralleled at day 20, showing interactive effects of the co-exposure.

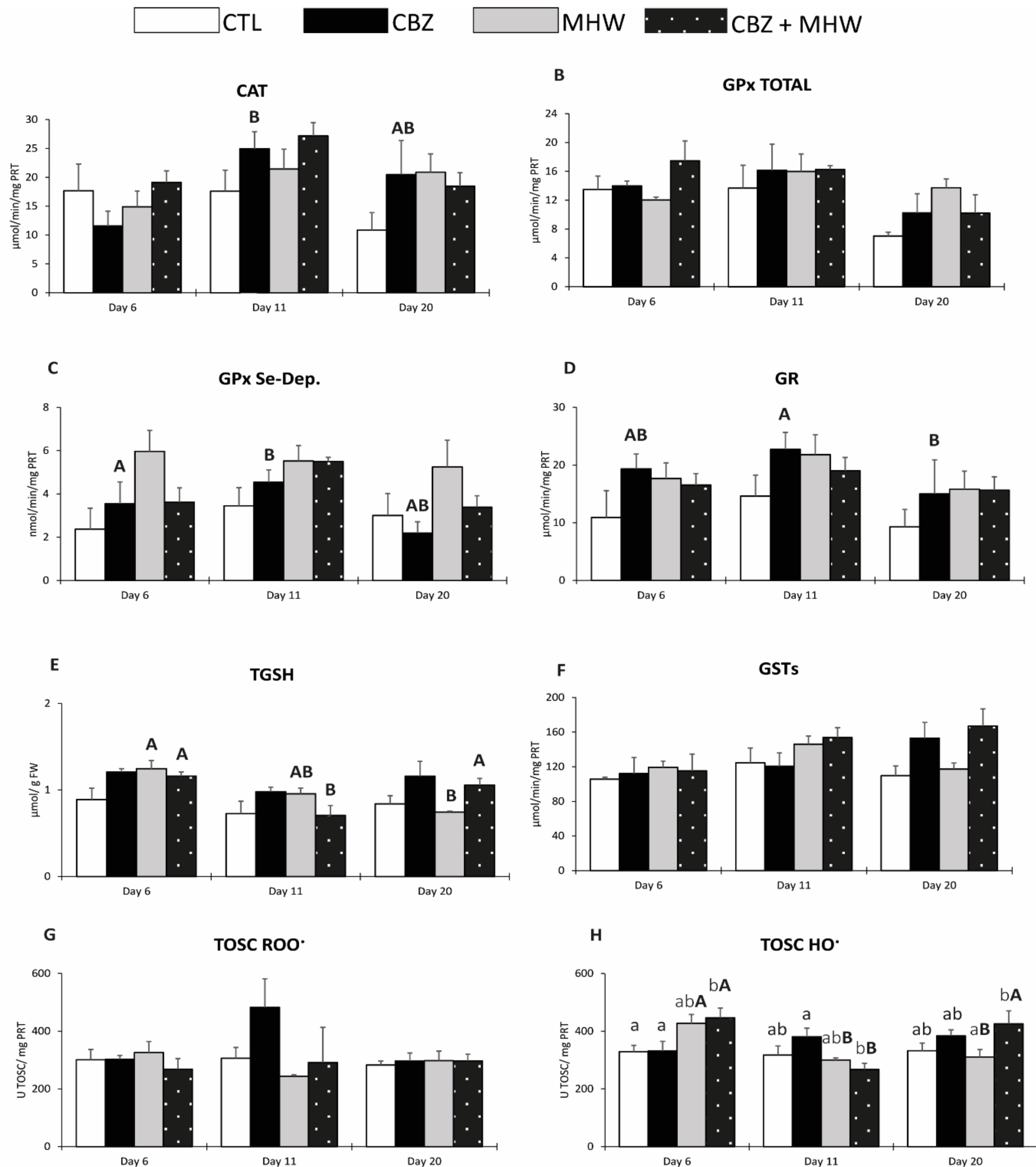

Fig. 5- Antioxidant defences and oxidative stress biomarkers in gills of mussels exposed to various treatments. **A:** Catalase activity (CAT), **B:** Sum of Se-dependent and Se-independent glutathione peroxidases activities (GPx TOTAL), **C:** Selenium dependent glutathione peroxidase activity (GPx Se-Dep.), **D:** Glutathione reductase activity (GR), **E:** Total glutathione content (TGSH), **F:** Glutathione S-Transferases activity (GSTs), **G:** Total oxyradical scavenging capacity toward peroxyl radical (TOSC ROO·) and **H:** Total oxyradical scavenging capacity toward hydroxyl radical (TOSC OH·). Data are given as mean values (n=3) ± standard deviations. Significant differences between group of means (ANOVA and SNK post-hoc) within the same sampling (Day 6, Day 11, Day 20) are marked with lower case letters. Significant differences within the same treatment (ANOVA and SNK post-hoc) along time (Day 6, Day 11, Day 20) are marked with upper case letters. Only significant differences are represented in the figures. CTL= Control; CBZ= Carbamazepine; MHW= Marine heatwave scenario; CBZ + MHW= Combination of Carbamazepine and Marine heatwave treatments.

3.3.3. Metabolic alterations and oxidative damage

A decrement of acyl-CoA oxidase activity (AOX, Fig. 6A), though not significant, was perceptible for organisms exposed to MHW scenario, regardless CBZ presence.

Malondialdehyde content (MDA, Fig. 6B) significantly increased in MHW treated organisms at day 11 (end of the heatwave) compared to day 6 (peak of the heatwave). Interestingly, a marked although not significant decrement was observed at day 20, with levels comparable to day 6.

After 6 days of experiment, a significant accumulation of lipofuscin in tertiary lysosomes was observed in all the exposure treatments (CBZ-exposure, MHW and their combination) when comparing to CTL (LIPO, Fig. 6C). The pattern continued at day 11, led by CBZ-exposure in CBZ and CBZ + MHW organisms, while at day 20 a significant decrement of lipofuscin content was observed in all the CBZ treated organisms (CBZ and CBZ + MHW) (LIPO, Fig. 6C).

At day 6 MHW exposed organisms presented statistically higher neutral lipids content (NL, Fig. 6D) in comparison to CTL treatment, but no differences occurred between CBZ and CBZ+MHW treatments. At day 11 and 20, CBZ was the main factor causing the significant increase when comparing with CTL and MHW treatments. When comparing trends of the same treatment, CBZ alone caused a significant increase along time, while MHW caused the opposite trend. No significant differences over time were observed in organisms co-exposed to both stressors.

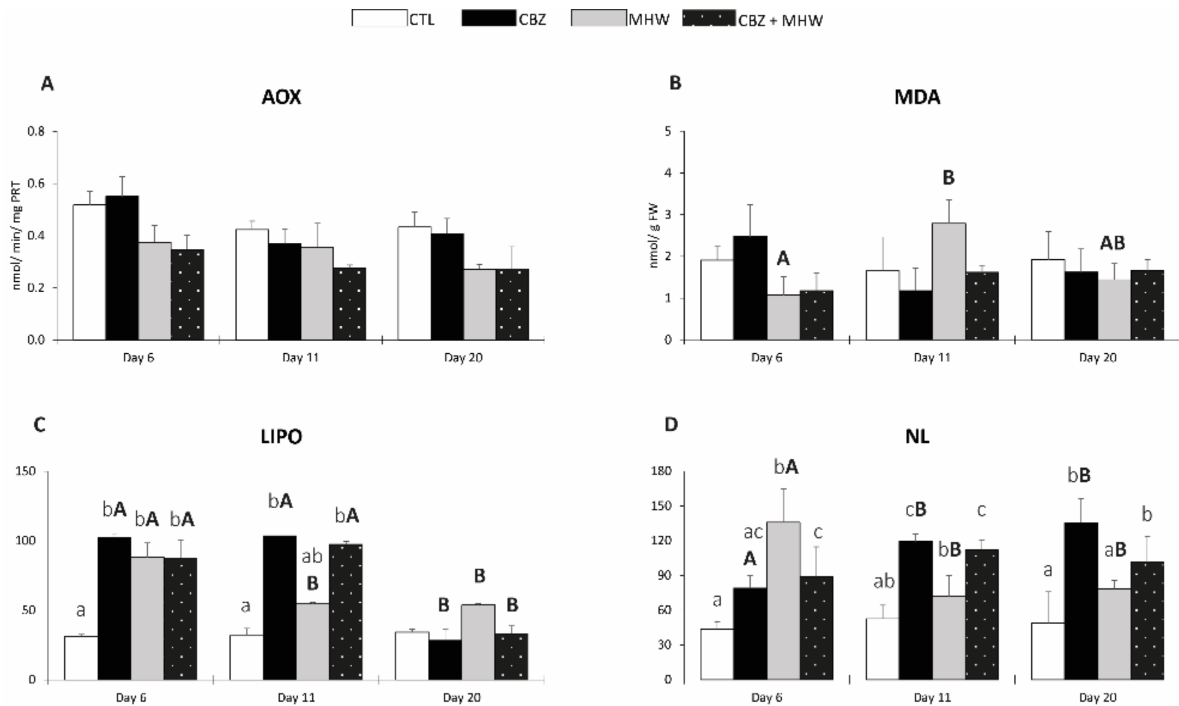

Fig. 6- **A:** Acyl-CoA enzyme activity in haemolymph (AOX), **B:** Malondialdehyde content (MDA), **C:** Lipofuscin content (LIPO) and **D:** Neutral Lipids content (NL) in digestive gland of mussels exposed to various treatments. Data are given as mean values (n=4 or n=5 for AOX) ± standard deviations. Significant differences between group of means (ANOVA and SNK post-hoc) within the same sampling (Day 6, Day 11, Day 20) are marked with lower case letters. Significant differences within the same treatment (ANOVA and SNK post-hoc) along time (Day 6, Day 11, Day 20) are marked with upper case letters. Only significant differences are represented in the figures. CTL= Control; CBZ= Carbamazepine; MHW= Marine heatwave scenario; CBZ + MHW= Combination of Carbamazepine and Marine heatwave treatments.

3.3.4. DNA integrity

DNA fragmentation, measured through the Comet assay, shown time-dependent variations affecting organisms exposed to MHW and CBZ+MHW (Fig. 7A). After experiencing the heatwave (day 11), organisms exposed to MHW showed lower levels of DNA damage compared to day 6 and day 20. On the other hand, the co-exposure to the heatwave and CBZ caused an increase of the DNA fragmentation at day 20 compared to day 6 and 11. Differences between treatments were observed at day 20, 10 days after the heatwave end, when organism exposed to the heatwave (MHW and CBZ+MHW) showed a significant increase of DNA damage compared to CTL treatment.

Significant micronuclei increase was observed at day 6 in organisms co-exposed to MHW and CBZ (CBZ+ MHW, Fig. 7B) and at day 20 in organisms exposed to CBZ independently on the temperature regime (CBZ and CBZ+MHW, Fig. 7B). Nonetheless, time-

dependent variations affected both CBZ and CBZ+MHW exposed organisms: in organisms co-exposed to both stressors the highest levels of these nuclear abnormalities was measured at day 6, at the peak of the heatwave, while a linear and time-dependent increase was observed for organisms exposed to CBZ alone; after 20 days of exposure, no significant difference was observed between organisms exposed to CBZ at constant temperature or under marine heatwave scenario.

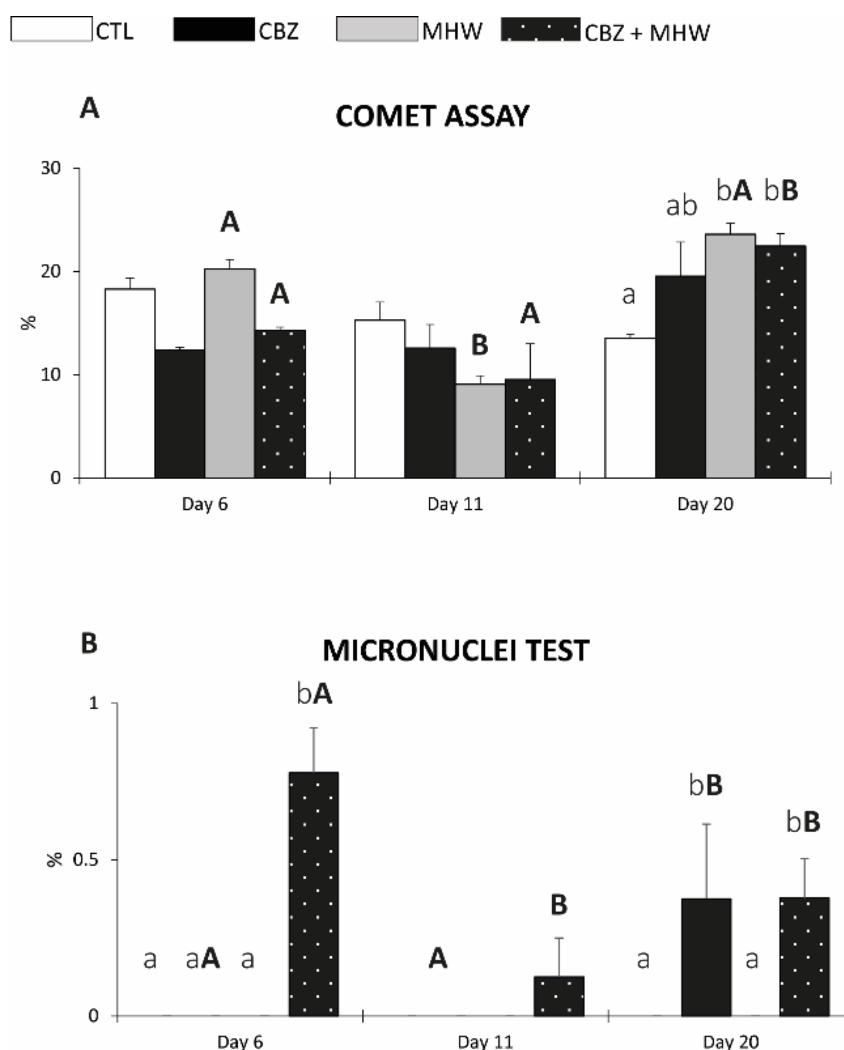

Fig. 7- **A:** DNA damage (COMET ASSAY) and **B:** Frequency of micronuclei (MICRONUCLEI TEST) in haemocytes of mussels exposed to various treatments. Data are given as mean values (n=4) ± standard deviations. Significant differences between group of means (ANOVA and SNK post-hoc) within the same sampling (Day 6, Day 11, Day 20) are marked with lower case letters. Significant differences within the same treatment (ANOVA and SNK post-hoc) along time (Day 6, Day 11, Day 20) are marked with upper case letters. Only significant differences are represented in the figures. CTL= Control; CBZ= Carbamazepine; MHW= Marine heatwave scenario; CBZ + MHW= Combination of Carbamazepine and Marine heatwave treatment.

4. DISCUSSION

4.1. CARBAMAZEPINE CONCENTRATIONS

The developed research demonstrates ecotoxicological consequences of a largely unexplored aspect of climate changes, marine heatwaves. Moreover, this is the first study revealing the effects of MHWs on the accumulation and biological effects of carbamazepine (CBZ), chosen as model contaminant of emerging concern (CECs).

Seawater warming and acidification can alter contaminants' bioavailability in sediments and in water as well as the marine specie's physiological status, which may affect the way that marine organisms cope with the presence of chemical contaminants like compound uptake, retention and detoxification rates. Several studies highlighted the role of seawater warming on the chemical speciation and accumulation of pollutants in the mussel species *Mytilus galloprovincialis* (Maulvault et al., 2018a, 2018b; Serra-Compte et al., 2018; Freitas et al., 2020). The present study showed that organisms exposed to increasing temperatures tend to present higher CBZ uptake rates. These findings are in accordance with the study conducted by Serra-Compte et al. (2018) which performed a 20-day experiment assessing the differential bioaccumulation rates of a drug mixture (15.7 µg/L) containing CBZ. The authors determined the concentration of each mixture's compound on organisms' tissues and verified that *M. galloprovincialis* accumulated more CBZ under warming conditions (22 °C) compared to control values (18 ºC). Moreover, the trend to bioaccumulate higher concentration of contaminants under warming conditions was previously observed in literature for bivalves exposed to compounds from different classes (Coppola et al., 2018; Maulvault et al., 2018a; Serra-Compte et al., 2018; Costa et al., 2020a; Pirone et al., 2020). Nevertheless, it is very difficult to stablish comparisons with other studies on aquatic species evaluating the effect of the temperature alone, as a factor controlling bioaccumulation, given the high variability, and even controversial data. For example, Sokolova and Lannig (2008) reviewed metals accumulation in bivalves and observed that metals' uptake will highly depend on the element and on the species, tissue analysed and experimental conditions. Pirone et al. (2019) evaluated the impacts of triclosan (1 µg/L TCS), lead (50 µg/L Pb) and the mixture of both compounds under warming (22 ºC) and control (17 ºC) conditions upon mussels *M. galloprovincialis*. The authors observed higher tissues' concentrations for organisms exposed to Pb and increased temperature but not for TCS. Pirone et al. (2019) hypothesized that the increase in toxicity of metals enhanced by high temperature- may be due to limiting the scope of aerobic metabolism (oxygen extraction, transport and utilization), since when the metabolism of aquatic species is increased and oxygen concentration in water is reduced, the inflow rate of water into the animal increases in order to extract more oxygen, which can

simultaneously increase the entry of dissolved pollutants into the body, highlighting cumulative CBZ effects and higher bioavailability increased temperature, for the present study. One of the most interesting results of the present research is that the highest CBZ bioaccumulation was measured in organisms exposed to CBZ under heat wave scenario at the end of the recovery period (day 20), allowing to hypothesize that effects of the thermal stress may last even after temperature reestablishment, highlighting footprint of the event on organisms metabolism. Based on this result, marine heat waves should be considered issue of concern not only for their direct effect on organisms' health, but even for their indirect capability to affect organisms' bioaccumulation of contaminants from the environment.

4.2. BIOMARKERS RESPONSES

4.2.1. Immune system and neurotoxic alterations

The present study revealed haemocytes as one of the main target of the tested treatments. Circulating haemocytes are involved in important functions, among which immune defence is one of the most important (Gorbi et al., 2013). The results obtained here showed a significant decrease of lysosomal membrane stability in mussels co-exposed to CBZ and MHW, with major effects measured at the end of 11 days. Lysosomes may be implicated in cell autophagy, a cellular process involving in the remove of oxidative damaged organelles and proteins. Autophagic mechanisms can be triggered by several stressors (e.g., restricted nutrients, hyperthermia, hypoxia and salinity increase), being recognized as a primary survival strategy in multicellular organisms, protecting the cells against oxidative stress (Moore et al., 2006a, 2006b; Moore et al., 2008). By breaking down longer-lived proteins and organelles and recycling the products into protein-synthesis and energy-production pathways, this process allows cells to be temporarily self-sustaining during periods when nutrients are restricted (Cuervo, 2004; Levine, 2005; Moore et al., 2006a). Previous studies assessing the effects of pharmaceuticals on mussel's lysosomal membrane stability already demonstrated negative impacts after short (7 days) and long (35 days) term exposures to different pharmaceuticals (Aguirre-Martínez et al., 2013; Juhel et al., 2016; Shaw et al., 2019). In particular, *Perna viridis* mussels exposed for 7 days to CBZ, alone (1, 10 and 96 ng/L) and in a mixture (1, 9, 95 ng/L, for each compound) with bisphenol A and atrazine, showed reduced lysosomal membrane

stability regardless the CBZ dose (Juhel et al., 2016). Studies conducted by Shaw et al. (2019) also demonstrated a significant decrease on lysosomal membrane stability in clams *Ruditapes philippinarum* exposed for 35 days to caffeine, ibuprofen, carbamazepine and novobiocin (0.1, 1, 10, 50 µg/L). The study conducted by Dimitriadis et al. (2012) also showed that the exposure to increased temperature was responsible for similar impacts in the species *Modiolus barbatus* maintained for 30 days to 18, 24, 28 or 30 °C. Decrement of lysosomal membrane stability remarks higher efflux rates of lysosomal contents reflecting membrane damage by the combined exposure of drugs and increased temperature and therefore, cellular oxidative stress occurrence.

In *M. galloprovincialis*, usually two haemocytes subpopulations are recognized: granulocytes, with nucleus surrounded by cytoplasmic granules involved in phagocytic activity, and hyalinocytes, with small cytoplasm and undifferentiated cell aspect, involved in haemocytes turnover, coagulation and encapsulations processes (Gorbi et al., 2013; Carballal et al., 1997). In the present study, the ratio between granulocytes and hyalinocytes was found to be negatively affected by temperature variations, especially when comparing organisms exposed to CBZ under the two temperature regimes at the peak of the MHW (day 6) and 10 days after this was ended (day 20). The negative impact on granulocytes and hyalinocytes ratio parameter in the present study may explain the significant decrement of phagocytosis activity only after the MHW: at day 20 the decrease of phagocytosis activity in organisms co-exposed to CBZ and MHW (CBZ + MHW) could be, at least partially, explained by a reduction of circulating granulocytes, evidencing higher pressures upon organisms when exposed to coupled stressors. Published studies also demonstrated the same pattern for mussels exposed to different contaminants (Mezzelani et al., 2018; Nardi et al., 2018; Giuliani et al., 2019). Both ratio between granulocytes and hyalinocytes and phagocytosis were used to assess the impacts of cadmium (20 µg/L Cd) upon mussels *M. galloprovincialis* (Nardi et al., 2018). Also Nardi et al. (2018) verified that organisms exposed to Cd and increased temperature showed lower granulocytes and hyalinocytes ratio and phagocytosis rate, regardless pH conditions, highlighting the temperature effect in cellular processes.

Acetylcholinestrease (AChE) is an enzyme involved in the degradation of the neurotransmitter acetylcholine and has key function in bivalve's nervous system. Several studies targeted AChE activity in shellfish to be sensitive to metals and pesticides exposure (Lionetto et al., 2011; Canty et al., 2007), and more recently pharmaceuticals effects on this enzyme has been described (Munari et al., 2014; Aguirre-Martínez et al., 2016). In the present study, CBZ exposure at constant temperature affected AChE activity in a biphasic mode: compared to control treatment, an inhibitory effect was measured after 11 days of exposure,

followed by an induction after 20 days. In accordance with the obtained data, Aguirre-Martínez et al. (2016) short-term exposure (14 days) to 1 µg/L CBZ led to AChE activity inhibition in *R. philippinarum* clams with decreasing trend along the increasing exposure concentrations. Overall, the present findings indicate that CBZ can effectively affect AChE in *M. galloprovincialis*, with duration of exposure as a key factor in modulating the behaviour of this enzyme. The pharmaceutical did not affect AChE activity when dosed during and after the MHW event, suggesting antagonistic role of temperature variations on responsiveness toward CBZ exposure. Temperature impacts on AChE activity are already described in the literature. For example, Morosetti et al. (2019) assessed the impacts of cerium oxide nanoparticles (1 µg/L) and mercury (10 µg/L) upon mussels *M. galloprovincialis* for 28 days. The author observed the effects under a control condition (17 ºC) and a warming scenario (22 ºC) and observed an inhibition of AChE enzyme for non-contaminated organisms exposed to increased temperature. Previous studies with mussels under increased temperature demonstrated the inhibition of AChE enzyme.

4.2.2. Single antioxidants and Total Oxyradical Scavenging Capacity

One of the main mechanisms of toxicity of chemicals is mediated through increased intracellular production of reactive oxygen species (ROS) or impaired efficiency of the antioxidant system (Regoli and Giuliani, 2014). Several studies already demonstrated that exposure to pharmaceuticals and/or temperature rise may impact bivalves, and in particular mussels, oxidative status with changes on their antioxidant capacity (Almeida et al., 2014; 2015; Costa et al., 2020a; 2020b; Freitas et al., 2016; 2019; 2020). In the present study the antioxidant status of mussels was assessed through the integration of single antioxidants with the overall capability to neutralize specific pro-oxidants. Limited variations of single antioxidant defences involved catalase (CAT), glutathione reductase (GR) and glutathione peroxidase Se-dep (GPx Se-dep) activity in organisms exposed to CBZ at constant temperature. Antioxidant mechanisms' activation in presence of CBZ is already documented in literature (Almeida et al., 2014, 2015; Freitas et al., 2015, 2016; Oliveira et al., 2017). In addition, the significant decrease of total glutathione content (TGSH), coupled with trends of increased GR and Se-Dep. GPx Se-dep increase activities may suggest an increased oxidative pressure for MHW exposed organisms deriving from increased cellular ROS concentration under thermal stress. Nevertheless, data regarding antioxidant activity is not consistent and of difficult interpretation. This may be due whether to the low CBZ concentration or the short period of exposition. For

example, Almeida et al. (2015) assessed the CBZ (0.00, 0.03, 0.30, 3.00 and 9.00 µg/L) effects upon clams *R. philippinarum* for a longer period (28 days). The author observed that the concentration of 3.00 µg/L seemed to be a “threshold” concentration, on which antioxidant machinery was compromised with growing effects until the highest CBZ concentration (9.00 µg/L), suggesting that experimental conditions may not be enough to observe consistent responses in the present study.

Variations of single antioxidants defences were associated with the total oxyradical scavenging capacity (TOSC) which, by quantifying the overall capability of the organism to neutralize specific oxyradicals, gives a comprehensive information on the overall organism oxidative status. The results obtained on hydroxylic TOSC (TOSC $OH^{\cdot}$) showed an increased capability to counteract the oxyradical at the peak of the MHW (MHW and CBZ+MHW), followed by a sudden decrease at the end of the heatwave (day 11) and then increased only in organisms co-exposed to MHW and CBZ 10 days after the end of the MHW. This trend, which resembles the one observed for TGSH, suggests that mussels respond immediately to thermal stress and delayed CBZ effects occurred only in pre-conditioned organisms subjected to thermal stress by using the most abundant cytosolic scavenger, reduced glutathione (GSH). Results here presented evidenced that GSH acted directly in the neutralization of reactive species through its oxidation to GSSG. Mismatches related with single antioxidant analysis and TOSC results suggests that other antioxidant defences apart of those analysed could be involved in organism’s response to oxidative stress, such as ascorbic acid (vitamin C), uric acid or α-tocopherols (vitamin E) (Regoli and Giuliani, 2014).

4.2.3. Metabolic alterations and oxidative damage

The sensitivity of mussels to the heatwave was confirmed by the negative trend observed for acyl-CoA oxidase (AOX) activity. On the other hand, no effect of CBZ was observed on this enzyme involved in fatty acids metabolism, despite AOX inhibition was previously observed in mussels exposed to other pharmaceuticals (Mezzelani et al., 2018; Mezzelani et al., 2016b). This could be explained by both the exposure doses (25 µg/ L each) and the typology of pharmaceuticals tested: acetaminophen (AMP), diclofenac (DIC), ibuprofen (IBU), ketoprofen (KET) and nimesulide (NIM) modulates key pathways of lipid metabolism, such as β-oxidation. The metabolism of fatty acids chains and important lipid derivatives like prostaglandins and leukotrienes will be allowed, originating plasmalogens and cholesterol, which act as precursor of steroid hormones (Mezzelani et al., 2016b).

In order to understand if variations observed for the antioxidant status were paralleled by oxidative insult, malondialdehyde (MDA) content was assessed. The results here presented demonstrated that, at the end of the heatwave, MDA content increased in organisms exposed to MHW, and paralleled the lowest TOSC HO•. Given that MDA is end product of lipid peroxidation (LPO), data evidenced higher oxidative insult confirmed by the lower capacity to counteract HO• radicals. Usually, oxidative insult can be highlighted by accumulation of lipofuscin which might reflect autophagic and peroxidative processes occurrence (Mezzelani et al., 2016b). In the present study, an early and generalized increase of lipofuscin was registered, which was then eliminated more slowly in organisms exposed to CBZ. A response variation closely related to temperature variation was observed for neutral lipids: increasing temperature caused accumulation of these energy reserve molecules, followed by a sharp decrease when control temperature was re-established; on the other hand, CBZ exposure caused an accumulation of energetic reserves along time, with no additional effect of thermal stress. For MHW treated organisms the content of neutral lipids was paralleled by the decreasing trend of acyl-CoA oxidase activity, highlighting a clear influence of temperature on lipids metabolism. The impairment of total oxyradical scavenging capacity and lipofuscin content confirm the importance of oxidative pathways in modulating the responsiveness of marine organisms to CBZ exposure. On the other hand, MWH scenario was accompanied by lower AOX levels and neutral lipids contents which may suggest lower metabolism rates of fatty acids under this treatment. The same pattern was observed for both lipofuscin and neutral lipids contents by Mezzelani et al. (2016b) for *M. galloprovincialis* contaminated with single exposures of AMP, DIC, IBU, KET and NIM.

4.2.4. DNA integrity

Investigated stressors revealed moderate genotoxic effects measured through the Comet assay and micronuclei frequency. Short-term temperature increase was shown to affect DNA integrity, with cumulative effects that were manifested mainly after the end of the marine heatwave, independently of the presence or not of CBZ. This can be explained by an increased oxidative pressure in organisms exposed to the heatwave probably due to increased metabolic rate, confirmed by the sensitivity of the overall antioxidant capacity (TOSC) in the early phase of the event (day 6). Nonetheless, despite observed variations, measured values of DNA integrity always remained in a range of moderate impact and that can be measured in control and wild organisms. The increased metabolic rate as trigger of oxidative insult can even

partially explain the early increase of micronuclei which was not paralleled during the late phase of the experiment: the values observed in organisms co-exposed to MHW+CBZ can be explained by higher mitotic rate as already observed in *M. galloprovincialis* exposed to other environmental stressors (Nardi et al., 2018; Mezzelani et al., 2020). Nonetheless, CBZ-exposure revealed to have slight genotoxic effects independently of temperature regime after 20 days of exposure, leading to a very slight increase of this unrepairable nuclear abnormalities compared to the control condition. Similar values were previously observed in *M. galloprovincialis* exposed to CBZ alone or in combination to reduced pH (7.6) (Mezzelani et al., 2020).

5. CONCLUSION

The present study provides innovative information concerning the potential harms deriving from marine heat waves and carbamazepine for the aquatic environments and inhabiting organisms. Moreover, this research demonstrated *M. galloprovincialis* as a good sentinel species for monitoring presence and ecotoxicological hazard of pharmaceuticals.

The conducted experiment represents a valuable information regarding the potential interactions between chemical and physical insults towards aquatic biota. In particular, CBZ bioaccumulation showed to be enhanced after a MHW event despite the end of this. Moreover, some parameters such as Granulocytes *vs* Hyalinocytes and AChE evidenced that haemocytes immune function and neurotransmission are strongly affected when exposed to multiple stressors. In a general way, both CBZ and MHW condition revealed to induce ROS production given the general antioxidant machinery activation (such as GR and Se-Dep. GPx) which was well impaired with the TGSH content. Furthermore, TOSC $OH^{\cdot}$ presented a lined impairment with both TGSH, MDA contents and presented consistency with the DNA damage pattern. Considering impacts on organisms' metabolism, AOX decreasing trends showed to be consistent with the lower neutral lipids content for MHW exposed specimens. Overall, mussels showed a wider responsiveness to CBZ contamination, although MHW scenario showed to have a synergistic or antagonistic effect in some parameters such as Granulocytes *vs* Hyalinocytes and AChE, respectively.

Though significant information was produced, further investigation is required in order to understand how intensity, duration or frequency of heatwaves can enhance the toxicological impact of contaminants. Moreover, it is important to test diverse compounds in combination with CBZ, mimicking the natural environment.

It is possible to conclude that contaminants of emerging concern (CECs) gains even more ecotoxicological relevance when considering their effect under extreme events caused by anthropogenic CO_2-driven climate changes, like MHW events. These interactions could have profound ecological and socio-economic consequences, such as alterations of mussels reproductive and feeding capacity and growth, resulting in a potential loss of biodiversity in cultures of this species.

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