Assessment of the impact of aquaculture facilities on transplanted mussels (*Mytilus galloprovincialis*): Integrating plasticizers and physiological analyses as a biomonitoring strategy

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Abstract

The growing plastic production and its continuous use is a significant problem. In addition, aquaculture practices have experienced a considerable growth and plastic is widely used in these activities, hence plasticizers must be considered due to their potential ecotoxicological impacts on species. Mussels placed inside an Integrated Multi-Trophic Aquaculture (IMTA) system and at two control locations were employed to quantify the ingestion of anthropogenic particles and associated chemical plasticizers, such as bisphenol A (BPA) jointly to bisphenol F (BPF) and bisphenol S (BPS), and phthalates represented by diethyl phthalate (DEP), dibutyl phthalate (DBP) and bis(2-ethylhexyl) phthalate (DEHP). In addition, some metabolism and oxidative stress related parameters were measured in mussels' whole soft tissue. Anthropogenic particle ingestion of mussels increased over time at the three locations and the following order of abundance of pollutants was observed: BPA> BPF> DEHP> DBP> BPS> DEP. Even though no differences according to location were found for pollutants' occurrence, time trends were evidenced for BPA and DEHP. On the other hand, a location effect was observed for biomarkers with highest values detected in mussels located at the vicinities of the aquaculture facility. In addition, a reduced detoxification activity was observed over time parallel to BPA decrease.

Key words: sentinel species; chemical additives; anthropogenic particles ingestion; microplastics; biomarkers

1. INTRODUCTION

Worldwide aquaculture has rapidly expanded in recent decades, experiencing a continuous growth and reaching a global production of 82.1 million tons of commercial fish in 2018 (FAO, 2020). Impacts associated with aquaculture practices are diverse and include the release of waste derived from plastics (Wu et al., 2020). In particular, gears such as tanks, fishing nets, buoyant material, ropes and cable ties are necessary materials in aquaculture facilities (Krüger et al., 2020) and these are mainly made up of plastic polymers such as polyethylene (PE) (nets, buoyant materials), polypropylene (PP) (fibers in ropes and fishing nets), high density polyethylene (HDPE) or polyvinyl chloride (PVC) (tanks) (Kumar and Karnatak, 2014; Oxvig and Hansen, 2007; Park et al., 2016). Despite that aquaculture has not been identified as a major contributor of plastic debris (Schoof and DeNike, 2017), it is estimated that up to 41,000 tons of debris can be released from this activity into the marine environment every year (Sherrington et al., 2016). Consequently, the presence of debris in the marine environment can affect biota through entanglement with lost anchors, lines and nets, as well as through ingestion (Angiolillo and Fortibuoni, 2020; Consoli et al., 2019). In this sense, higher number of anthropogenic particles, understood as a broad category of particles produced directly or indirectly by human activities such as plastics, dyed particles or textile fibers (Collard et al., 2018) have been reported to be ingested by farmed mussels (Davidson and Dudas, 2016; Li et al., 2018; Mathalon and Hill, 2014). In addition, it is known that chemicals associated with plastic polymers can generate oxidative stress and inflammatory processes in species located in aquaculture facilities (Capo et al., 2021; Rios-Fuster et al., 2021), corroborating the harmful effects of plastics derived from these practices.

Several chemicals belonging to the category of phthalates and bisphenols have been intensively used in the manufacture and processing of plastic products such as plasticizers, with a wide range of industrial applications (Beltifa et al., 2017; Thompson et al., 2009). The occurrence of low molecular weight phthalates like diethyl phthalate (DEP) and dibutyl phthalate (DBP) in the marine environment can be attributed to their wide use in plastic bags but also in

pharmaceuticals and personal care products; whereas high molecular weight phthalates, like bis(2-ethylhexyl) phthalate (DEHP), are used in flexible PVC daily use products like food packaging and home furnishings amongst others (Net et al., 2015; Wormuth et al., 2006). Regarding bisphenols' family, bisphenol A (BPA), is widely used in food packaging as protective coating inside food cans, but also in thermal papers and in dentistry materials (Beltifa et al., 2017). In addition, manufacturers have begun to replace BPA from their products with bisphenol analogs such as bisphenol F (BPF) and bisphenol S (BPS) (Rochester and Bolden, 2015). Given that these chemicals are not covalently bounded to plastic polymers they are easily released to the environment (Net et al., 2015; Rochester and Bolden, 2015) and can act as endocrine disruptors (Fossi et al., 2006; Rochester and Bolden, 2015) causing immunotoxicity, neurotoxicity and oxidative stress disorders in marine species, including bivalves, exposed to this type of pollution (Jang et al., 2020; Seoane et al., 2021; Tang et al., 2020).

Organisms dispose of detoxification mechanisms to alleviate the consequences of exposure to toxic compounds. During detoxification process, enzymes as carboxylesterases (CEs) convert toxic compounds into more hydrophilic and more reactive molecules to facilitate their elimination. In a second step, glutathione transferase (GST) conjugate xenobiotics metabolites with glutathione in order to convert them into more hydrophilic and less reactive molecules (Falfushynska et al., 2019; Uno et al., 2012). During the detoxifying process, Reactive Oxygen Species (ROS) can be produced and in front of this, organisms have developed a complex antioxidant system to avoid oxidative stress damage (Livingstone, 2001). The antioxidant system is composed by enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GRd) (Capo et al., 2021; Capó et al., 2015; Regoli and Giuliani, 2014). However, if the ROS production is over the organisms elimination capabilities, ROS can damage several biomolecules such as lipids generating oxidative products as malondialdehyde (MDA) that can be used as biomarkers of oxidative damage (Bartoskova et al., 2013; Ding et al., 2018).

Mediterranean mussels (*Mytilus galloprovincialis*) have been widely used as a bioindicator species and considered sentinel organisms in coastal contamination and environmental pollution monitoring programs (Li et al., 2019; Vidal-Liñán et al., 2010; Zorita et al., 2007). This species tends to bioaccumulate anthropogenic particles and many persistent contaminants at higher concentrations than those found in the surrounding water (Beiras et al., 2003; Isobe et al., 2007). For these reasons, the present study aims to evaluate the exposure of *Mytilus galloprovincialis*, an important commercial filter feeder species and a potential bioindicator of pollution by plasticizers, to pollution in aquaculture facilities. The ingestion of anthropogenic particles in mussels was quantified as well as the concentration of phthalates (DEP, DBP and DEHP) and bisphenols (BPS, BPF and BPA) and the condition index (CI) was determined as a measure of the nutritional and general health status of mussels. In order to evaluate the biochemical response of mussels towards anthropogenic particle ingestion and associated contaminants detoxification enzymes activities (CEs, GST) and metabolic (ETS, GLY), antioxidant system (CAT, SOD, GRd, GPx, GSH) and oxidative stress damage (LPO, CARB) biomarkers were measured.

2. MATERIAL AND METHODS

2.1. Study area

In order to determine the concentrations of phthalates and bisphenols in mussels, as well as assessing the presence of anthropogenic particle ingestion, wild mussels were deployed in shallow coastal waters of Port d'Andratx, Mallorca (Fig. 1). Specifically, three different locations were selected: a) an impacted location by an Integrated Multi-Trophic Aquaculture (IMTA) system (aquaculture), b) a control site of the aquaculture facility at the entrance of Port d'Andratx (control 1) and c) a reference non-impacted location at a distance of 2 km outside of Port d'Andratx (control 2) (Fig. 1).

Mussels used for the study were acquired from an aquaculture farm located at the Port of Mahón (Menorca). Prior to the study and the deployment of the mussels at the different study locations,

111 mussels went through a depuration period of 5 days in large tanks filled with seawater. During
112 these 5 days, mussels were not fed. Tanks were located inside the experimental facilities of
113 LIMIA (*Laboratorio de Investigaciones Marinas y Acuicultura*) and protected from climate and
114 weather conditions, ensuring the stability of physical and chemical conditions of the seawater.
115 After this depuration period, mussels were placed inside anti-predator nets and deployed at
116 approximately 5 meters from the seafloor at the three study locations. In the aquaculture
117 location, mussel nets were hanged from the aquaculture facilities. For both controls, mussels'
118 nets were moored with a cotton rope to the seabed and in the other extreme a buoy was placed
119 to maintain the neutral floatability of mussel cages. To assess the ingestion of anthropogenic
120 particles and the bioaccumulation of phthalates and bisphenols along with the associated
121 biomarker responses, the study was carried out at the above mentioned three locations during
122 three sampling periods: at the start of the study, before the deployed of mussels at the study
123 locations (T0), and after 60 days (T60) and 120 days (T120) of the deployment of mussels at the
124 selected locations. The experiment was carried out during summer months (20/05/2019 to
125 25/09/2019) with mean seawater temperatures ranging from 18 to 26 ºC as indicated in Table 1.

126 *2.2. Sample collection*

127 Before the deployment of the mussels at the different locations (T0) 10 mussels were analyzed
128 to determine initial anthropogenic particle ingestion, 7 individuals to assess initial phthalates
129 and bisphenols levels and 30 individuals to evaluate the initial values of biochemical markers.
130 At each location and for each sampling period (T60 and T120), the following samples were
131 analyzed: 10 mussels for the evaluation of the ingestion of anthropogenic particles, 5 mussels
132 for the determination of phthalates and bisphenols, and for biomarkers, 30 mussels were taken
133 from aquaculture facilities jointly to 10 mussels from each control location. Consequently, a
134 total of 237 mussels were analyzed: 70 were used for anthropogenic particle ingestion detection,
135 37 individuals for phthalates and bisphenols occurrence and 130 specimens for biomarker
136 determinations.

2.3. Biological parameters

Length and width of the shell were measured to the nearest mm for each organism, and the total weight (shell and soft tissue) and the fresh weight of the soft tissue were recorded to the nearest g. The Condition Index (CI) was calculated for all individuals sampled during the study. The formula applied was:

- Condition Index (CI) = (fresh weight of the soft tissue / total weight) × 100.

2.4. Anthropogenic particle ingestion

For the study of anthropogenic particle ingestion the whole soft tissue of each mussel was stored at -20 ºC until posterior analyses at the laboratory. For anthropogenic particle isolation and identification, samples were subjected to a chemical digestion of organic matter using potassium hydroxide (KOH 10 %) applying the method described by Duflos et al. (2017). After digestion, the remaining solutions were filtered through a vacuum filtration tramp using polycarbonate filters (i.e. FILTER-LAB Polycarbonate membrane filters, pore size 20.0 µm, diameter 47 mm) and filters were transferred to glass Petri dishes for the posterior visual sorting of anthropogenic particles under the stereomicroscope. To prevent contamination, all instruments were previously rinsed with 96% alcohol before each analysis.

2.5. Analytical methods for phthalates and bisphenols quantification

2.5.1. Reagents and materials

Commercial analysis standards bisphenol A (BPA, purity ≥ 99.9%), bisphenol F (BPF, purity ≥ 98%), bisphenol S (BPS, purity ≥ 98%), diethyl phthalate (DEP, purity ≥ 99%), dibutyl phthalate (DBP, purity ≥ 99%), bis(2-ethylhexyl) phthalate (DEHP, purity ≥ 99.5%)) were supplied by Sigma-Aldrich (Madrid, Spain). High Performance Liquid Chromatography (HPLC) grade organic solvents (acetonitrile and methanol) were purchased from Scharlab (Barcelona, Spain). Analytical grade formic acid was purchased in Sigma Aldrich (Madrid,

Spain). Ultrapure water (18 MΩ/cm) used for the preparation of all aqueous solutions was obtained using a Milli-Q water system (Millipore Ibérica, Madrid, Spain).

Stock standard solutions of individual compounds at concentrations of 1000 mg/L for BPA, BPS and phthalates (DBP, DEP and DEHP); and 100 mg L^{-1} for BPS, were prepared by exact weight of these compounds and dissolved in methanol and then stored in darkness at 4 °C until use. In these conditions, all solutions remained stable for at least three months. The working standard solution at desired concentration of each analyte was daily prepared by appropriate dilution of the mentioned solutions with the dilution mixture methanol/water (85:15, v/v).

Florisil (< 200 mesh) from Sigma-Aldrich (Madrid, Spain), sodium sulfate anhydrous (Na_2SO_4, purity ≥ 99.9 %) from Panreac (Barcelona, Spain) and washed sea sand (0.25-0.30 mm) from Symta (Madrid, Spain) were used as solid phase materials for matrix-solid phase dispersion (MSPD). The glass wool silanized used was supplied by Panreac (Barcelona, Spain).

2.5.2. Mussel sample preparation for matrix-solid phase dispersion (MSPD) extraction

For sample preparation, mussels were dissected with a clean scalpel blade to separate the soft tissue from the shell. The whole raw material, previously drained, was ground, homogenized, and stored at 4 °C prior to the chromatographic analyses. Precautionary measures were taken to avoid the contamination of samples during the collection, transportation and sample preparation.

2.5.3. Matrix-solid phase dispersion (MSPD) procedure

The analytes under consideration were extracted from the biomatrix by performing MSPD extraction procedure. An amount of 0.1 g of homogenized raw mussels was poured into a glass mortar containing 0.5 g of dispersing sorbent Florisil, 0.5 g of sodium sulfate as anhydrous agent and 0.2 g of washed sea sand and the simultaneous extraction of all analytes was carried out following the MSPD method described in a previous work (Cañadas et al., 2021). Additionally, a solvent blank was prepared to check for background contamination due to the use of plastic laboratory material.

186 *2.5.4. Chromatography analyses*

187 Analyses were performed using an HPLC-MS system composed of a HPLC model 1200 series
188 (Agilent Technologies, Germany) equipped with a diode array spectrophotometric detector
189 (DAD), a quaternary pump, a thermostatted column compartment and autosampler controlled by
190 HP Chemstation software of Agilent technologies. It is coupled to a 6110 simple quadrupole
191 mass spectrometer (Agilent), using an electrospray ionization (ESI) interface. The analytical
192 column was an ACE 5 C18-PFP HPLC column (150 × 4.6 mm, 5 μm) from Symta (Madrid,
193 Spain). Chromatographic analyses were provided by applying an elution gradient using as
194 mobile phase ultrapure water Milli-Q as component A and acetonitrile as component B. The
195 composition of eluent varied from 45 to 80% B in 30 min, 80-100% B from 30 to 31 min and
196 100% B for 9 min, the flow rate was kept at 0.8 mL min^{-1}. Subsequently, the column was
197 equilibrated for 10 min with 45% B isocratic for 10 min at the same flow rate. Column
198 temperature was kept constant at 20 ºC and the injection volume was 40 μL. The quantification
199 of the analytes was performed using external calibration and peak area measurements, selecting
200 as optimum wavelength for all them 210 nm.

201 For HPLC-ESI-MS analysis, the experiments were carried out in the positive ion mode for
202 phthalates, while bisphenols were detected in negative ion mode. The LC flow rate was 0.8 mL
203 min^{-1}. The operating conditions for the ESI interface were as follows: positive and negative
204 ionization modes for phthalates and bisphenols, respectively; temperature of the capillary, 350◦
205 C; capillary voltage, 5000 V and sheath gas (N_2) flow, 11 L min^{-1}.

206 *2.6. Biomarkers analysis*

207 For biomarkers analysis, the parameters measured were: metabolic biomarkers [electronic
208 transport System activity (ETS) and glycogen reserves (GLY)], antioxidant enzymes [catalase
209 (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase
210 (GRd)], detoxifying enzymes [carboxilesterase activity (CE) and glutathione-*S*-Transferases
211 activity (GSTs)], and oxidative damage markers [lipid peroxidation (LPO) and Protein Carbonyl

derived levels (CARB)]. Reduced glutathione levels (GSH) due to its antioxidant properties, was also included. All mussel soft tissues were homogenised individually (10 mussels per location) with a specific extraction buffer (in a proportion of 1:2 w/v) for each biochemical parameter: for ETS activity quantification, supernatants were extracted in homogenizing buffer [0.1 M Tris-HCl pH 8.5 with 15% (w/v) PVP, 153 µM magnesium sulphate (MgSO4) and 0.2% (v/v) Triton X-100]; for LPO determination supernatants were extracted using 20 % (w/v) trichloroacetic acid (TCA); for SOD, CAT, GSTs, CEs, GPx, GRd, CARB and GLY assays supernatants were extracted in potassium phosphate buffer [50 mM potassium phosphate; 1 mM ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1 mM dithiothreitol (DTT); pH 7.0]; for GSH concentrations were determined in supernatants extracted with KPE buffer [0.1 M potassium phosphate; 5 mM ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA) with 0.1 % (v/v) Triton X-100 and 0.6% (w/v) sulfosalicylic acid; pH 7.5] (Andrade et al., 2018; Lompré et al., 2021). After the addition of the specific buffers, samples were submitted to 90 s of high-speed shaking in a tissue-lyser (TissueLyser II, Qiagen) and afterwards to 20 min of centrifugation at 10000 *g* or 3000 *g* (depending on the biomarker) at 4 °C. Also depending on the biomarker, the supernatant was then stored at − 80 °C or immediately used. Two replicates per individual (i.e., per supernatant) were used to determine each biochemical parameter.

2.6.1. Metabolic capacity and energy reserves content

Electronic transport System (ETS) was measured following the method described by King and Packard (1975), with some modifications. Absorbance was measured during 10 min at 490 nm with intervals of 25 s. Molar extinction coefficient of 15.9 $mM^{-1}cm^{-1}$ was used to calculate formazan produced. Results were expressed in µkat per g of fresh weight (FW).

Glycogen levels (GLY) were measured applying the method described by DuBois et al. (1956) using glucose standards (0-2 mg/L). Absorbance was measured at 492 nm after an incubation during 30 min at room temperature. Results were expressed in mg per g FW.

238 *2.6.2. Detoxifying enzyme activities*

239 Carboxylesterases (CEs) were measured using 2 different commercial substrates: ρ-nitrophenyl
240 acetate (ρNPA) and ρ-nitrophenyl butyrate (ρNPB). Activity was recorded
241 spectrophotometrically at 405 nm for 5 min as the formation of ρ-nitrophenol from ρNPA and
242 ρNPB as described by Hosokawa and Satoh (2002). Activities were expressed in µkat per g of
243 FW. In the present study values of CEs are reported as those corresponding to the activity with
244 ρNPB due to the high correlation between them.

245 Activity of GSTs was quantified following Habig et al. (1974) protocol with some
246 modifications. GST activity was measured spectrophotometrically at 340 nm (ε = 9.6 mM^{-1}
247 cm^{-1}). The enzymatic activity was expressed in µkat per g of FW.

248 *2.6.3. Antioxidant enzyme activities*

249 The activity of CAT was quantified following the method describe by Johansson and Borg
250 (1988). Absorbance was measured at 540 nm. Formaldehyde standards (0-150 µM) were used to
251 perform the standard curve. Results were expressed in µkat per g of FW.

252 Regarding SOD, its activity was quantified based on the method previously described by
253 Beauchamp and Fridovich (1971). Absorbance was measured at 560 nm. SOD standards (0.25-
254 60 U/mL) were used to generate a standard curve. Results were expressed in µkat per g of FW.

255 Activity of GRd was determined using the method described by Carlberg and Mannervik
256 (1985). The absorbance was measured at 340 nm and the enzymatic activity was determined
257 using ε = 6.22 mM^{-1} cm^{-1}. The results were expressed as µkat per g of FW.

258 The activity of GPx was determined following the method described by Paglia and Valentine,
259 (1967). Absorbance was measured at 340 nm (ε =6.22 mM^{-1} cm^{-1}) and results were expressed
260 in µkat per g of FW.

Levels of GSH were determined following the method previously described Rahman et al. (2016). Absorbance was measured at 412 nm and the results were expressed in μmol per g of FW.

2.6.4. Oxidative damage

Levels of LPO were measured according to the method described by Carregosa et al. (2014). Absorbance was measured at 535 nm (ε = 156 mM^{-1} cm^{-1}) and the results were expressed in nmol of MDA equivalents formed per g of FW.

The quantification of carbonyl protein levels (CARB) levels followed the DNPH alkaline method described by Mesquita et al. (2014). Absorbance was measured at 450 nm (ε = 22.308 mM^{-1} cm^{-1}) and the results were expressed in nmol of protein carbonyl groups formed per g of FW.

2.7. *Statistical analyses*

In order to study the concentration of phthalates and bisphenols in whole soft tissue of *M. galloprovincialis* as indicators of plastic pollution in the marine environment and anthropogenic particle ingestion, six different plastic additives belonging to the categories of phthalates and bisphenols and a total of ten different oxidative markers were analyzed. Before the analyses, the dataset was checked for normal distribution (Shapiro-Wilk Test) and homogeneity of variances (Levene's test). A series of two-way ANOVA with interaction effect between sampling locations (fix factor and 3 levels: aquaculture, control 1 and control 2) and sampling period (fix factor and 3 levels: T0, T60 and T120) were performed for condition index, fresh weight of the soft tissue, anthropogenic particle ingestion, phthalates, bisphenols, and oxidative stress and metabolic biomarkers, separately. Following, a Tukey's multicomparison post-hoc test was performed to discriminate statistically significant locations or sampling periods when ANOVA results indicated significant differences amongst them.

In addition, a series of Pearson' correlations were carried out between levels of phthalates and bisphenols with the condition index, the number of anthropogenic particles ingested and with each of the biochemical biomarkers assessed. Given that Pearson's correlation requires an equal number of samples between variables, a random number of individuals were selected when this was necessary.

For both the ANOVA and posterior post-hoc test as well as for the correlations, a $p < 0.05$ was considered as significant. All analyzes were conducted using R studio version 3.4.0.

3. RESULTS

3.1. Biological parameters

The soft tissue weight showed a decreased trend throughout the study. Values related to the weight of the soft tissue of the mussels range from 6.55 ± 1.70 g in T0 to a general mean value of 5.11 ± 1.60 g in T120. In addition, mussels from control 2 with a mean value of 4.41 ± 0.97 g in T60 and 4.76 ± 1.31 g in T120 have a statistical lower weight than mussels from aquaculture facilities with a mean value of 6.31 ± 1.30 g in T60 and 5.53 ± 1.89 g in T120 (Fig. 2).

According to the Condition Index (CI), in this study mussels showed a similar trend than the soft tissue weight with statistically higher values at T0 (29.58 ± 7.66) and lowest values at T120 ranging from 21.30 ± 6.45 in mussels from aquaculture facilities to 19.99 ± 3.47 in mussels from control 2 ($p < 0.001$; Fig. 2). CI was not statistically different among locations ($p > 0.05$) and CI was significantly positively correlated with ETS, CE, GSTs, CAT and GSH ($p < 0.05$; Table 6).

3.2. Anthropogenic particle ingestion

From a total of 70 mussels analyzed, 529 anthropogenic particles were identified with a mean value (± SD) of 7.56 ± 10.09 particles per individual. The maximum number of particles was 57 in one individual and only two individuals had no items. Anthropogenic particles were found at all sampling locations and periods (Table 2) being statistically higher at T120 than in T60 (Two-way ANOVA, $p < 0.05$) without statistically differences between locations. No correlation was

observed between anthropogenic particles ingestion and the condition index ($p > 0.05$; Table 6). On the other hand, a negative correlation was found between anthropogenic particles ingestion and BPA (Pearson's correlation, $p < 0.05$; Table 6) but a positive correlation was observed with several biomarkers such as ETS, CE, GST, CAT and GSH ($p < 0.05$; Table 6).

Most of the anthropogenic particles found in this study were identified as fibers (846 particles), followed by fragments (23 particles) and finally, only two films were identified (Table 2). Regarding size, most of the anthropogenic particles had a diameter smaller than 2 millimeters (603 particles; 69.2 %) and a total of 45 particles (5.2 %) had a diameter larger than 5 millimeters (Table 3). Regarding color, transparent was the most predominant color with a total of 649 of the particles identified as such (74.5 %), followed by black (65 particles; 7.5 %), red (63 particles; 7.2 %) and blue (58 particles; 6.7 %; Table 3).

3.3. Phthalates and bisphenols levels

The results obtained in the quantification of mussel samples for plasticizers determination showed that mean levels of phthalates and bisphenols differed between the contaminants assessed. The order of abundance of contaminants in mussels was BPA> BPF> DEHP> DBP> BPS> DEP. All bisphenols were detected in all individual mussels, but not all phthalates. DEP could be detected in 36 samples (92.30 %), DBP in 35 samples (94.59 %) while DEHP only in 20 samples (54.05 %).

In general, regarding the content of bisphenols, no differences were found among the different locations. The concentrations of BPA at the beginning of the experiment (T0) and at a time of 60 days (T60) in aquaculture facilities' mussels were similar, with mean values of 8.14 ± 1.35 μg/g and 8.05 ± 1.05 μg/g respectively. However, after 120 days of the beginning of the study (T120) the concentration of BPA detected was significantly lower, between 6.51 ± 2.91 μg/g in aquaculture mussels and between 4.95 ± 1.53 μg/g in control 2 mussels ($p < 0.05$; Table 4-5). Regarding BPS and BPF, no differences were found between locations ($p > 0.05$; Table 5). However, in general, the BPF content was of the order of 10 times higher than that of BPS at all

337 sampling locations. Mussels from control 1 showed a maximum value of BPS and BPF at T120
338 of 0.41 ± 0.76 μg/g. and 2.13 ± 1.27 μg/g respectively. On the other hand, only levels of BPA
339 were significantly and positively correlated with the condition index and also with ETS, GSH,
340 LPO and CARB but BPS was negatively correlated with GLY and CE ($p < 0.05$; Table 6).

341 The maximum value of DEP was observed in mussels located at the control 2 at T120 with
342 mean values of 0.67 ± 1.04 μg/g (Table 4). However, no differences among locations or
343 sampling periods were found for this contaminant ($p > 0.05$; Table 5). Similarly, for DBP values
344 no differences were found among locations nor sampling periods ($p > 0.05$; Table 5). DBP
345 concentrations were found in the order of 0.94 ± 1.45 μg/g at the beginning of the experiment
346 (T0) and 0.83 ± 0.15, 0.91 ± 0.35 and 1.27 ± 1.39 μg/g at T120 in mussels located in
347 aquaculture facilities, control 1 and control 2, respectively. Finally, DEHP values at T60 ranged
348 from 1.16 ± 2.59 to 4.06 ± 0.82 μg/g being significantly higher at the beginning of the study
349 (T0) with a mean value of 0.23 ± 0.61 μg/g ($p < 0.05$; Table 5). No differences in mussels from
350 aquaculture facilities and both control locations were found for DEHP despite interaction
351 between locations and sampling periods was found. This statistical interaction was a
352 consequence of the increase of DEHP from T60 to T120 in mussels located at the aquaculture
353 facilities and of the simultaneous decrease in both control locations (Table 4). According to the
354 relation between the different plasticizers, only DBP was significantly negatively correlated
355 with CARB ($p < 0.05$; Table 6).

356 *3.4. Biomarkers levels*

357 *3.4.1. Metabolic capacity and energy reserves*

358 ETS and GLY as metabolism-related biomarkers are present in Fig. 3. Significantly higher
359 levels of both biomarkers were found in mussels from T0 in comparison with mussels of T120
360 ($p < 0.0001$), and from mussels from aquaculture in T60 with mussels of T120 from the three
361 locations ($p < 0.0001$). Nevertheless, no significant differences were found between mussels in

362 T0 with mussels in T60. In addition, the three locations display similar levels of these
363 biomarkers and no differences between locations were found in T60 neither T120 ($p > 0.05$).

364 On the other hand, a statistical positive correlation between ETS and GLY was observed with
365 almost all the other biomarkers assessed, and negatively only with LPO ($p < 0.05$; Table 6). The
366 strongest correlation was observed between ETS and GLY ($p < 0.05$, rho = 64; Table 6).

367 *3.4.2. Detoxifying enzyme activities*

368 Activities of CEs are presented in Fig. 3. Mussels from T0 presented higher CEs levels than
369 mussels in T60 from control 1 and aquaculture facilities locations ($p < 0.01$), but not with
370 mussels from control 2 in T60. Nevertheless, mussels from T0 and from T60 had statistical
371 higher values than mussels from the three locations in T120 ($p < 0.0001$). No statistical
372 differences were found between locations in T60 or T120 ($p > 0.05$).

373 The activity of GSTs as the marker of detoxification process is presented in Fig. 3. Mussels
374 from T60 presented lower GSTs activity than mussels from T0 ($p < 0.001$; Fig. 3). In addition,
375 at T60 GSTs activity in mussels from the aquaculture site was significantly higher than mussels
376 from control 1 ($p < 0.05$), but no difference with control 2 were found. Similarity, mussels from
377 T120 showed a significantly lower GSTs activity than mussels from T0 and T60 in the three
378 sampling locations ($p < 0.001$). Mussels from control 2 at T120 showed a significantly lower
379 activity than mussels from the aquaculture cage at T120 ($p < 0.01$). On the other hand, both
380 detoxifying enzyme activities (CEs and GSTs) were correlated with CAT, but only GSTs was
381 also correlated with GRd, GPx and GSH ($p < 0.05$; Table 6).

382 *3.4.3. Antioxidant enzyme activities*

383 Fig. 4 represents the evolution of antioxidant enzymes along the study in different sampling
384 locations. CAT and GRd enzymes presented a significantly higher activity at the beginning of
385 the study ($p < 0.001$). However, a progressive decrease in the activity of both enzymes was
386 observed throughout the study in the three locations (Fig. 4). SOD activity presented

387 significantly higher activity in mussels from the beginning of the study (T0; 0.12 ± 0.05 µkat/g
388 FW) and aquaculture facilities at T60: 0.14 ± 0.04 µkat/g FW, in relation to both control
389 locations at T60, with 0.06 ± 0.01 and 0.05 ± 0.01 µkat/g FW in control 1 and control 2,
390 respectively; and was also significantly higher than the three locations at T120 where values
391 ranged from 0.08 ± 0.04 to 0.06 ± 0.02 µkat/g FW. GPx showed a similar response, with
392 significantly higher GPx activity in mussels from the aquaculture location at T60 in relation to
393 T0. Nevertheless, no differences between T0 and both control locations (control 1 and control 2)
394 were observed at T60. In addition, significantly lower values were found in mussels from
395 control 1 at T120 with a mean value of 0.002 ± 0.00 µkat/g FW.

396 GSH, as non-enzymatic antioxidant molecule, was measured and values are presented in Fig. 4.
397 No significant differences were observed between mussels from the beginning of the study (T0)
398 and mussels from T60. However, a significant decrease in GSH levels was detected in mussels
399 from T120 in comparison to mussels from T0 and T60 ($p < 0.0001$; Fig. 4). In addition, GSH
400 levels in mussels at T120 from aquaculture were of 1247 ± 325 µmol/g FW and were
401 significantly higher than mussels from control 1 that showed a mean value of 1021 ± 322
402 µmol/g FW ($p < 0.05$). Statistical positive correlations between different biomarkers were
403 observed ($p < 0.05$; Table 6).

404 *3.4.4. Oxidative damage*

405 Biomarkers of oxidative stress damage as LPO and CARB derivates are presented in Fig. 5.
406 Significantly higher levels of LPO were found in mussels from the aquaculture facilities at T60
407 and T120 ($p < 0.001$). However, no effects of sampling location or period were observed on
408 mussels from control 1 and control 2 locations. CARB levels were modulated by both time and
409 location. No differences in CARB were found between T0 and T60 samples. However, samples
410 from T120 showed significantly lower CARB levels than samples from T0 and T60 ($p < 0.001$).
411 In addition, samples from control 1 and control 2 at T120 showed significantly lower CARB
412 levels than samples from aquaculture at T120 ($p < 0.01$).

4. DISCUSSION

As far as we know, there are no studies including the ingestion of anthropogenic particles in the Mediterranean mussel, *Mytilus galloprovincialis*, the bioaccumulation of selected environmental bisphenols and phthalates, and the associated metabolic and oxidative stress responses. This particular study was conducted taking into consideration the proximity to aquaculture facilities and over a four month period. This novel, comprehensive and complementary approach revealed that the intake of anthropogenic particles increased over time, as well as the concentrations of DEHP in mussels. This increase was concomitant to a decrease of BPA, to the mussels' fitness reflected by the CI and to biochemical biomarkers activities. Nonetheless, less clear differences were observed among locations.

4.1. Fresh weight of the soft tissue and Condition index

The Condition Index (CI) decreased through the four months of the study at the three locations and was positively correlated to BPA levels; meanwhile the correlation with the other pollutants assessed in general is negative and negligible. This result may seem contradictory as mussels from polluted areas displayed a lower CI than individuals from a reference site (Pampanin et al., 2005). In addition, individuals from control 2 had a high decrease in the fresh weight after two months of the experiment. Other external factors such as the oligotrophy of the waters of the study area (D'Ortenzio and D'Alcalà, 2009) at the time of the deployment and that this species is not naturally found in this area of the Mediterranean Sea may account for the reduced CI index.

4.2. Anthropogenic particles ingestion

The number of anthropogenic particles found in mussels increased during the experiment, being more than double at the end of the experiment but with no differences between locations. Previous studies also found no site differences between the presence of ingested particles between cultured and wild clams (Davidson and Dudas, 2016). Moreover, fibers and particles with a size smaller than two millimeters appear to be the most predominant particles identified

in mussels from this study. Formerly, a strong correlation between anthropogenic particle levels in water and in mussels was described (Qu et al., 2018), hence we estimated that the presence of these anthropogenic particles in mussels are representative of its abundances in the surrounding water.

Unexpectedly, an absence of correlation between the presence of anthropogenic particles and levels of phthalates and bisphenols was observed, and a negative correlation with BPA. Similar to our results, a previous study did not find microplastics in the gastrointestinal tract of a commercial fish, the silver scabbard fish (*Lepidopus caudatus*), although chemical analysis revealed the presence of BPA, DEHP and other phthalates (Salvaggio et al., 2019). These results evidence that these lipophilic compounds are freely present in relatively high concentrations in marine waters and can be transferred to tissues as a consequence of biota's filtering activity (Gobas et al., 2003). In addition, the negative statistical correlation between the presence of anthropogenic particles and BPA could be suggesting that the particles identified are not the direct source of the assessed chemicals. A plausible explanation to justify the lack of relationship between anthropogenic particles and plasticizers could be that the increase of ingested particles corresponds to an acute entrance of suspended particles from land based origin due to tourism and have remained long enough in the environment facilitating the desorption of some chemicals. In fact, the anthropogenic discharges would be expected to increase in the summer period at vicinity of the harbour where the aquaculture facilities were located; whereas the plasticizer's presence would reflect chronic presence in the soluble and particulate water matrix. In this sense, these chemical additives are not covalently bound to the plastic polymers and are easily released from the particles into the environment (Fikarová et al., 2019; Net et al., 2015; Rochester and Bolden, 2015). As a result of the behavior of these chemicals in seawater, in the moment in which these particles are ingested the most probably is that plasticizers have already been released from the particles into the environment, hence plastic particles are expected to be a direct source of these chemicals only when these plastic particles are ingested shortly after being released into the sea.

On the other hand, the correlation between the ingested anthropogenic particles and several of the evaluated biomarkers belonging to the metabolic (ETS), detoxifying (CE and GST) and antioxidant system (CAT and GSH) suggests that the ingestion of anthropogenic particles activates the detoxification systems related to the presence of toxic compounds and highlights the consequences that the ingestion of inedible particles can trigger on mussels. The activation of the detoxification system was already reported in mussels exposed to different microplastics under controlled conditions (Avio et al., 2015; Hariharan et al., 2021; Paul-Pont et al., 2016).

4.3. Phthalates and bisphenols

4.3.1. Sampling location effect of phthalates and bisphenols

The lack of differences in terms of plasticizers bioaccumulation among locations suggests that the targeted aquaculture facilities are not an additional source for most of the analyzed additives. Nevertheless, the increase of DEHP in mussels located at the aquaculture facilities jointly to the decrease observed in both control locations throughout the experiment suggests that aquaculture facilities could be an additional source of this particular phthalate. In this sense, a previous study observed that DEHP was out of other 13 phthalates analyzed the dominant one at the surroundings of aquaculture fish ponds (Cheng et al., 2019).

Overall, values obtained in mussels from the present study were intermediate compared with the literature. In *M. galloprovincialis* from South and South East Asia recorded levels were as high as 0.0137 μg/L for BPA (Isobe et al., 2007). In *M. edulis*, BPA was found in concentrations from 0.22 to 1.8 ng/g WW (wet weight) (Vethaak, 2014). Fewer studies are available regarding the concentration of BPS and BPF in the environment, notwithstanding no study assessed their presence in mussels or other species.

With regard to phthalates, as observed in our study, no significant differences were either reported between mussels collected from farms and those from the open sea in the North Adriatic Sea (Cerkvenik et al., 2018). The literature shows that DEHP is the most frequently reported phthalate in the aquatic environment (Fromme et al., 2001). Moreover, in the present

492 study low values were obtained for DEP which are in concordance with those values found in
493 mussels collected in the Netherlands, with DEP values ranging from < 6.7 to 320 ng/g WW
494 (Vethaak, 2014). On the other hand, in this study we report values of DBP and DEHP higher
495 than mussels collected in Netherlands with DBP values ranging from < 0.7 to 150 ng/g WW and
496 DEHP from < 2.2 to 400 ng/g WW (Vethaak, 2014).

497 *4.3.2. Temporal effect of phthalates and bisphenols*

498 The pollutants assessed in this study are hydrophobic compounds; therefore, they are expected
499 to have a relatively high tendency to bioaccumulate in mussels. With respect to bisphenols,
500 BPA levels were found to be relatively higher only at the beginning of the study and then
501 decreased throughout the study period. This trend was observed at the three locations suggesting
502 that mussels were already exposed to an initial BPA contamination burden at the start of
503 experiment, highlighting the ubiquity of this chemical. Taking into account that the biological
504 half-life of BPA in the Mediterranean mussel is 26 days (Gatidou et al., 2010) the present
505 results show that individuals were undergoing a BPA detoxification during the study that is
506 supported by the activity and levels of the different biomarkers assessed. In this sense, several
507 enzymes such as carboxylesterases or other detoxification enzymes, convert xenobiotics
508 compounds into more soluble products to facilitate their elimination. However, in this process,
509 reactive ROS can also be formed as it was suggested by a positive correlation between LPO
510 levels and BPA concentration.

511 *4.4. Biochemical biomarkers*

512 The activation of antioxidant enzymes is closely associated with metabolic parameters
513 (increased ETS activity and expenditure of GLY), while, at the same time, the increase in the
514 ETS activity can induce an important increase in ROS production (Mazat et al., 2020)
515 explaining the high correlation reported between the metabolic, especially ETS, and the
516 antioxidant system biomarkers. On the other hand, antioxidant enzyme activities may avoid the
517 oxidative damage associated with the ROS production. In addition, the direct correlation

between antioxidant enzymes activities (CAT, SOD and GRd) and GSH levels could be explained by the fact that oxidative stress situation induces an activation of antioxidant mechanisms. Biotransformation enzymes (GSTs and CEs) are involved in pollutants detoxification, with the involvement of GPx in the case of GSTs. For this reason, responses of the biomarkers selected for this study can be correlated amongst them.

4.4.1. Sampling location effect on biochemical biomarkers

Even though the concentrations of phthalates and bisphenols in mussels' soft tissues did not show a sampling location trend, a particular aquaculture site effect was observed in some of the measured biomarkers such as CAT, SOD, GPx and LPO. The higher levels found in mussels from aquaculture facilities suggest that aquaculture facilities are a source of stressors that can trigger the antioxidant system and cause cellular damage. In addition to the damage caused by the presence of BPA and explained by the significant correlation reported between LPO and BPA in this study, problems associated to the aquaculture production are diverse. In this sense, it is also well established that aquaculture farms are an important source of organic material coming from the feed supplied to the cultivated species as well as their faeces. In addition, the elevated concentration of nutrients cannot easily be managed, as most is in dissolved form and released directly to the marine environment. The release of dissolved and particulate nutrients results in increasing nutrient loads, and changes in nutrient stoichiometry (Bouwman et al., 2013; Cao et al., 2007) affecting the individuals and activating the mussel's antioxidant system.

4.4.2. Temporal effect on biochemical biomarkers

The present study showed a clear reduction of the activities and levels on ETS, GLY, CAT, GRd, CEs and GST in the three samplings locations suggesting a general temporal effect. These results are surprising, because an increase in antioxidant and detoxifying enzymes as consequence of exposure to a polluted marine environment, with levels increasing with the summer period, was expected (Box et al., 2007; Regoli et al., 2004). Furthermore, an increase in

oxidative damage marker throughout the study period was also expected as it is described in previous studies (Box et al., 2007; Faggio et al., 2018; Sureda et al., 2011).

These results could be further explained if we analyzed together biomarkers and BPA mussels' content as BPA shows also a decrease with time. It is largely described that BPA is a toxic compound with deleterious effects in liver, brain and kidney in model animals (Aslanturk and Uzunhisarcikli, 2020; Gyimah et al., 2021; Mukherjee et al., 2020), and that, even at low concentrations, induce oxidative stress (Bindhumol et al., 2003; Kazemi et al., 2017) triggering toxicity problems (Park et al., 2020; Sharifinia et al., 2020; Shmarakov et al., 2017). The highest BPA levels found in mussels at the beginning of the study could involve an activation of detoxification mechanisms increasing GSTs and CEs activities. In addition, during phase I detoxification process, hyper reactive compounds are produced (Uno et al., 2012). In this sense, the activity of ETS trigger the activity of ROS with a consequent increase in LPO (Freitas et al., 2020). This increase in the ROS production due to the detoxification process could explain the highest CAT, GRd and GPx activities found at the beginning of the study. However, the increase in antioxidant enzymes would not be enough to avoid oxidative damage, since higher levels of carbonyls were observed at the beginning of the study (Capó et al., 2015; Sureda et al., 2018). Overall all the metabolic parameters decrease over time, including CI which suggests a lower performance towards the summer period. However, this decrease had a more negative impact near the aquaculture facilities as levels of LPO and carbonyl proteins were significantly higher.

On the other hand, the higher activity of SOD and GPx at T60 in mussels from the aquaculture facilities can be explained by the initial activation of the antioxidant system to a polluted location. In this sense, previous studies have reported the capacity of the individuals to return to initial values of the activity of different biomarkers after the exposure to additional stress (Capó et al., 2015; Sureda et al., 2013, 2011).

CONCLUSIONS

The ubiquity of anthropogenic particles in the marine environment due to human activities was confirmed by an increase throughout the study at the three locations. Results from this study showed biochemical alterations over time and among locations, with greater impacts in mussels from aquaculture facilities. Nevertheless, the absence of a correlation between anthropogenic particles and levels of phthalates and bisphenols in mussels suggests that plasticizers bioaccumulation was not by direct transfer from the anthropogenic particles ingested. On the other hand, with respect to the occurrence of phthalates and bisphenols, a time trend has been observed, with a decrease in BPA and an increase in DEHP in mussels from aquaculture facilities; and with regards to biomarkers, in addition to a clear time trend with decreased enzymatic responses, a location effect was also observed as mussels from aquaculture facilities showed induced oxidative stress damage. Results suggest that the condition index is probably more correlated to environmental conditions such as the oligotrophy environment of the study area. Altogether evidence the complex interactions between the anthropogenic pollutants assessed and the biological and physiological parameters of marine mussels in field conditions.

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FIGURES AND TABLES

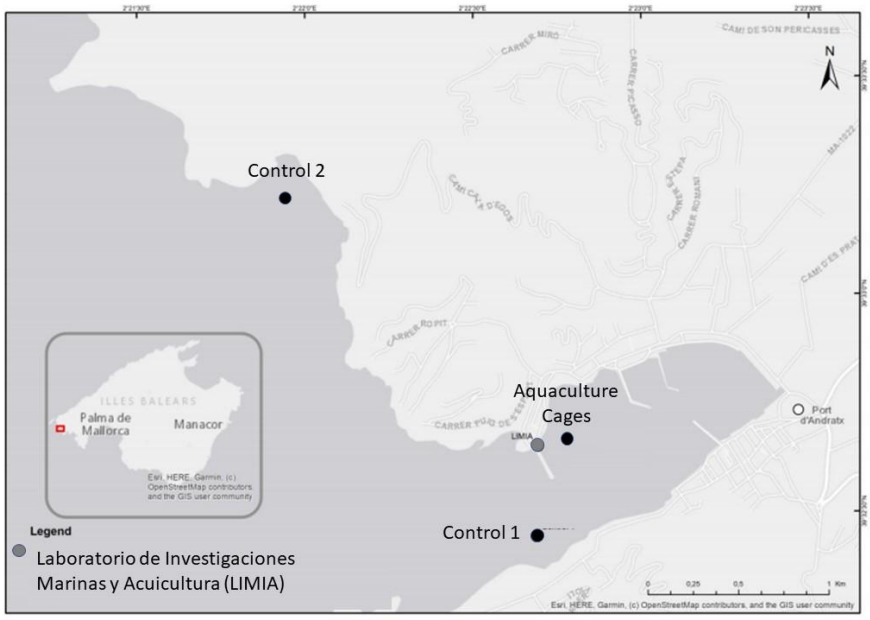

Fig. 1. Locations selected to assess phthalates and bisphenols concentration under three different anthropogenic impacts: the vicinity of cages of scientific aquaculture (aquaculture), the mouth of the bay near to the aquaculture installations (control 1) and an exposed reference beach (control 2)

Table 1. Summary of temperatures (º C) in the different sampling periods (T0, T60 and T120) and at the different locations (aquaculture, control 1 and control 2) expressed as mean value ± standard deviation (SD).

	Sampling period		
Location	**T0**	**T60**	**T120**
Aquaculture	19.57	23.91 ± 2.44	26.60 ± 1.19
Control 1	18.40	23.01 ± 2.65	26.30 ± 1.08
Control 2	17.90	22.68 ± 2.75	26.14 ± 0.97

Table 2. Summary of the anthropogenic particles per individual identified and classified per typology in fibers, fragments and films represented as mean value (± SD). In brackets, total number of particles identified.

Sampling period	Location	Fibers	Fragments	Films
Time 0		5.47 ± 3.16 (82)	0.33 ± 0.82 (5)	0.13 ± 0.35 (2)
T60	Aquaculture	4.93 ± 3.83 (74)	0.07 ± 0.26 (1)	0
	Control 1	6.53 ± 4.81 (98)	0.67 ± 1.84 (10)	0
	Control 2	3.53 ± 3.64 (53)	0.13 ± 0.35 (2)	0
Mean		5 ± 4.22 (225)	0.29 ± 1.10 (13)	0
T120	Aquaculture	12.47 ± 13.57 (187)	0.27 ± 0.80 (4)	0
	Control 1	11 ± 7.28 (165)	0.07 ± 0.26 (1)	0
	Control 2	12.47 ± 7.51 (187)	0	0
Mean		11.98 ± 9.69 (539)	0.11 ± 0.49 (5)	0

Table 3. Summary of the total items identified and categorized by size range in millimeters (mm) and by color, and their percentage (%).

	Total items	Percentage (%)
Size range		
< 1	298	34.2
1 – 2	305	35.0
2 – 3	141	16.2
3 – 4	49	5.6
4 – 5	33	3.8
> 5	45	5.2
Color		
Transparent	649	74.5
Black	65	7.5
Red	63	7.2
Red	58	6.7
Other	36	4.1

908 Table 4. Summary of the bisphenols (µg/g ww): bisphenol S (BPS); bisphenol F (BPF); and
909 bisphenol A (BPA); and phthalates (µg/g ww): diethyl phthalate (DEP); dibutyl phthalate
910 (DBP); and bis(2-ethylhexyl) phthalate (DEHP) in mussels in the different sampling periods
911 (T0, T60 and T120) and at the different locations (aquaculture, control 1 and control 2). All
912 values are expressed as mean value ± standard deviation (SD).

Sampling period	Location	BPS	BPF	BPA	DEP	DBP	DEHP
Time 0		0.14 ± 0.13	1.87 ± 0.47	8.14 ± 1.35	0.13 ± 0.15	0.94 ± 1.45	0.23 ± 0.61
T60	Aquaculture	0.08 ± 0.04	2.16 ± 0.51	8.05 ± 1.35	0.04 ± 0.01	0.58 ± 0.13	1.16 ± 2.59
	Control 1	0.07 ± 0.04	1.41 ± 0.30	6.56 ± 0.90	0.05 ± 0.04	0.91 ± 0.27	2.72 ± 2.11
	Control 2	0.07 ± 0.05	2.47 ± 0.42	7.59 ± 0.62	0.05 ± 0.02	0.56 ± 0.11	4.06 ± 0.82
Mean		0.07 ± 0.04	2.01 ± 0.60	7.40 ± 1.13	0.05 ± 0.03	0.68 ± 0.24	2.65 ± 2.21
T120	Aquaculture	0.07 ± 0.03	1.78 ± 0.37	6.51 ± 2.91	0.05 ± 0.01	0.83 ± 0.15	2.10 ± 2.04
	Control 1	0.41 ± 0.76	2.13 ± 1.27	5.20 ± 2.54	0.07 ± 0.04	0.91 ± 0.35	1.05 ± 1.05
	Control 2	0.20 ± 0.14	1.92 ± 0.40	4.95 ± 1.53	0.67 ± 1.04	1.27 ± 1.39	1.32 ± 1.42
Mean		0.23 ± 0.44	1.94 ± 0.75	5.56 ± 2.33	0.27 ± 0.63	0.98 ± 0.73	1.49 ± 1.51

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Table 5. Results from the two-way ANOVA analysis of the levels of bisphenols: bisphenol S (BPS); bisphenol F (BPF); and bisphenol A (BPA); and phthalates: diethyl phthalate (DEP); dibutyl phthalate (DBP); and bis(2-ethylhexyl) phthalate (DEHP) in soft tissue of *M. galloprovincialis*.

	BPS					**DBP**				
	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Location	3	0.1343	0.04478	0.530	0.665	3	0.301	0.1004	0.162	0.921
Time	1	0.1818	0.18182	2.153	0.153	1	0.675	0.6753	1.090	0.305
Location:Time	2	0.1552	0.07758	0.919	0.410	2	0.616	0.3082	0.498	0.613
Residuals	30	25.333	0.08444			28	17.344	0.6194		

	BPF					**DEP**				
	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Location	3	0.975	0.3250	0.886	0.459	3	0.628	0.2092	1.393	0.264
Time	1	0.037	0.0372	0.101	0.752	1	0.357	0.3569	2.376	0.134
Location:Time	2	2.357	11.785	3.214	0.054 .	2	0.618	0.3091	2.058	0.145
Residuals	30	11.000	0.3667			30	4.506	0.1502		

	BPA					**DEHP**				
	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Location	3	26.10	8.701	2.831	0.055 .	3	25.24	8.415	3.213	0.037 *
Time	1	25.41	25.408	8.266	0.007 **	1	10.06	10.058	3.840	0.059 .
Location:Time	2	2.39	1.197	0.389	0.681	2	17.93	8.967	3.424	0.046 *
Residuals	30	92.21	3.074			30	78.57	2.619		

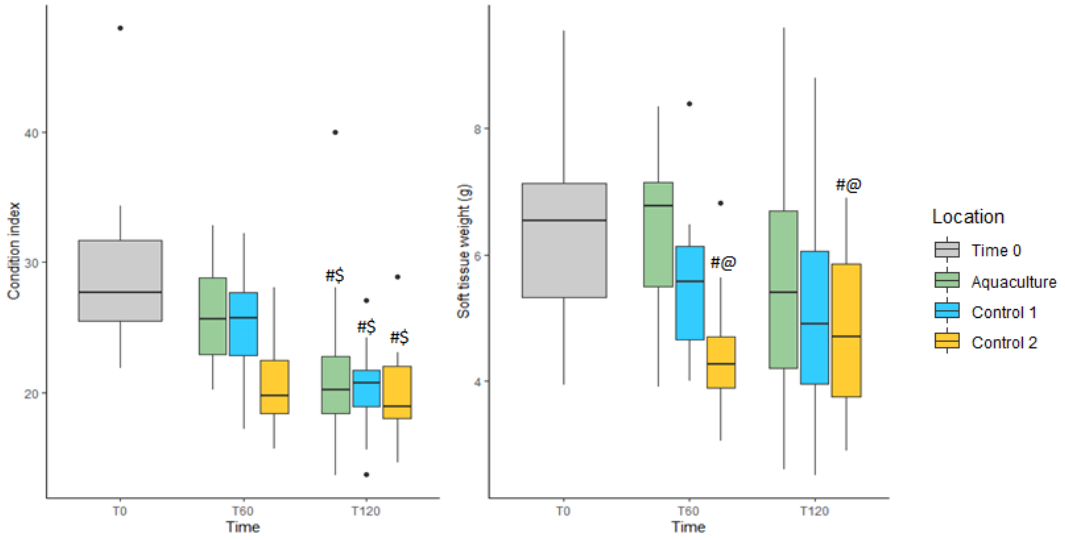
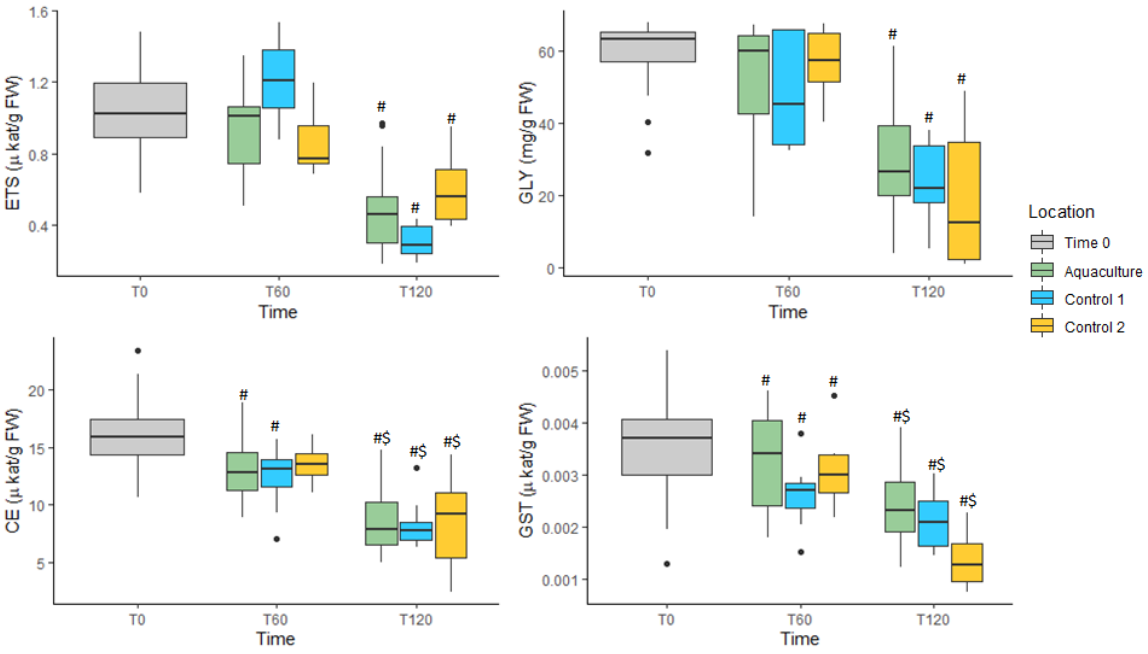
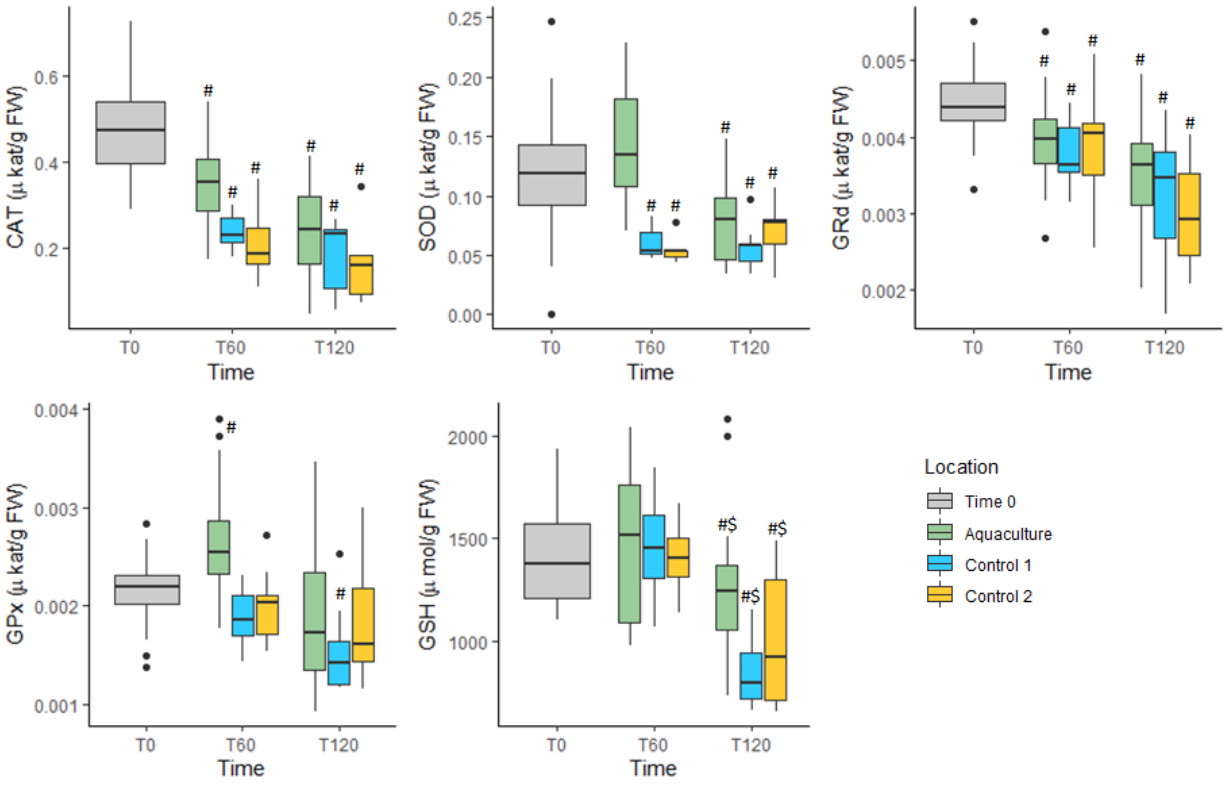


Fig. 2. Boxplot of condition index and the soft tissue fresh weight (g) of mussels in the different sampling periods (T0, T60 and T120) and at the different locations (aquaculture, control 1 and control 2). # indicates differences respect to T0; $ indicates differences respect T60; and @ indicates differences respect aquaculture location.



927 Fig. 3. Boxplots of the metabolic biomarkers: electronic transport system (ETS); and glycogen
928 levels (GLY); and the detoxification enzymes activities in phase I: carboxylesterase activity
929 (CE); and in phase II: glutathione-*S*-transferase (GST) assessed in mussels in the different
930 sampling periods (T0, T60 and T120) and at the different locations (aquaculture, control 1 and
931 control 2). # indicates differences respect to T0; and $ indicates differences respect T60.



932 Fig. 4. Boxplots of the antioxidant system biomarkers: catalase (CAT); superoxide dismutase
933 (SOD); glutathione reductase (GRd); glutathione peroxidase (GPx); and reduced glutathione
934 (GSH) assessed in mussels in the different sampling periods (T0, T60 and T120) and at the
935 different locations (aquaculture, control 1 and control 2). # indicates differences respect to T0;
936 and $ indicates differences respect T60.

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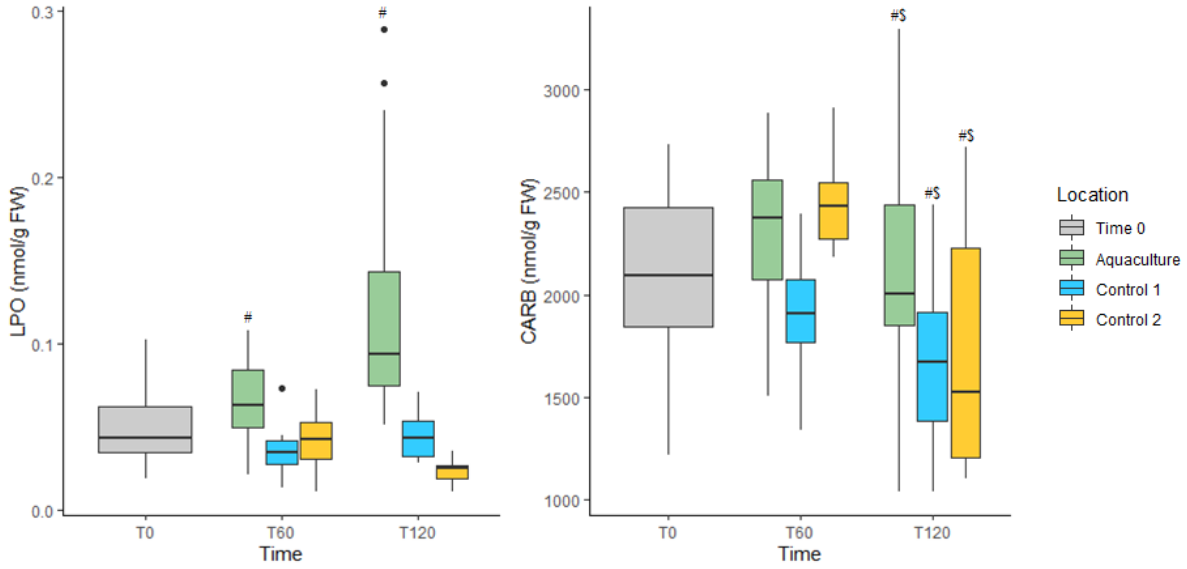

938 Fig. 5. Boxplots of the oxidative stress damage biomarkers: lipid peroxidation (LPO); and
939 proteins carbonyls derivates (CARB) assessed in mussels in the different sampling periods (T0,
940 T60 and T120) and at the different locations (aquaculture, control 1 and control 2). # indicates
941 differences respect to T0; and $ indicates differences respect T60.

Table 6. Pearson correlation between anthropogenic particles (AP), and levels of phthalates and bisphenols with condition index (CI) and biomarkers assessed in the whole soft tissue of *Mytilus galloprovincialis* (N = 35). The metabolic biomarkers were: electronic transport system (ETS); and glycogen levels (GLY); the detoxification enzymes: carboxilesterases (CE); and glutathione-*S*-transferase (GST); the antioxidant system biomarkers were: catalase (CAT); superoxide dismutase (SOD); glutathione reductase (GRd); glutathione peroxidase (GPx); and reduced glutathione (GSH); and the oxidative stress damage biomarkers in lipids: lipid peroxidation (LPO); and in proteins: protein carbonyls derivates (CARB). Asterisks represent significant levels: '·' $p < 0.1$, '*' $p < 0.05$, '**' $p < 0.01$, '***' $p < 0.001$.

	AP	**BPS**	**BPF**	**BPA**	**DEP**	**DBP**	**DEHP**	**CI**	**ETS**	**GLY**	**CE**	**GST**	**CAT**	**SOD**	**GRd**	**GPx**	**GSH**	**LPO**	**CARB**
AP	--	0.16	0.11	-0.34 *	-0.10	-0.03	0.00	-0.18	0.39 *	0.32	0.42 **	0.34 *	0.41 *	0.11	0.21	0.23	0.40 *	-0.04	-0.05
BPS		--	0.62 ***	0.28 ·	0.16	0.05	-0.04	-0.04	-0.12	-0.39 *	-0.35 *	-0.19	-0.07	-0.14	-0.02	-0.14	-0.25	0.01	0.03
BPF			--	0.41 *	0.11	0.06	0.20	0.08	0.08	-0.10	-0.23	0.12	0.22	0.20	0.14	-0.02	0.15	-0.02	0.10
BPA				--	-0.01	0.02	0.04	0.44 *	0.36 *	0.06	0.18	0.25	0.33 ·	0.14	0.21	0.09	0.43 **	0.38 *	0.37 *
DEP					--	0.83 ***	0.02	-0.08	-0.12	0.07	-0.19	-0.32 ·	-0.20	-0.16	-0.29 ·	0.023	-0.11	-0.16	-0.04
DBP						--	-0.17	0.26	-0.22	-0.13	0.26	-0.27	0.07	-0.21	-0.13	-0.24	-0.16	-0.02	-0.37 *
DEHP							--	-0.20	0.07	0.17	-0.07	-0.08	-0.16	-0.04	0.003	-0.16	0.11	0.03	0.20
CI								--	0.39 *	0.32 ·	0.42 **	0.34 *	0.41 *	0.11	0.21	0.23	0.40 *	-0.04	-0.5
ETS									--	0.64 ***	0.49 **	0.49 **	0.61 ***	0.52 **	0.18	0.39 *	0.59 ***	-0.35 *	0.24
GLY										--	0.36 *	0.42 **	0.47 **	0.28	0.18	0.34 *	0.41 *	-0.45 **	0.35 *
CE											--	0.24	0.42 *	0.16	0.14	0.10	0.14	-0.33	-0.16
GST												--	0.51 **	0.30	0.40 *	0.37 *	0.55 ***	-0.02	0.33 ·
CAT													--	0.52 **	0.68 ***	0.27	0.57 **	-0.10	0.26
SOD														--	0.34	0.69 ***	0.45 *	-0.16	0.34
GRd															--	0.32	0.47 **	-0.08	0.45 **
GPx																--	0.44 **	-0.20	0.44 **
GSH																	--	0.10	0.44 **
LPO																		--	0.10
CARB																			--