Marine heatwaves hamper neuro-immune and oxidative tolerance toward carbamazepine in Mytilus galloprovincialis

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1   Marine heatwaves hamper neuro-immune and oxidative tolerance toward carbamazepine in *Mytilus*

2   *galloprovincialis*

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21   **Abstract**

22   The increased frequency and intensity of short-term extreme warming phenomena have been

23   associated to harsh biological and ecosystem outcomes (*i.e.,* mass mortalities in marine organisms).

24   Marine heatwaves (MHWs), occurring when seasonal temperature threshold is exceeded for at least

25   5 consecutive days, may reduce the tolerance of coastal species toward additional pressures, but

26   interactions between such multiple stressors are virtually unexplored. The present study aimed to

27   characterize in *Mytilus galloprovincialis* the influence of a simulated MHW scenario on the

28   toxicological effects of the pharmaceutical carbamazepine (CBZ), ubiquitously detected in the marine

29   environment and chosen as model compound for this relevant class of emerging contaminants. The

30   bioaccumulation of CBZ and responsiveness of various biological parameters, including immune

31   system, antioxidant status, lipid metabolism and cellular integrity, were analyzed in exposed mussels

32   both during and after the end of the heatwave.

33   MHW appeared to strongly modulate accumulation of CBZ, paralleled by weakened

34   immunocompetence and onset of oxidative disturbance that finally evolved to cellular damages and

35   lipid metabolism disorders. Elaboration of the overall results through a quantitative Weight of

36   Evidence model, revealed the highest hazard in organisms exposed to both the stressors 10 days

37   after the end of the heatwave, suggesting that MHWs could leave a footprint on the capability of

38   mussels to counteract CBZ toxicity, thus affecting their vulnerability and predisposition to adverse

39   effects toward multiple stressors.

Introduction

The global ocean warming trend, with a 0.06°C temperature increase per decade since 1900, is a direct consequence of anthropogenic CO_2 (Garcia-Soto et al., 2021; Bindoff et al., 2013). Ocean adsorbed almost 90% of excess heat from Earth system since the industrial revolution, reaching an unprecedented heat content in 2021, the hottest year ever recorded (Cheng et al., 2022). This long-term and gradual warming is superimposed to an increased frequency of marine heatwaves (MHWs), short-term extreme events with seawater temperature exceeding a seasonally varying threshold (usually the 90th percentile) for at least 5 consecutive days (Collins et al., 2019; Frölicher et al., 2019; Oliver et al., 2019; Hobday et al., 2018; Perkins et al.; 2012).

The number of anomalous warm days per year has increased globally by 50% since the early twentieth century and average intensity of MHWs showed a linear trend of almost +0.1°C per decade since 1982 (Oliver et al., 2019, 2018). These phenomena, attributed to the anthropic impact 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 (Collins et al., 2019; Oliver et al., 2019). The Mediterranean Sea represents one of the most endangered areas, where frequency and intensity of MHWs are predicted to increase, causing severe consequences on marine ecosystems structure, functioning and services (Ainsworth et al., 2020; Darmaraki et al., 2019; Genevier et al., 2019; Oliver et al., 2019; Galli et al., 2017). In this respect, MHWs have already been linked to harsh outcomes and mass mortality events of Mediterranean invertebrates (Strydom et al., 2020; Smale et al., 2019; Rubio Portillo et al., 2016; Garrabou et al., 2009).

The consequence of environmental temperature changes can seriously affect organisms health condition since temperature is fundamental in shaping aerobic capacity and physiological homeostasis of marine ectotherms (Sokolova, 2021; Hemraj et al., 2020). Exposure to constantly elevated temperature caused adverse effects ranging from impairment of molecular and cellular

64 pathways (mitochondria functioning, oxidative stress, signalling), up to energy trade-offs and

65 alterations of physiological processes, as immune system efficiency, embryonic development,

66 fertilization and reproduction (Arribas et al., 2022; Crespo et al., 2021; Armstrong et al., 2020; Balogh

67 and Byrne, 2020; Velez et al., 2017; Bartolini et al., 2013; Byrne et al., 2013; Parker et al., 2009;

68 Monari et al., 2007; Abele et al., 2002). Biological responsiveness to stress influences the possibility

69 that organisms resist (maintain performance), recover (restore performance after a decline) or

70 collapse after thermal stress deriving from marine heatwaves (Leung et al., 2019), and possible

71 delayed outcomes of such stress have been reported (Minuti et al., 2021; Amorim et al., 2020).

72 In addition to direct effects, MHWs are of interest also for biological consequences of their

73 interaction with other co-occurring stressors (Arrigo et al., 2020; Przeslawski et al., 2015; Kroeker et

74 al., 2013), including harmful algal blooms and emerging contaminants (ECs) such as perfluorinated

75 compounds, microplastics and pharmaceuticals (Carniero et al., 2021, Lim et al., 2021, Reichert et al.,

76 2021). The widespread occurrence of pharmaceuticals represents a growing risk for coastal areas

77 (COM/2019/128 final), being a direct consequence of the limited removal by wastewater treatment

78 plants, inadequate waste handling and accidental discharges, associated to a global increase of drugs

79 consumption in human medicine and animal husbandry farms (Boxall et al., 2012). Of the 4000

80 pharmaceuticals released in natural ecosystems, the antiepileptic carbamazepine (CBZ) is one of the

81 most environmentally relevant, being ubiquitously detected in water column (up to tens of $\mu g\ L^{-1}$)

82 and frequently measured in aquatic invertebrates (Mezzelani et al., 2020; Miller et al., 2019; Álvarez-

83 Muñoz et al., 2018). Among these organisms, previous studies showed that dissolved and particulate-

84 bound carbamazepine can be accumulated in *Mytilus galloprovincialis* through both gills and

85 digestive gland, being rapidly uptaken and metabolized (Serra-Compte et al., 2018; Boillot et al.,

86 2015). Carbamazepine was also reported to affect several physiological and reproductive processes

87 in aquatic non-target species with potential deleterious consequences on wild populations and

88 ecosystem services (Mezzelani and Regoli, 2022; Mezzelani et al., 2021; Almeida et al., 2018).

89 Despite previous studies revealed that ocean and acidification can modulate the bioavailability and

90 effects of several classes of contaminants, *e.g.* trace metals and polycyclic aromatic hydrocarbons

91 (Giuliani et al., 2021; Kibria et al., 2021; Nardi et al., 2021, 2018, 2017; Wu et al., 2020; Moreria et

92 al., 2016; Sokolova and Lannig, 2008), similar interactions were only recently confirmed for

93 pharmaceuticals, including carbamazepine (Mezzelani et al., 2021; Costa et al., 2020; Almeida et al.,

94 2021). In addition, the majority of studies were carried out at constant or linearly increasing

95 temperature values, while the effects of pulsed thermals stress (MHWs) or the possibility of carry-

96 over alterations, have been so far poorly investigated.

97 In the present study, Mediterranean mussel *Mytilus galloprovincialis* were exposed to carbamazepine

98 under a realistic MHW scenario, to test the hypothesis that marine heatwave has detrimental or

99 delayed outcomes on drug accumulation and effects. *M. galloprovincialis* was selected as test species

100 due to its ecological, economic and ecotoxicological value (Musella et al., 2020), being recognized as

101 a suitable bioindicator in the Mediterranean Sea also toward the increasing occurrence of MHWs and

102 pharmaceuticals, as carbamazepine (Almeida et al., 2021; Mezzelani et al., 2020; Darmaraki et al.,

103 2019; Galli et al., 2017). Drug bioaccumulation and a wide panel of biochemical, cellular and

104 histological markers were selected to represent the main pathways of cellular disturbance and stress-

105 response: the neuroendocrine-immune system, antioxidant defenses, lipid metabolism and oxidative

106 damages were measured in mussels tissues during, at the end and ten days after the MHW event.

107 The overall results were finally integrated through a quantitative Weight Of Evidence (WOE) model

108 that elaborates specific hazard indices based on the number, magnitude and toxicological relevance

109 of observed responses (Regoli et al., 2019). This approach provides quantitative insights on multiple

110   stressors, useful to highlight vulnerability of marine species to interactions between commonly found

111   pharmaceuticals and the increasing intensity, frequency and duration of marine heatwaves.

Materials and Methods

Animal collection and experimental design

Mussels, *Mytilus galloprovincialis* (5.3 ± 0.5 cm shell length), were obtained in October 2019 from a shellfish farm in an unpolluted area of central Adriatic Sea (Mezzelani et al., 2020). As soon as their arrival to laboratory facilities, the whole soft tissues of 15 organisms were dissected for the determination of CBZ levels in wild specimens before the acclimation period. Organisms were maintained for 7 days in aquaria with aerated artificial seawater (ASW; Instant Ocean®) at local seasonal environmental conditions of salinity 35, temperature 18 °C and pH 8.20.

Mussels were then randomly assigned to eight tanks, each containing 60 organisms in 20 L, and exposed for 20 days to clean or carbamazepine contaminated ASW either at constant seasonal environmental temperature or under marine heatwave scenario, according to the four following treatments, performed in duplicate: CTL, control condition at seasonal climatological sea surface temperature (T_{CSST} = 18°C); CBZ, carbamazepine exposure (1 µg L^{-1}) at T_{CSST}=18°C; MHW, marine heatwave scenario (peaking temperature = 22.5 °C, further details are given below and in Supplementary Information Figure SF1); MHW + CBZ, carbamazepine exposure (1 µg L^{-1}) under marine heatwave scenario. The exposure dose of carbamazepine reflects environmental levels in coastal areas (Mezzelani et al., 2018, 2020; Almeida et al., 2021), while marine heatwave scenario was reconstructed based on oceanographic data of events occurred in the Adriatic Sea in late summer – early autumn of 2014 and 2019 (www.marineheatwaves.org/tracker, coordinates: 43.625, 13.625; cumulative intensity of events: 26.8°C and 40.4°C, respectively; duration: 12 and 15 days, respectively). Temperature for marine heatwave scenario was increased daily by 0.9 °C until the peak was reached (22.5 °C, day 5 and 6), then it was daily decreased with the same slope until the climatological mean temperature was restored (18 °C, day 11), and maintained constant for 10 additional days, as "recovery" phase from the marine heatwave. Water was changed every other day,

136 and carbamazepine re-dosed. Organisms in each tank were fed 24h prior the water change with 500

137 µL of an unpolluted commercial mixture of zooplankton (Brightwell Zooplanktos-S, size range 50-300

138 µm) for filter-feeding organisms, according to manufacturer indications.

139 From each experimental condition, 40 organisms (20 per duplicate tank) were randomly sampled

140 and dissected at three experimental times: T1 (day 6), T2 (day 11) and T3 (day 20) which represented

141 the peak of heatwave (T1), the end of heatwave (T2), and 10-days post heatwave (T3) respectively.

142 The choice of 10 days after the end of the heatwave was considered appropriate to highlight

143 persistence or onset of adverse outcomes after a comparable period of pulsed thermal stress.

144 The whole soft tissues of 15 individuals were used for analyses of CBZ bioaccumulation (5 pools,

145 each constituted by 3 organisms, stored at -20°C after dissection) while the haemolymph was

146 withdrawn (5 pools, each constituted by 3 organisms), partly immediately processed for in vivo

147 analyses and partly fixed in Carnoy's solution until nuclear alterations analysis. For biomarker

148 analyses, digestive glands and hemolymph were collected from other 20 individuals (5 pools, each

149 constituted by tissues of 4 organism, rapidly frozen in liquid nitrogen and maintained at -80°C). For

150 histological analyses, digestive glands of 5 additional organisms were excised, flash frozen separately

151 and maintained at -80°C.

152

153 *Chemical analyses*

154 Concentrations of CBZ in mussels whole soft tissues (n=5) were determined by High Performance

155 Liquid Chromatography with fluorometric and diode array detectors. Information on reagents, as well

156 as detailed extraction and analytical protocols, including QA/QC procedures, are given in

157 Supplementary Information.

158

159 *Biomarkers analyses*

160   Validated protocols were used to analyze the following classes and typologies of biomarkers

161   (n=5, details are given in Supplementary Information): immunological responses (haemocytes

162   lysosomal membrane stability, granulocytes vs hyalinocytes ratio and phagocytosis rate), cholinergic

163   effects (acetylcholinesterase activity in haemolymph); levels of antioxidant defenses (activity of

164   catalase, Se-dependent glutathione peroxidases, total glutathione peroxidases, glutathione S-

165   transferases, glutathione reductase, content of total glutathione) and total oxyradical scavenging

166   capacity (TOSC) toward peroxyl radical ROO•, hydroxyl radical HO• and peroxynitrite ONOO-, in the

167   digestive gland; lipid metabolism (acyl-CoA oxidase activity and neutral lipids content) and

168   peroxidation (malondialdehyde concentration and lipofuscin content) in the digestive gland; onset

169   genotoxic damage (DNA fragmentation and micronuclei frequency, in haemocytes).

170

171   *Statistical analyses and weighted elaboration*

172   Data visualization and statistical analyses for CBZ bioaccumulation and biomarkers data were

173   performed using RStudio (version 1.2.5033). Data were checked for normal distribution (Shapiro-Wilk

174   test) and homogeneity of variances (Levene's test); when assumptions were not fulfilled, data were

175   transformed using Box-Cox transformation (power transformation, R package "MASS"). Effect of the

176   factors "CBZ Exposure" (two levels: 0 and 1 µg L^{-1}), "Temperature Scenario" (two levels: constant and

177   marine heatwave), "Experimental Time" (three levels: T1, T2 and T3) and their interactions on CBZ

178   bioaccumulation and biological parameters were assessed using a generalized linear model (three-

179   way ANOVA, Table 1); Tukey HSD (HSD) *post-hoc test* was applied for comparing the means of interest

180   between different treatments at each sampling time and between different sampling times within

181   the same treatment. Multivariate principal component analysis (PCA) of bioaccumulation and

182   biomarkers results was applied to visualize the relationships among the different treatments at each

183   sampling time.

184 The results on bioaccumulation and biomarkers analyses were further elaborated through a

185 quantitative Weight Of Evidence (WOE) model that provides synthetic hazard indices for each

186 typology of data (or Line of Evidence, LOE) before their final integration (Regoli et al., 2019).

187 Independent elaborations for bioaccumulation (LOE-2) and biomarkers (LOE-3) were based on

188 magnitude of observed variations, statistical significance compared to controls and weights assigned

189 to toxicological relevance of measured endpoints. After normalization of indices to a common scale,

190 individual hazard indices were integrated through a classical weight of evidence approach, and level

191 of risk assigned to 1 of 5 classes, from Absent to Severe. Whole calculations, detailed flow-charts,

192 rationale for weights, thresholds and expert judgements have been described in detail in

193 Supplementary Information (Regoli et al., 2019).

Results

Carbamazepine bioaccumulation

Carbamazepine in tissues of mussels was below the limit of detection of 1.03 ng g^{-1} (d.w.) at the moment of collection (data not shown). Levels of carbamazepine in whole soft tissues of exposed mussels highlighted significant interactions between CBZ exposure, temperature scenario and experimental time (Table 1, Fig. 1). Average CBZ values in MHW+CBZ organisms were almost double at the peak of heatwave (T1) and 10-days post heatwave (T3) compared to those of mussels exposed to CBZ-alone: at control temperature, the peak of accumulation was reached at T2, then remaining almost constant at T3.

Immunological and cholinergic alterations

Lysosomal membrane stability in haemocytes was affected by CBZ exposure under the MHW scenario (Table 1, Fig. 2a): the significant reduction of LMS observed at T1 (peak of heatwave), persisted even at the end of heatwave (T2), and 10-days post heatwave (T3). No differences among time were reported for any of the treatments. Significant interactions between CBZ, temperature scenario and exposure time occurred in granulocytes vs. hyalinocytes ratio (Table 1, Fig. 2b): a bell-shaped variation characterized MHW+CBZ treatment, which showed a significant increase at T2 followed by a consistent decrease at T3. Similarly, phagocytosis rate (Table 1, Fig. 2c) was significantly reduced in MHW+CBZ organisms compared to CBZ alone at T1 and T3. Significant differences of AChE activity were measured in organisms exposed to CBZ alone compared both to control condition (CBZ vs. CTL) and to MHW+CBZ organisms, with significantly lower activity in these treatments at T1 and T3 (Table 1, Fig. 2d).

Single antioxidant defenses and total oxyradical scavenging capacity

Catalase activity significantly varied along time with a bell-shaped trend (Table 1, Fig. 3a) in organisms exposed to CBZ and MHW+CBZ. Similar effects were observed for Se-dep. glutathione peroxidases (Table 1, Fig. 3b), with enhanced activity measured at T2 compared to T1 and T3; in addition, MHW alone caused a significant induction of Se-dep- GPx activity persisting from the peak up to 10 days after the end of heatwave. In MHW+CBZ organisms, total glutathione peroxidases activity significantly declined at T3 compared to T1 (Table 1; Fig. 3c), while glutathione S-transferases showed a trend of increasing activity reaching a peak of significant induction at T3 in CBZ-exposed organisms, independently of temperature scenario (Table 1; Fig. 3d). Glutathione reductase was enhanced by single and combined stressors, particularly evident at T2 (Table 1; Fig. 3e). Total glutathione increased in all treatments at T1 remaining elevated at T3 in organisms exposed to CBZ, particularly in MHW+CBZ condition (Table 1; Fig. 3f). Significant effects of carbamazepine and MHW were highlighted on TOSC ROO•, TOSC HO• and TOSC ONOO- (Table 1, Fig. 4a, b and c respectively). TOSC ROO• increased at T2 in organisms exposed to CBZ at control temperature, while in marine heatwave scenarios (MHW and MHW + CBZ) this trend was inverted and exacerbated by the presence of the drug. An increased capability to counteract HO• was observed in organisms exposed to marine heatwave (MHW and MHW + CBZ) at T1 and to MHW+CBZ also at T3. Organisms co-exposed to MHW+CBZ showed significantly lowered capability to counteract peroxynitrite (TOSC ONOO-) compared to single stressors both at T1 and T2.

235   *Lipid metabolism and peroxidation*

236   CBZ exposure under MHW scenario resulted in a lowered activity of Acyl CoA oxidase compared to

237   control temperature at T1 (Table 1, Fig. 5a).

238   The content of neutral lipids generally raised in all the treatments with significant interactions

239   between CBZ exposure, MHW and time (Table 1, Fig. 5b). CBZ showed a prolonged and increasing

240   effect for the whole duration of exposure under constant temperature scenario (CBZ treatment),

241   while the effect was not time dependent when CBZ exposure was combined to MHW. Different

242   effects of MHW and CBZ were highlighted by divergent responses when organisms were exposed to

243   single or combined stressors: organisms exposed to MHW also exhibited a marked increase of neutral

244   lipids at the peak of heatwave (T1), followed by a rapid decrement at T2 and T3. CBZ and MHW (Table

245   1, Fig. 5c) caused slight and irregular changes of malondialdehyde (MDA) at T1 and T2, respectively,

246   while no variations were observed in co-exposed organisms, which showed MDA content always

247   comparable to CTL.

248   Lipofuscin content was significantly affected by interactions of CBZ exposure, MHW and experimental

249   times (Table 1, Fig. 5d). The drug, independently of the temperature scenario, led to increased levels

250   of lipofuscin at T1 and T2 in CBZ and MHW+CBZ organisms, while values were comparable to CTL at

251   T3. A biphasic trend was observed also in MHW treated organisms which showed an initial increase

252   of lipofuscin at T1, followed by the decrement at T2 and T3 with values which, however, remained

253   higher compared to CTL even after 10 days of recovery.

254   *Genotoxic damage*

255   Exposure to CBZ and heatwave, alone or in combination determined a significant loss of DNA integrity

256   at T3 (Table 1, Fig. 6a); in addition, a three phases trend was observed in MHW scenario treatments

257   (MHW and MHW + CBZ): haemocytes experienced DNA damage accumulation at T1, which dropped

258   at T2 and furtherly increased at T3. CBZ-exposure also led to an enhancement of micronuclei

259   frequency at T3 (Table 1, Fig. 6b): interestingly, in MHW+CBZ organisms, the highest frequency of

260   MN was observed at T1 when no effects were caused by single stressors.

261   *Principal components analysis and weighted elaboration*

262   Principal components analysis carried out for each experimental time on the whole dataset of results

263   (Fig. 7) provided two-dimensional patterns of separation between treatments, always explaining

264   more than 50% of total variance. At T1 (Fig. 7a), divergence was observed between the two

265   temperature scenarios, at T2 single and combined stressors were separated (Fig. 7b), while at T3 a

266   relevant discrimination was further observed for CBZ exposure along Dimension 1 and for MHW along

267   Dimension 2, producing a split between MHW+CBZ and other treatments (Fig. 7c).

268   Synthetic hazard indices for each experimental treatment and time of exposure were provided by the

269   weighted elaboration of bioaccumulation (LOE-2) and biomarkers (LOE-3) results (Fig. 8 and details

270   in Supplementary Information). Bioaccumulation hazard (LOE-2) for CBZ treatment was classified as

271   "Slight" at T1, "Major" at T2 and "Moderate" at T3, while for MHW+CBZ treatment was classified as

272   "Moderate" at T2 and "Major" at T1 and T3. The absence of CBZ exposure and bioaccumulation in

273   MHW treatment resulted in an "Absent" hazard classification for this treatment. The elaboration of

274   biomarkers (LOE-3) based on the magnitude of variations compared to CTL organisms and the

275   toxicological relevance of each analyzed parameter produced for CBZ-treated organisms (CBZ and

276   MHW+CBZ) a "Moderate" hazard classification at T1 and T2 and a "Slight" hazard classification at T3.

277   In MHW organisms, hazard was classified as "Moderate" at T1 and "Slight" at T2 and T3. The

278   contribution of each investigated parameter to such hazard classifications, is summarized in

279   Supplementary Information Table ST2. The final weighted integration of LOE-2 and LOE-3, shown in

280   Figure 8, revealed a level of risk constantly classified as "Moderate" or "Slight" for the carbamazepine

281   treatment (CBZ) and marine heatwave scenario (MHW), respectively. When carbamazepine was

282   dosed under marine heatwave scenario (MHW+CBZ), a "Moderate" risk was assigned at the peak and

283   at the end of heatwave (T1-T2), while it increased to "Major" after 10 days of recovery from the event

284   (T3).

285   Discussion

286   This study provided clear evidence of the capability of marine heatwaves to modulate the susceptibility to

287   the pharmaceutical CBZ in mussels.

288   Beside the confirmed capability of *M. galloprovincialis* to accumulate this drug, organisms exposed under

289   MHW scenario exhibited the highest CBZ tissue levels, even after a recovery period from the heatwave: to

290   our knowledge this is the first study observing delayed effects on pharmaceuticals uptake after the exposure

291   to a pulsed thermal stress, which characterizes MHWs. Additional evidence on the modulation of the toxicity

292   of pharmaceuticals in future oceans derives from the onset of immune system alterations caused by

293   combined rather than single stressors. This was particularly evident on haemocytes lysosomal membranes,

294   which lost integrity in co-exposed organisms during all phases of the heatwave and recovery period,

295   consistently with the elevated accumulation of CBZ. We hypothesize an early disturbance triggered by CBZ

296   (Mezzelani et al., 2021; Franzellitti et al., 2019; Aguirre-Martínez et al., 2013; Martin-Diaz et al., 2009) and

297   sustained thereon by thermal stress (Marigómez et al., 2017; Parisi et al., 2017), supported by the reduced

298   capability of lysosomes to recover heat-damaged membranes, as previously observed in *M. galloprovincialis*

299   exposed to a secondary stressor (*e.g.* cadmium, Múgica et al., 2015). Synergistic effects of CBZ and MHW

300   were evident also on haemocytes sub-populations and their functional activity. In co-exposed organisms,

301   granulocytes-hyalinocytes ratio and phagocytosis activity showed a bell-shaped trend of variation over

302   MHW phases: a counteracting phase, characterized by an increase of these immunological parameters at

303   the end of the heatwave, was followed by a significant inhibition observed after 10 days of recovery from

304   the event, confirming that MHWs modulate CBZ toxicity and the immune system of mussels, potentially

305   increasing their long-term vulnerability to stressors. The mechanisms behind these effects may involve CBZ-

306   mediated alterations of the neuroendocrine-immune system (Liu et al., 2018): CBZ was shown to increase

307   ACh synthesis in non-target organisms (Mizuno et al., 2000) and this neurotransmitter has been recently

308   suggested to suppress bivalves phagocytosis through the alteration of NF-kB and Ca^{2+} signaling pathways

309   (Cao et al., 2021; Du et al., 2020). The differences of AChE activity observed in this study between organisms

310 exposed to CBZ alone or under marine heatwave scenario, support the hypothesis that the impairment of

311 immune system in co-exposed organisms is, at least partially, caused by a reduced ACh hydrolysis. Such

312 alterations might have been exacerbated by a reduced functionality of haemocytes due to an increased

313 oxidative pressure caused by thermal stress of MHW (Benedetti et al., 2022; Rahman et al., 2019), and

314 confirmed in co-exposed organisms by the appreciable responsiveness of antioxidant defenses in the

315 digestive gland.

316 Catalase and Se-dependent glutathione peroxidases in CBZ-treated mussels (CBZ and MHW+CBZ) showed a

317 temporary increase at the end of the heatwave suggesting a delayed demand of hydrogen peroxide

318 detoxification (Regoli and Giuliani, 2014), possibly reflecting CBZ-metabolism: in non-target species, this

319 drug is biotransformed through phase I and II pathways, that may thus promote an intracellular formation

320 of ROS (Mezzelani et al., 2021; Benedetti et al., 2022). The increased activity of the phase II enzymes

321 glutathione S-transferases observed in the present study, along with variations of glutathione reductase and

322 levels of total glutathione, support the hypothesis of biotransformation as a source of ROS worsened by

323 thermal stress and an increased demand of glutathione metabolism to neutralize derived ROS.

324 The responsiveness of individual antioxidants was paralleled by alterations of the total oxyradical scavenging

325 capacity: in co-exposed organisms the capability to neutralize hydroxyl and peroxyl radicals (TOSC HO• and

326 TOSC ROO•, respectively), showed trends of variations complementary to CAT and Se-dependent GPx and

327 analogous to TGSH levels. The early increase of defenses against these ROS and the following variations,

328 confirm non-synchronous responsiveness of single antioxidants and oxidative challenge during exposure.

329 On the other hand, the initial depletion of capability to neutralize peroxynitrite (TOSC ONOO-) was followed

330 by its relevant increase after the thermal stress, suggesting that the acute disturbance of this redox pathway

331 was partly compensated by the mutual interplay with other antioxidant mechanisms (Regoli and Giuliani,

332 2014). Overall, the persistence of antioxidants alterations after the recovery from the heatwave, further

333 corroborates the additional impact of MHWs on oxidative effects of CBZ, with potential energetic costs and

334 metabolic trade-offs to restore redox homeostasis (Sokolova, 2021).

335   In this respect, a disturbance of lipid metabolism was evidenced in co-exposed organisms, which showed a

336   moderate decrease of Acyl-CoA oxidase and accumulation of neutral lipids (NL). Both carbamazepine and

337   thermal stress have been previously suggested to have a role on neutral lipids accumulation (Múgica et al.,

338   2015; Mezzelani et al., 2021; Martin-Diaz et al., 2009; Dimitriadis et al., 2004), but the effects observed in

339   this study for combined stressors were different from those of single ones. In particular, co-exposed

340   organisms accumulated NL in the early phases of the heatwave, and these remained constant throughout

341   the exposure, despite the end of thermal stress; however, after ten days of recovery, levels of NL were lower

342   compared to those of organisms exposed to CBZ alone, allowing to hypothesize that lipid metabolism is

343   affected by reciprocal but still unknown interactions between thermal stress and carbamazepine.

344   Lipofuscin content in tertiary lysosomes was increased by all stressors (alone or in combination) highlighting

345   that the sustained activation of the antioxidant system could not prevent lipid peroxidation processes in the

346   early phases of stress, and confirming the progression of oxidative imbalance from the activation of

347   biochemical defenses to cellular disturbance.

348   Oxidative damages were not limited to lipid peroxidation but also involved DNA integrity and micronuclei

349   formation: exposure to single stressors promoted DNA fragmentation, particularly at the end of recovery

350   period, with similar outcomes but no synergistic effects in co-exposed organisms. Conversely, a subtle

351   enhancement of micronuclei frequency was observed in CBZ-treatments (CBZ and MHW+CBZ), with an

352   earlier and more evident effect in co-exposed organisms, highlighting a hastening of temperature-mediated

353   effect on CBZ genotoxicity (Nardi et al., 2017; Yao et al., 2013; Salazar et al., 2009).

354   Principal components analysis provided a detailed perspective on the prevalence of different stressors in

355   each phase of the experiment. At the peak of the heatwave (T1) a major separation occurred between

356   organisms exposed at control temperature and those under MHW scenario independently of CBZ exposure,

357   indicating the early onset of disturbance due to the extreme thermal stress; conversely, at the end of the

358   heatwave (T2), a major disturbance of CBZ was revealed, suggesting that the need to cope thermal stress

359   was rapidly met but may be disadvantageous in terms of protection toward chemical disturbance. Lastly,

360   after 10 days from the end of the heatwave (T3), a clear separation occurred between single and combined

361   stressors, supported by the synergic effects in co-exposed organisms.

362   Differences among treatments and experimental phases were summarized by the weighted elaboration of

363   bioaccumulation and biological responses data through the Weight of Evidence model (Sediqualsoft). The

364   elaboration of bioaccumulation results (LOE-2), based on the levels of CBZ and the magnitude of variation

365   compared to CTL organisms, revealed a consistently higher hazard in co-exposed organisms (MHW+CBZ).

366   For this treatment, the overall integration of the wide panel of analyzed biological traits (LOE-3) revealed a

367   "Moderate" hazard at the heatwave peak, classification supported by the accumulation of lipid peroxidation

368   products, immune parameters alterations and effects on lipid metabolism and redox homoeostasis; this

369   "Moderate" hazard classification persisted until the end of the heatwave, sustained by the effects on lipid

370   peroxidation and metabolism, immune parameters and antioxidant pathways; the hazard decreased to

371   "Slight" after the recovery phase, with still evident effects on certain immune and antioxidant responses.

372   The final integration of chemical and biological results through a WOE approach, provided a synthetic hazard

373   index with the worst classification ("Major") in co-exposed organisms after 10 days of recovery from the

374   heatwave, thus highlighting long-lasting synergic interaction of stressors and corroborating the hypothesis

375   of marine heatwave footprint in organisms coping with CBZ.

376

377   Conclusions

378   This study showed that the occurrence of short-term and pulsed events of temperature extremes may

379   enhance the accumulation of CBZ in mussels, causing effects that persist even 10 days after the end of the

380   heatwave. Interactions among stressors synergistically impaired both the neuroendocrine-immune and

381   oxidative system of organisms, addressing the intensification of extreme events as a key environmental

382   challenge, which can exacerbate the consequences of the increasing release of pharmaceuticals. Due to the

383   ecological relevance of this study referred to Mediterranean conditions, where both MHWs and

384   pharmaceuticals represent emerging challenges, our findings encourage further investigations to better

385   elucidate reciprocal interactions of such multiple stressors, recovery capacity and long-term perspectives

386   for biodiversity conservation, environmental impact and climate change risk assessment.

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Data availability

The data supporting the conclusions of this article are available at the following link: https://figshare.com/s/9c28068c95c8d5661f86.

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CBZ

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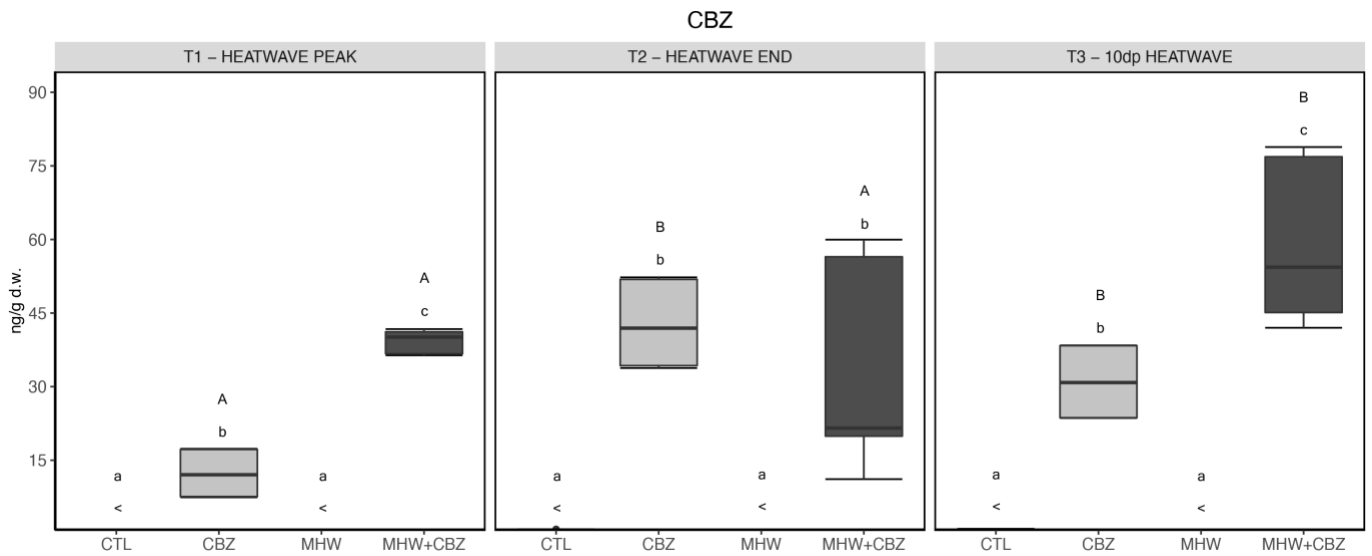
Figure 1 - Carbamazepine concentration in whole soft tissues of exposed organisms. Data are given in ng g^{-1} dry weight. Lower and upper boundaries of each box represent 25th and 75th percentile, respectively, line within the box indicates the median and whiskers above and below each box mark highest and lowest values, respectively. CTL (white boxes), control condition: constant temperature; CBZ (light grey boxes), CBZ-exposure at constant temperature; MHW (grey boxes), marine heatwave scenario; MHW+CBZ (dark grey boxes), CBZ-exposure under marine heatwave scenario. Letters are used to highlight significant differences between treatments within the same sampling time (lower case) and among the same treatment along time (upper case). "<" indicates concentrations below limit of detection, LOD (1.03 ng g^{-1} d.w.).
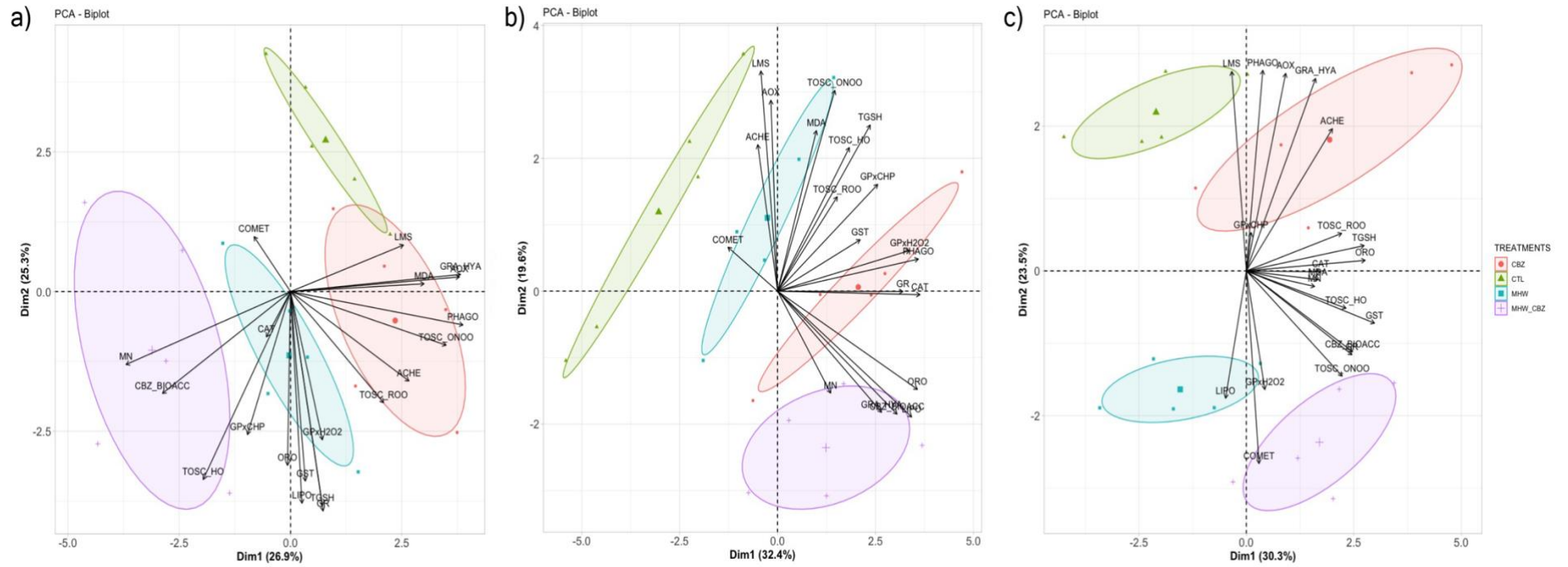
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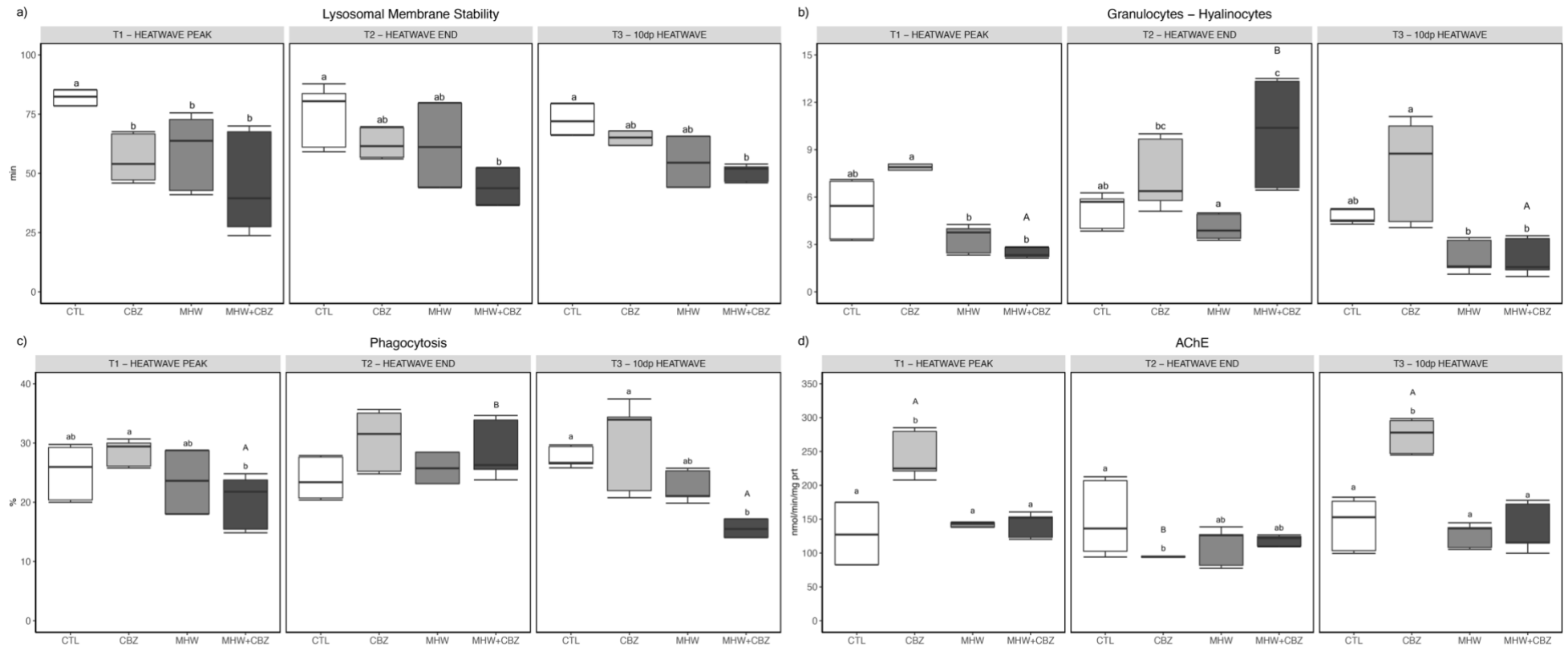
Figure 2 – **Immune and cholinergic alterations.** Lysosomal membrane stability (a), granulocytes/hyalinocytes ratio (b), phagocytosis rate (c), acetylcholinesterase activity (d) in haemocytes of exposed mussels. Lower and upper boundaries of each box represent 25th and 75th percentile, respectively, line within the box indicates the median and whiskers above and below each box mark highest and lowest values, respectively. CTL (white boxes), control condition: constant temperature; CBZ (light grey boxes), CBZ-exposure at constant temperature; MHW (grey boxes), marine heatwave scenario; MHW+CBZ (dark grey boxes), CBZ-exposure under marine heatwave scenario. Letters are used to highlight significant differences between treatments within the same sampling time (lower case) and among the same treatment along time (upper case).

Figure 3 – **Single antioxidant defenses.** Activity of catalase (a), Se-dependent glutathione peroxidases (b), total glutathione peroxidases (c), glutathione S-transferases (d), glutathione reductase (e) and content of total glutathione (f). Lower and upper boundaries of each box represent 25th and 75th percentile, respectively, line within the box indicates the median and whiskers above and below each box mark highest and lowest values, respectively. CTL (white boxes), control condition: constant temperature; CBZ (light grey boxes), CBZ-exposure at constant temperature; MHW (grey boxes), marine heatwave scenario; MHW+CBZ (dark grey boxes), CBZ-exposure under marine heatwave scenario. Letters are used to highlight significant differences between treatments within the same sampling time (lower case) and among the same treatment along time (upper case).
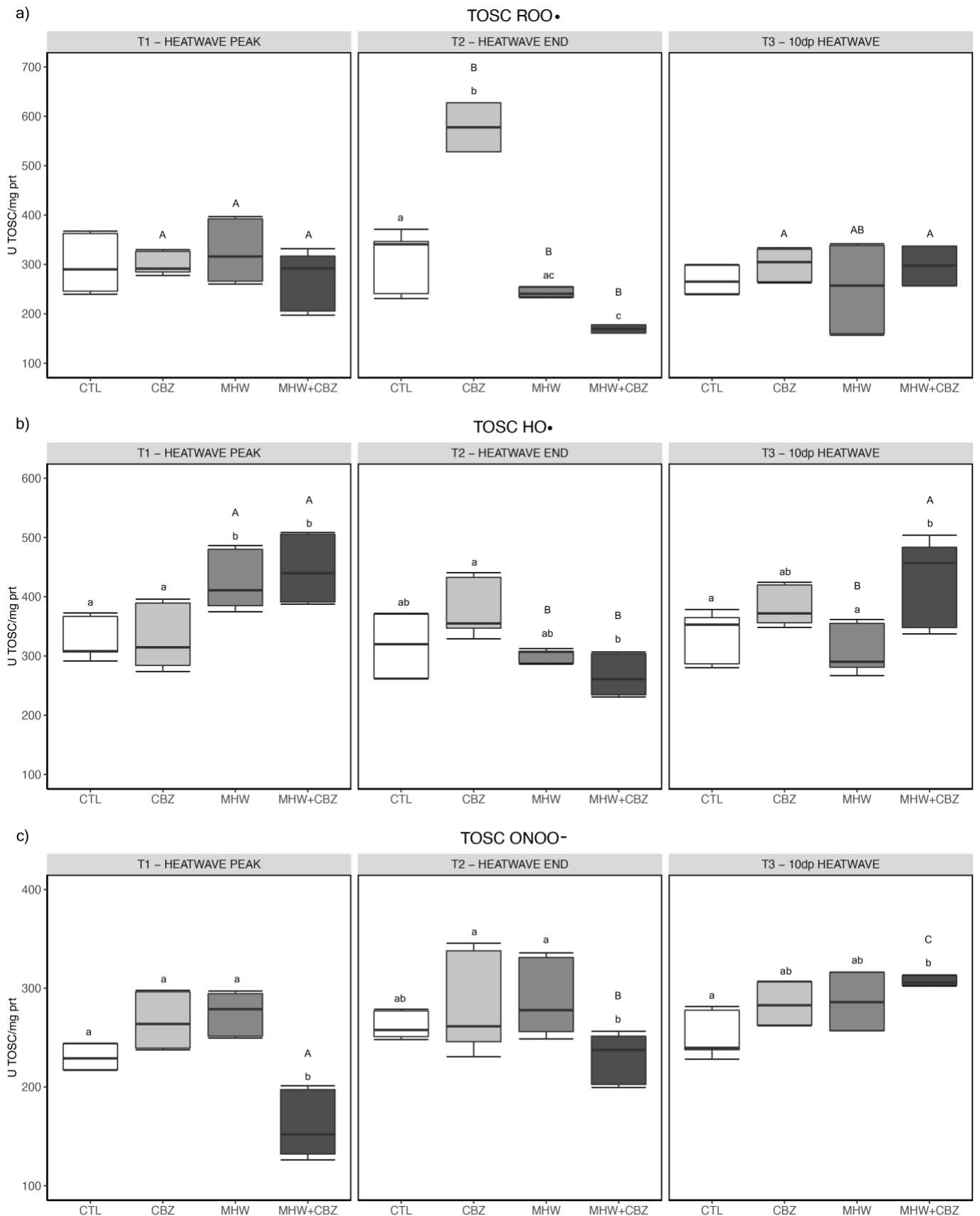
Figure 4 - **Total oxyradical scavenging capacity.** Overall defenses against peroxyl radical (a), hydroxyl radical (b) and peroxynitrite (c). Lower and upper boundaries of each box represent 25th and 75th percentile, respectively, line within the box indicates the median and whiskers above and below each box mark highest and lowest values, respectively. CTL (white boxes), control condition: constant temperature; CBZ (light grey boxes), CBZ-exposure at constant temperature; MHW (grey boxes), marine heatwave scenario; MHW+CBZ (dark grey boxes), CBZ-exposure under marine heatwave scenario. Letters are used to highlight significant differences between treatments within the same sampling time (lower case) and among the same treatment along time (upper case).

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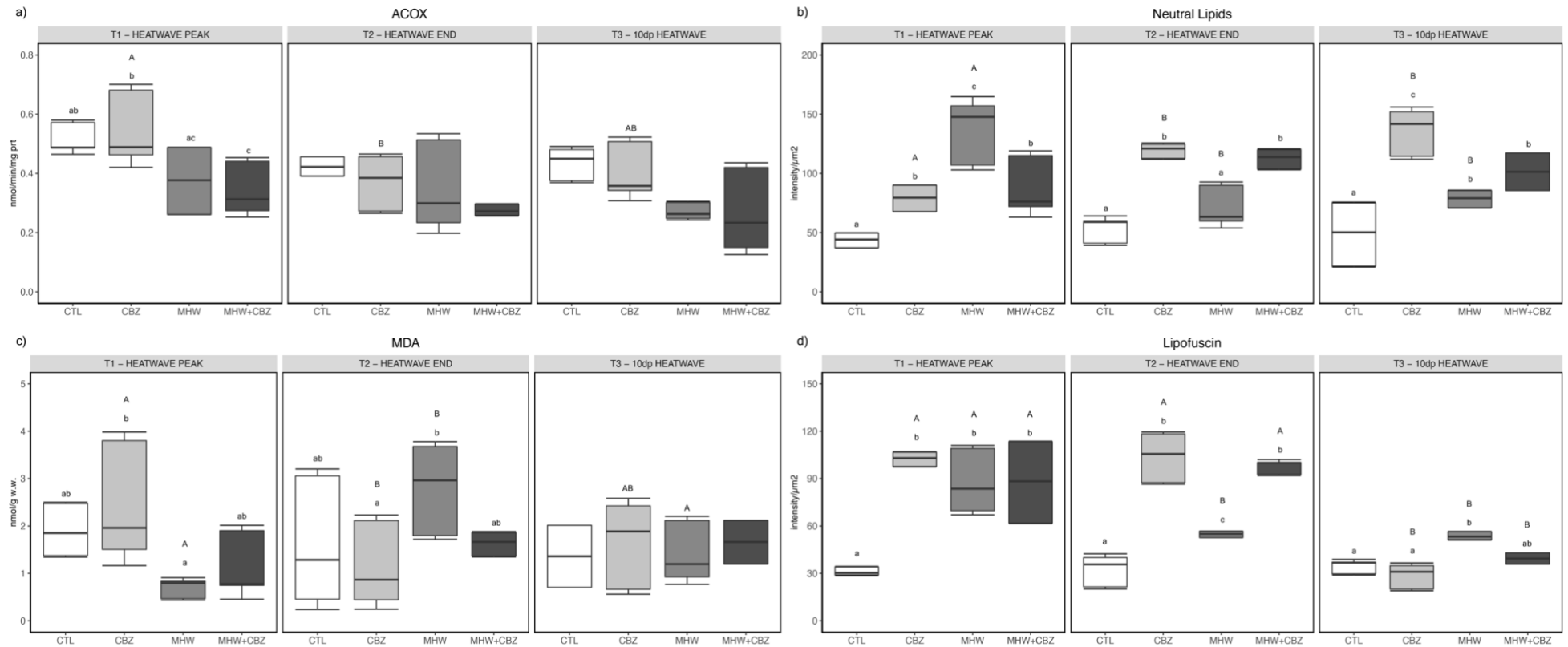
Figure 5 -   Lipid metabolism and peroxidation. Acyl Co-A oxidase activity (a), neutral lipids content (b), malondialdehyde concentration (c) and lipofuscin content (d). Lower and upper boundaries of each box represent 25th and 75th percentile, respectively, line within the box indicates the median and whiskers above and below each box mark highest and lowest values, respectively. CTL (white boxes), control condition: constant temperature; CBZ (light grey boxes), CBZ-exposure at constant temperature; MHW (grey boxes), marine heatwave scenario; MHW+CBZ (dark grey boxes), CBZ-exposure under marine heatwave scenario. Letters are used to highlight significant differences between treatments within the same sampling time (lower case) and among the same treatment along time (upper case).
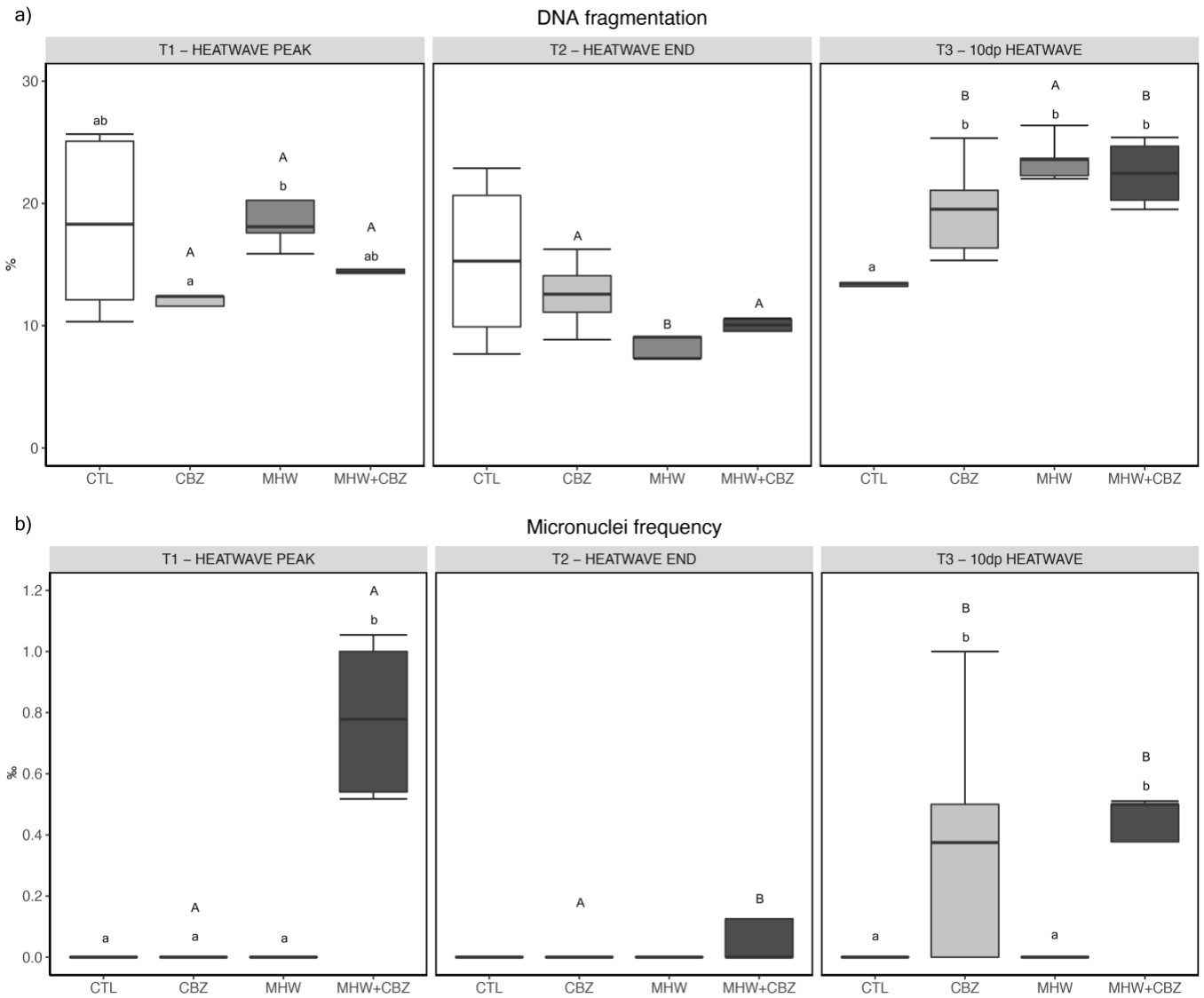
Figure 6 - Genotoxic damage. DNA fragmentation (a) and frequency of micronuclei (b) in haemocytes of exposed mussels. Lower and upper boundaries of each box represent 25th and 75th percentile, respectively, line within the box indicates the median and whiskers above and below each box mark highest and lowest values, respectively. CTL (white boxes), control condition: constant temperature; CBZ (light grey boxes), CBZ-exposure at constant temperature; MHW (grey boxes), marine heatwave scenario; MHW+CBZ (dark grey boxes), CBZ-exposure under marine heatwave scenario. Letters are used to highlight significant differences between treatments within the same sampling time (lower case) and among the same treatment along time (upper case).

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Figure 7 - Two-dimensional representation of Principal Components Analysis (PCA) of whole dataset for each experimental checkpoint. T1, day 6, peak of heatwave (a); T2, day 11, end of heatwave (b); T3, day 20, 10-days after heatwave (c).

Treatment	Experimental Checkpoint	LOE-2 (Bioaccumulation)		LOE-3 (Biomarkers)		WOE	
CBZ	T1	SLIGHT		MODERATE		MODERATE	
	T2	MAJOR		MODERATE		MODERATE	
	T3	MODERATE		SLIGHT		MODERATE	
MHW	T1 (peak)	ABSENT		MODERATE		SLIGHT	
	T2 (end)	ABSENT		SLIGHT		SLIGHT	
	T3 (10dp)	ABSENT		SLIGHT		SLIGHT	
MHW+CBZ	T1 (peak)	MAJOR		MODERATE		MODERATE	
	T2 (end)	MODERATE		MODERATE		MODERATE	
	T3 (10dp)	MAJOR		SLIGHT		MAJOR	

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Figure 8 - Weighted elaboration of whole dataset for each treatment in all experimental checkpoints. Classes of hazard (Absent, white; Slight, light blue; Moderate, yellow; Major, red; Severe, black) are given for each treatment-experimental checkpoint combination for CBZ bioaccumulation (LOE-2), biological effects (LOE-3) and their weighted integration (WOE).

Table 1 – Three-way ANOVA results for single factors and their interactions; F-values with degrees of freedom and p-values are reported for each tested stressors and interactions for each analyzed parameter. Significant effects are highlighted in bold and italic.

Parameter	CBZ Exposure	Temperature Scenario	Experimental Time	CBZ Exp. x Temp. Scen.	CBZ Exp. x Exp. Time	Temp. Scen. x Exp. Time	CBZ Exp. x Temp. Scen. x Exp.Time
CBZ bioaccumulation	$F_{(1,48)} = 236.1$ $p < 0.001$	$F_{(1,48)} = 10.76$ $p < 0.01$	$F_{(2,48)} = 5.979$ $p < 0.01$	$F_{(1,48)} = 11.05$ $p < 0.01$	$F_{(2,48)} = 5.858$ $p < 0.01$	$F_{(2,48)} = 6.771$ $p < 0.01$	$F_{(2,48)} = 6.933$ $p < 0.01$
Lysosomal membrane stability	$F_{(1,48)} = 19.73$ $p < 0.001$	$F_{(1,48)} = 28.45$ $p < 0.001$	$F_{(2,48)} = 0.001$ $p = 0.999$	$F_{(1,48)} = 0.286$ $p = 0.596$	$F_{(2,48)} = 1.672$ $p = 0.1987$	$F_{(2,48)} = 0.014$ $p = 0.986$	$F_{(2,48)} = 0.724$ $p = 0.490$
Granulocytes - Hyalinocytes ratio	$F_{(1,48)} = 22.48$ $p < 0.001$	$F_{(1,48)} = 25.55$ $p < 0.001$	$F_{(2,48)} = 10.64$ $p < 0.001$	$F_{(1,48)} = 1.114$ $p = 0.296$	$F_{(2,48)} = 4.664$ $p < 0.05$	$F_{(2,48)} = 11.70$ $p < 0.001$	$F_{(2,48)} = 6.602$ $p < 0.01$
Phagocytosis rate	$F_{(1,48)} = 0.480$ $p = 0.492$	$F_{(1,48)} = 18.62$ $p < 0.001$	$F_{(2,48)} = 3.707$ $p < 0.05$	$F_{(1,48)} = 8.163$ $p < 0.01$	$F_{(2,48)} = 3.587$ $p < 0.05$	$F_{(2,48)} = 6.229$ $p < 0.01$	$F_{(2,48)} = 0.542$ $p = 0.585$
AchE activity	$F_{(1,48)} = 18.24$ $p < 0.001$	$F_{(1,48)} = 28.32$ $p < 0.001$	$F_{(2,48)} = 16.12$ $p < 0.001$	$F_{(1,48)} = 12.53$ $p < 0.001$	$F_{(2,48)} = 13.27$ $p < 0.001$	$F_{(2,48)} = 6.033$ $p < 0.01$	$F_{(2,48)} = 14.19$ $p < 0.001$
Catalase activity	$F_{(1,48)} = 1.9024$ $p = 0.174$	$F_{(1,48)} = 2.025$ $p = 0.161$	$F_{(2,48)} = 6.743$ $p < 0.01$	$F_{(1,48)} = 0.057$ $p = 0.812$	$F_{(2,48)} = 2.122$ $p = 0.131$	$F_{(2,48)} = 0.115$ $p = 0.891$	$F_{(2,48)} = 2.584$ $p = 0.086$
Se-dep.glutathione peroxidase activity	$F_{(1,48)} = 1.071$ $p = 0.306$	$F_{(1,48)} = 24.90$ $p < 0.001$	$F_{(2,48)} = 8.486$ $p < 0.001$	$F_{(1,48)} = 9.220$ $p < 0.01$	$F_{(2,48)} = 3.076$ $p = 0.055$	$F_{(2,48)} = 0.566$ $p = 0.572$	$F_{(2,48)} = 0.656$ $p = 0.523$
Total glutathione peroxidase activity	$F_{(1,48)} = 0.005$ $p = 0.943$	$F_{(1,48)} = 0.255$ $p = 0.616$	$F_{(2,48)} = 3.13$ $p = 0.053$	$F_{(1,48)} = 0.379$ $p = 0.541$	$F_{(2,48)} = 4.086$ $p < 0.05$	$F_{(2,48)} = 0.300$ $p = 0.742$	$F_{(2,48)} = 0.928$ $p = 0.402$
Glutathione S-transferases activity	$F_{(1,48)} = 9.044$ $p < 0.01$	$F_{(1,48)} = 7.998$ $p < 0.01$	$F_{(2,48)} = 8.069$ $p < 0.001$	$F_{(1,48)} = 0.063$ $p = 0.803$	$F_{(2,48)} = 7.443$ $p < 0.01$	$F_{(2,48)} = 1.163$ $p = 0.321$	$F_{(2,48)} = 0.37$ $p = 0.693$
Glutathione reductase activity	$F_{(1,48)} = 17.56$ $p < 0.001$	$F_{(1,48)} = 12.27$ $p < 0.01$	$F_{(2,48)} = 9.137$ $p < 0.001$	$F_{(1,48)} = 22.75$ $p < 0.001$	$F_{(2,48)} = 0.036$ $p = 0.964$	$F_{(2,48)} = 0.964$ $p = 0.389$	$F_{(2,48)} = 2.309$ $p = 0.110$
Total glutathione	$F_{(1,48)} = 6.572$ $p < 0.05$	$F_{(1,48)} = 0.740$ $p = 0.394$	$F_{(2,48)} = 14.17$ $p < 0.001$	$F_{(1,48)} = 7.350$ $p < 0.01$	$F_{(2,48)} = 2.090$ $p = 0.135$	$F_{(2,48)} = 1.618$ $p = 0.210$	$F_{(2,48)} = 5.185$ $p < 0.01$
TOSC ROO•	$F_{(1,48)} = 7.529$ $p < 0.01$	$F_{(1,48)} = 39.46$ $p < 0.001$	$F_{(2,48)} = 3.903$ $p < 0.05$	$F_{(1,48)} = 23.94$ $p < 0.001$	$F_{(2,48)} = 7.692$ $p < 0.01$	$F_{(2,48)} = 33.07$ $p < 0.001$	$F_{(2,48)} = 17.27$ $p < 0.001$
TOSC HO•	$F_{(1,48)} = 8.07$ $p < 0.01$	$F_{(1,48)} = 1.789$ $p = 0.187$	$F_{(2,48)} = 9.645$ $p < 0.001$	$F_{(1,48)} = 0.046$ $p = 0.831$	$F_{(2,48)} = 3.342$ $p < 0.05$	$F_{(2,48)} = 15.04$ $p < 0.001$	$F_{(2,48)} = 3.434$ $p < 0.05$
TOSC ONOO-	$F_{(1,48)} = 1.87$ $p = 0.178$	$F_{(1,48)} = 0.488$ $p = 0.488$	$F_{(2,48)} = 14.72$ $p < 0.001$	$F_{(1,48)} = 28.20$ $p < 0.001$	$F_{(2,48)} = 6.288$ $p < 0.01$	$F_{(2,48)} = 5.349$ $p < 0.01$	$F_{(2,48)} = 7.001$ $p < 0.01$
Acyl-CoA oxidase activity	$F_{(1,48)} = 1.056$ $p = 0.309$	$F_{(1,48)} = 28.49$ $p < 0.001$	$F_{(2,48)} = 6.621$ $p < 0.01$	$F_{(1,48)} = 0.159$ $p = 0.692$	$F_{(2,48)} = 0.705$ $p = 0.499$	$F_{(2,48)} = 1.228$ $p = 0.302$	$F_{(2,48)} = 0.255$ $p = 0.776$
Neutral Lipids	$F_{(1,48)} = 56.51$ $p < 0.001$	$F_{(1,48)} = 16.45$ $p < 0.001$	$F_{(2,48)} = 0.266$ $p = 0.768$	$F_{(1,48)} = 39.79$ $p < 0.001$	$F_{(2,48)} = 19.24$ $p < 0.001$	$F_{(2,48)} = 13.24$ $p < 0.001$	$F_{(2,48)} = 3.277$ $p < 0.05$
Malondialdehyde	$F_{(1,48)} = 0.004$ $p = 0.947$	$F_{(1,48)} = 0.397$ $p = 0.532$	$F_{(2,48)} = 0.679$ $p = 0.512$	$F_{(1,48)} = 0.530$ $p = 0.399$	$F_{(2,48)} = 3.521$ $p < 0.05$	$F_{(2,48)} = 7.601$ $p < 0.01$	$F_{(2,48)} = 0.232$ $p = 0.794$
Lipofuscin	$F_{(1,48)} = 82.17$ $p < 0.001$	$F_{(1,48)} = 24.29$ $p < 0.001$	$F_{(2,48)} = 62.96$ $p < 0.001$	$F_{(1,48)} = 35.89$ $p < 0.001$	$F_{(2,48)} = 42.88$ $p < 0.001$	$F_{(2,48)} = 1.480$ $p = 0.238$	$F_{(2,48)} = 9.439$ $p < 0.001$
DNA fragmentation	$F_{(1,48)} = 2.322$ $p = 0.134$	$F_{(1,48)} = 1.576$ $p = 0.215$	$F_{(2,48)} = 21.93$ $p < 0.001$	$F_{(1,48)} = 0.440$ $p = 0.511$	$F_{(2,48)} = 5.786$ $p < 0.01$	$F_{(2,48)} = 10.21$ $p < 0.001$	$F_{(2,48)} = 2.270$ $p = 0.114$
Micronuclei	$F_{(1,48)} = 41.69$ $p < 0.001$	$F_{(1,48)} = 12.48$ $p < 0.001$	$F_{(2,48)} = 6.240$ $p < 0.01$	$F_{(1,48)} = 12.48$ $p < 0.001$	$F_{(2,48)} = 6.240$ $p < 0.01$	$F_{(2,48)} = 7.926$ $p < 0.01$	$F_{(2,48)} = 7.926$ $p < 0.01$