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# Realistic environmental exposure to microplastics does not induce biological effects in the Pacific oyster *Crassostrea gigas*

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**Abstract**

The aim of the present study was to evaluate the presence and potential toxic effects of plastic fragments (< 400 µm) of polyethylene and polypropylene on the Pacific oyster *Crassostrea gigas.* Oysters were exposed to environmentally relevant concentrations (0, 0.008, 10, 100 µg of particles/L) during 10 days, followed by a depuration period of 10 days in clean seawater. Effects of microplastics were evaluated on the clearance rate of organisms, tissue alteration, antioxidant defense, immune alteration and DNA damage. Detection and quantification of microplastics in oyster's tissues (digestive gland, gills and other tissues) and biodeposits using infrared microscopy were also conducted. Microplastics were detected in oyster's biodeposits following exposure to all tested concentrations: 0.003, 0.006 and 0.05 particles/mg of biodeposits in oysters exposed to 0.008, 10 and 100 µg of particles/L, respectively. No significant modulation of biological markers was measured in organisms exposed to microplastics in environmentally relevant conditions.

**Keywords**: Microplastics, *Crassostrea gigas*, biodeposits, biomarkers, bivalves

## 1. Introduction

Plastic debris have been identified in marine ecosystems worldwide and are recognized as a global threat for diverse marine organisms (Eriksen et al., 2014). The identification of microplastics (MPs) which is mostly the result of the fragmentation of larger pieces of plastic has been described in aquatic environments from Pole to Pole (Cózar et al., 2014). Microplastics are plastic debris < 5 mm (NOOA, 2008) which are expected to interact with marine organisms such as invertebrates. Ingestion and negative impact of MPs have been evaluated in various marine species such as zooplankton (Cole et al., 2013), worms (Wright et al., 2013), bivalves (Paul-Pont et al., 2016; Ribeiro et al., 2017; Sussarellu et al., 2016), fish (Digka et al., 2018; Giani et al., 2019) and marine mammals (Donohue et al., 2019; Hernandez-Gonzalez et al., 2018).

Oysters are commonly used in ecotoxicological studies as bioindicators (e.g. Palmer et al., 2015), as they are filter-feeding organisms , potentially ingesting large quantities of MPs which can potentially lead to physiological modification (Cole et al., 2013). One study conducted on *C. gigas* showed that oyster larvae had the capacity to ingest MPs and nanoplastics beads (70 nm - 20 µm) of polystyrene (PS) (Cole and Galloway, 2015). However, no effects on the development and feeding capacity was observed after 8 days of exposure to PS at concentrations lower than 100 MPs/mL (size of MPs: 1 and 10 µm). Sussarellu et al. (2016) exposed oysters to microbeads of plastic at 23 µg/L (2 - 6 µm) for 2 months during gametogenesis. They observed reproduction alterations such as a decrease in oocyte number and diameter, and delayed larval growth. Gardon et al. (2018) measured gonadal resorption and a decrease in assimilation efficiency of organic matter in pearl oysters *Pinctada margaritifera* exposed to PS microbeads (6-10 µm) for two months at 0.25, 2.5 and 25 µg/L. Other authors observed modulation of oxidative stress and DNA damage in clams *Scrobicularia plana* exposed to PS microbeads of 20 µm at 1 mg/L (Ribeiro et al., 2017). In

*Mytilus edulis*, tissue alterations were also observed after 6-h of exposure to polyethylene (PE) particles at 2.5 g/L (0-80 µm) (von Moos et al., 2012).

In most of the studies conducted on bivalves, organisms were exposed to mono-dispersed plastic microbeads. Moreover, the selected exposure concentrations are often very high (between 23 µg/L and 2.5 g/L) and not representative of the expected MPs concentrations in coastal waters. There is a lack of data obtained from laboratory exposures conducted at environmentally realistic concentrations. For locations on the coast, concentrations below 23 µg/L can be considered as representative of these areas (Goldstein et al., 2012; Sul et al., 2014). Plus, several of the previously mentioned studies worked with plastic particles under the form of microbeads which are less representative of MPs fragmented, of various shapes and sizes, identified in marine environments which can have a different biological impact on marine biota (Lenz et al., 2016). Few authors began to evaluate the impact of more environmentally realistic MP fragments, made from larger pieces of plastic (Rainieri et al., 2018; Weber et al., 2018; Revel et al., 2019). One study compared the effects of spherical and irregular shapes of MPs in fish sheepshead minnow (*Cyprinodon variegatus*). Differences were observed with the shape of MPs in antioxidant enzymes (activities and transcripts) and swimming behaviour, the biological effect being more important for MPs fragments (Choi et al., 2018).

In this study, the accumulation and potential toxicity of polypropylene (PP) and PE MPs were assessed in the oyster *Crassostrea gigas*. Commercial plastic products were bought and fragmented into MP particles through milling in the laboratory. PP and PE polymers were selected for this study in accordance with previous results demonstrating that in bivalves from the French Region Pays de la Loire the majority of detected MPs in their tissues were made of these two polymers (Phuong et al., 2017). Oysters were exposed to environmentally relevant concentrations of 0.008, 10 and 100 µg of MPs/L which are representative of coastal regions

or gyres (Su et al., 2014; Goldstein et al., 2012). To evaluate the effects of MPs on oysters, a multi-marker approach was implemented. The clearance rate was measured and tissue modifications were analysed by histopathology to determine the impact of MP exposure on the general physiological state of oysters. Detoxification and oxidative stress parameters, which are impacted by various xenobiotics, were evaluated through the measurement of Reactive Oxygen Species (ROS) in the hemocytes; antioxydant enzyme activities of glutathione-S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD), in gills and digestive gland, and gene expression level of these enzymes including glutathione peroxydase (GPx) in the same previous tissues. Potential alteration of the immune system was assessed in hemocytes, through acid phosphatase (AcP) activity previously proposed as a proxy of sublethal immune effect of contaminant exposure. It is also a good indicator of sensitivity to microbial infections (Gagnaire et al., 2007; Luna-Acosta et al., 2010). The possibility for MP exposure to damage the DNA was also evaluated on hemocytes by the comet assay, which is a widely used test for measuring the genotoxic effects of contaminants in bivalves (Akcha et al., 2012; Châtel et al. 2017). Finally, identification and quantitative analysis of MP content in oyster tissues (gills, digestive gland, and remaining tissues), and biodeposits (faeces and pseudofaeces) were performed using microscopy coupled with infrared spectroscopy.

## 2. Materials and methods

### 2.1 Microplastics

#### 2.1.1 Preparation and characterization of lab-made MP particles

The powders of MPs were prepared by cryo-milling several grams of green PP and purple PE samples from commercial products as described in Revel et al. (2019). Bright colors were chosen to facilitate their observation under the microscope. After grinding, each

powder with MP fragments was sieved, the fraction below 400 µm was selected and a granulometry analysis was performed with a particle laser diffractometer (Beckman Coulter® LS 130) to determine the size distribution of the particles.

### 2.1.2 Suspensions of microplastics

For each type of plastic particles, stock suspensions at concentrations of 1 and 0.1 mg/mL were prepared in milliQ water. Working solutions at 0.1 µg/mL were obtained by three serial dilutions of the stock suspension at 0.1 mg/mL in milliQ water. To obtain the expected final concentrations of the two polymers in each aquarium, a specific volume of either the stock suspension (1mg/mL or 0.1 mg/mL) or working suspension (0.1 µg/mL) was distributed in each aquarium to obtain half PE and half PP at 0.008, 10 and 100 µg of particles/L (details of the methodology are available in Revel et al. 2019). To prevent potential additional effects associated to the use of surfactant during MP preparations, none were used in this experiment: a manual and strong mixing was performed for each plastic suspension before adding the last aliquot to the aquariums in order to ensure the best reproducibility of the contamination levels. Additionally, so as to verify our technique of dispersion, an aliquot of the diluted suspension at 0.1µg/mL, corresponding to the volume added to the aquariums to obtain 0.008 µg/L, was analysed to confirm the presence of both PE and PP particles. During the experiment, all MPs suspension were made and spilled daily, after every water renewal.

## 2.2 Model organism and exposure setup

### 2.2.1 Oysters

Diploid oysters *Crassostrea gigas* of 18 months were obtained from an oyster producer located at Bouin, French Atlantic Coast. Organisms were maintained 15 days for

acclimatization in tanks (35 individuals per tank) filled with 35 L of artificial seawater (ASW), prepared with commercial salt (Tropic Marin ®, France) at 30 g/L and uninterrupted aeration. Oysters with similar size were selected (shell length 10 ± 0.9 cm). Every day, half of the water was changed and oysters were fed with a commercially available algal solution of *Tetraselmis suecica* (Teramer ®, France) following the product requirements for bivalve feeding (40 µL/L of water, $10^8$ cells/mL). During acclimatization and MP exposure, controlled temperature of 15 ± 1°C and photoperiod of 12 h :12 h were applied.

### 2.2.2 Exposure assay

Following the acclimatization period, 20 oysters were sampled (5 in each tank) to evaluate their physiological conditions at T0. Remaining oysters were then randomly separated into groups of 10 individuals and placed into 20 L glass aquariums filled with 10 L of ASW. Oysters were exposed for 10 days to 0 (negative control), 0.008, 10 and 100 µg/L (corresponding to about 9, 11200 and 112000 particles/L) of a mixture of PE and PP and a negative control (0 particle/L). Each experimental condition was made of 3 aquariums containing each 10 individuals (12 aquariums and 120 oysters in total). Oysters were fed every day during an hour before water change and MP contamination to prevent any interactions between algae cells and MPs as it was described in our previous study on *M. edulis* (Revel et al. 2019). Each day, just before the renewal of total water volume and MP contamination, biodeposits from each aquarium were gently removed with glass pipettes into glass tubes and conserved at -20°prior to analysis.

#### 2.2.1.1 Oysters sampling for analysis

Several analyses were conducted on oysters sampled at T0, T10 and T20 (Details about oysters sampling and analysis are described in supplementary material 1). At T0 (before

the beginning of the experiment), 20 oysters were used for a histopathological exam, AcP activity, oxidative stress, condition index using biometric measurements (total weight, shell weight and wet weight of soft tissues) and MP content in oysters' tissues. Following the 10 days of exposure (T10), 18 oysters per condition were sampled (6 per aquariums) for analysis of MP content in soft tissues and condition index (N=5 with individuals from different replicate aquarium); histopathology, AcP activity and condition index (N=5); genotoxicity and oxidative stress (N=5) ; and finally, the evaluation of clearance rate (N=5).

After the exposure, remaining oysters were gently washed to prevent any external binding of MPs, and disposed into glass aquariums for 10 days of depuration (T20) in the same controlled conditions (seawater, light, temperature, and food). At the end of the 10 days of depuration (T20), half of the oysters were used for evaluation of MP content in soft tissues and the other half for clearance rate assessment (N=5).

2.2.1.2 Tissue preparation for analysis

The total soft tissues of oysters were removed from the shell to perform histological examination. The tissues were separately placed into Davidson's solution (10% glycerol, 20% formaldehyde, 30% ethanol at 95%, 30% sterile seawater, 10% acetic acid) for 48 h, and conserved in 70% ethanol until analysis.

Collection of oysters hemolymph was operated by punction from the posterior adductor muscle sinus using a sterile hypodermic needle (23 G) in a 2 mL syringe pre-rinsed with the anticoagulant Alsever's solution (113.7 mM glucose, 27.2 mM sodium citrate, 58.44 mM sodium chloride) at pH 6.1. A part of the hemolymph was directly frozen in liquid nitrogen and stored at -80 °C for the measurement of AcP activity, the rest was kept on ice until comet assay and flow cytometry analyses.

Gills and digestive gland from each individual were dissected and stored at -20°C for analysis of MP content or frozen in liquid nitrogen for antioxidant enzyme activities and gene expression. Later on, tissues were reduced into powder with a mixer mill MM400 (Retsch®) at -196°C and directly kept at either -80°C for enzymatic measurements or in trizol for RNA extraction.

### 2.3 Microplastic analyses in oyster's tissues

#### 2.3.1 Quality control

In order to ovoid external contamination, a series of procedure were followed in every step of the exposure and the preparation of samples as it is described in Revel et al. (2019). Briefly, all the equipment used were washed with 70% ethanol, dried in a hood and kept under aluminium foil; 100% cotton laboratory coats and nitrile gloves were used; dissection of bivalves was operated under a hood, and samples for MP analysis were kept in aluminium foil.

Sample digestion was conducted in glass beakers covered with glass lids and after the filtration was performed, filters were conserved in glass Petri dishes until infrared microscopy analysis. To evaluate the external contamination level, one blank consisting of 10% KOH (m/v) without samples was added to every series of digestion (5 replicates per types of sample) and the filter analysed.

#### 2.3.2 Sample digestion and microscopy analysis

Analysis of MP content was conducted on individual tissues (gills, digestive gland and oyster's other tissues) of each oyster after the exposure (T10) and the depuration (T20) following a procedure adapted from the protocol published by Phuong et al. (2017). Each tissue sample was placed into a 50 mL beaker filled with 20 mL of 10% KOH (m/v) and

heated at 60°C with a 24 h agitation. Biodeposit samples were treated by pools of each replicate aquariums from each day for each condition (4 pools for the exposure and 4 pools for the depuration) in a 50 mL glass beaker, dried for 2 h at 60° and then weighted. A volume of 20 mL 10% KOH (m/v) was then added and a 24 h agitation with a temperature of 60°C was applied. The solutions (including blanks) were then filtered on a cellulose nitrate filter (12 µm, Whatman®, Germany) which was dried at room temperature in a closed glass Petri dish until identification of MPs.

For the analysis of PE and PP particles on the filters, the Fourier transform infrared microscopy system (µFT-IR; Spotlight 200i FT-IR microscopy system, PerkinElmer®) was used in reflection mode. The entire surface of the filter was observed and each identified particle was examined individually. A number of 8 accumulations ranging from 600 to 4000 $cm^{-1}$ were selected to record each spectrum which was then compared to the polymer database (PerkinElmer Polymer database) to validate the type of plastic.

### 2.4 Physiological markers

#### 2.4.1 Condition index

To calculate the condition index (CI), biometric measurements such as total weight and wet weight of soft tissues of 10 oysters per condition were determined at T0 and T10. CI was then determined following the formula: CI = [(wet weight of soft tissues/total weight) x 100] (AFNOR, 1985).

#### 2.4.2 Clearance rate

The clearance rate (CR) of oysters previously exposed to clean ASW (negative control) or to the mixture of PE+PP MPs (0.008, 10 and 100 µg/L) for 10 days (T10) or after the depuration (T20) was estimated with a static system (Pernet et al., 2008). Oysters were

individually placed in metabolic units of 1.3 L for 1 h before the measurements. An algal solution of *Tisochrysis lutea* at an initial concentration of 225000 cells/mL was then added to each unit. A fine bubble aeration was applied around the wall of the metabolic unit to prevent the dispersion of biodeposits. CR was evaluated by an indirect method based on the measurement of algal concentration every 15 min for 1 h according to standard algal cell counting technique (Utermöhl, H, 1958). Algal cells were fixed with a drop of Lugol's solution (I2, 5%; KI 10%, seawater) added to 10 ml of each sample and cells were counted with a Malassez counting chamber at 250X under a light microscope (Duchemin et al., 2008).

**2.5 Histological observations**

The oyster's reproductive status was evaluated to characterize the oysters' batch and because of its importance to interpret all studied biomarkers. Cross sections of oysters (5 mm-thick), previously fixed in Davidson's solution, were cut and 3-µm paraffin-embedded sections were stained following the trichrome protocol of Prenant Gabe (Gabe, 1968). Each specimen was sexed but also classified into different stages of gonadal maturation corresponding to sexual resting (0), early developing (gonial mitosis; I), developing (II), sexual maturity and spawning (IIIB) and finally late spawning/post-spawning (IIID) (Costil et al., 2005).

For each oyster, tissue alterations (eg. hemocytic infiltrations in the digestive tract, diffuse hemocytic infiltration in the connective tissue, hemocyte aggregations in the connective tissue and mantle and palps destructuration) were semi-quantified according to a scale with 4 levels from slight (score of 1) to severe (score of 4) (Buisson et al., 2008). An average index was then calculated for each condition and tissue alterations.

**2.6 Oxidative stress**

### 2.6.1 ROS production

ROS production was evaluated in hemolymph from oysters exposed to the mixture of PE+PP MPs (0.008, 10 and 100 µg/L) using a protocol adapted from Haberkorn et al. (2014). A stock solution of the fluorescent probe 2 ,7 -dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) at 10 mM in DMSO was used to prepare a 1 mM working solution in filtered sterile seawater (FSSW). A volume of 100 µL of each hemolymph sample was diluted to ¼ with FSSW and incubated at 18°C in the dark for 120 min with 10 µM $H_2$DFC-DA (Haberkorn et al., 2014). At the end of the incubation period, DCF fluorescence which is quantitatively related to the ROS content in hemocytes, was measured at 515–545 nm with a flow cytometer (Becton Dickinson Accuri™ C6).

### 2.6.2 Antioxidant enzyme activities

Frozen powder of gills and digestive gland were mixed in TRIS buffer (TRIS 50 mM, NaCl 150 mM and DTT 1 mM) with an antiprotease solution (Sigma® P8340, diluted in 1/1000) at pH 7.4 in a 1:3 ratio (w:v) and at 4°C to avoid the degradation of tissue components. Samples were centrifuged at 9,000 g (25min) and GST, SOD and CAT activities were measured spectrophotometrically as it is described in Revel et al. (2019). The activity of GST was measured at 340 nm and expressed as nmoles/min/mg of protein (Habig et al., 1974). CAT activity, expressed as µmoles/min/mg protein was determined at 240 nm (Claiborne, 1985). SOD activity was measured at 550 nm using the protocol of the inhibition of cytochrome C reduction by superoxide anion radicals (McCord and Fridovich, 1969) and expressed as SOD Unit per mg of protein (1 unit = the amount of sample inducing 50% inhibition in 1 mL reaction system per mg of protein). Protein content was previously measured using the Bradford protocol (Bradford, 1976).

### 2.6.3 Antioxidant enzyme gene expression

Total RNA was extracted from the frozen powder of gills and digestive gland with TRI Reagent following the manufacturer's instructions. Determination of RNA concentrations and integrity plus reverse transcription were conducted as previously described (Revel et al., 2019). The levels of SOD, CAT, GST and GPx transcripts were then measured by real-time PCR using two reference genes: Elongation Factor 1 alpha (EF1α) and GAPDH. The software Primer 3V 4.0.0 was used to design the specific primers (Untergasser et al., 2012) and the PCR efficiency (E) was determined for each primer and each organ on serial dilutions of a pool sample containing cDNA from all experimental conditions and was comprised between 95% < E < 104%. Genbank accession numbers, primers sequences and efficiency are in Supplementary Material 2. Real-time PCR reactions were conducted as it is described in Revel et al. (2019) and accurate amplification of the target amplicon was checked by performing a melting curve. During each run, three controls were processed: a cDNA inter-run, constituted of a pool sample with cDNA from all experimental conditions, a no-template, and a sample with water alone. In order to normalize gene expression, the geometric mean of the two reference genes was used with the application of the Pfaffl formula (Pfaffl, 2001) and specific amplification efficiencies.

## 2.7 Immune marker: acid phosphatase

Measurements of AcP activity were assessed according to manufacturer's instructions (Sigma kit CS0740) in pooled hemolymph samples of approximatively 1.5 oysters per tube (organisms from a different replicate aquarium in each tube), to obtain the adequate amount of tissue for the analysis (N=3). AcP activity is expressed as unit/mg of protein (1 unit = the quantity of enzyme required for the formation of 1 µmol of p-nitrophenol per minute).

### 2.8 Genotoxicity: Comet assay

DNA damage was evaluated using the comet assay following a previously described protocol (Akcha et al., 2003, Barranger et al., 2013). Directly after sampling, hemolymph samples were centrifugated (1500 rpm, 5 min) to recover individual hemocytes. A volume of 160 µL of 0.5% low melting point agarose was added to the hemocyte pellet for the preparation of 2 comet slides. After lysis and denaturation step in the electrophoresis buffer, DNA migration was carried out for 15 min at 23 V (390 mA, E = 0.66 V/cm). Slides were then washed three times by incubation for 5 min in neutralization buffer (Tris base 0.4 M, pH 7.5). So as to fix the preparations, slides were immersed in absolute ethanol for at least 10 min to dehydrate and then dried at room temperature. For the analysis, slides were stained with 75 µL of GelRed at 8 mg/L spread over each slide using a cover glass and stored for a minimum of 1 h in the dark at 4°C. The analysis was conducted using an optical fluorescence microscope (Olympus BX60, ×40) equipped with a CDD camera (Luca-S, Andor Technology®) and image analysis system (Komet 6, Kinetic Imaging Ltd. ®). The percentage of DNA present in the Comet tail (% Tail DNA) was measured for each observed nucleus and a minimum of 50 nuclei were analysed per slide.

### 2.9 Statistical analysis

The normality of the data was evaluated using a Shapiro-Wilk test and the homogeneity of the variances was verified with the Levene's test. The means of the different conditions were then compared with a 1-factor ANOVA test and if significant the Tukey's test was applied. Kruskal-Wallis tests followed by multiple comparisons were used instead of the ANOVA when the conditions of application were not verified and for the tissue alterations (expressed by ordinal values). For determination of reproductive status, the proportions of the

different gametogenesis stages between experimental conditions were analysed using a Chi-square test. The statistical analyses were processed with software R (R.3.3.1) or Statistica 6.0.

## 3. Results and discussion

### 3.1 Characterization of microplastics in suspensions

Granulometric analysis of MP dry particles showed the presence of all sizes between 0.4 and 500 µm in samples of PE and PP powders with a mean distribution of size close to 300 µm for PE and 200 µm for PP. When these values are in accordance with the mode of preparation of MPs (selection of particles below 400 µm), a small proportion of particles (less than 10 % in volume) with size larger than expected was also observed. These particles could be the result of agglomeration of some particles after the sieving step. In addition, the granulometric analysis showed the presence in small quantities (less than 0.5 % in volume) of particles below 1 µm and down to 400 nm which corresponds to the instrument's limit of detection. Nevertheless, there is a possibility that some particles either isolated or aggregated and smaller than 400 nm are also present.

In this study, environmental concentrations were selected which prevented the granulometric analysis of MP suspensions that were not concentrated enough. MP suspensions were then characterized by the microscopic counting of MPs contained in aliquots of each stock (1 mg/mL and 0.1 mg/mL) and diluted suspensions (0.1 µg/mL). The volume of aliquots analysed were corresponding to the volumes added into the aquariums to obtain 100, 10 and 0.008 µg/L. However, when deposited on a slide, particles aggregated rapidly and no measurement or counting (or weighting) of individual plastic particles could be performed accurately to eventually confirm the concentration of suspensions.

In MP studies, currently remain important methodological and analytical challenges (Paul-Pont et al., 2018). Most of the MPs studies use microbeads which are easier to

characterize, but fragments and fibres are environmentally more realistic. In aquatic systems, animals can interact with different sorts of polymers (PE, PP, etc.) with different sizes and shapes which is complicated to reproduce in the laboratory. Furthermore, the concentrations tested in controlled experiments are frequently higher than the ones reported from the field and the majority of these studies use particles of one or two sizes (microbeads). Some studies also employed chemical surfactants in order to stabilize the particles and avoid agglomeration. During this study, considering technical constraints, we tried to use the most possible relevant conditions for the experiment in terms of plastic type (PE and PP), origin (particles obtained from milling of commercial products) and range of particle sizes. With these conditions, a fine characterization of particles is more difficult to obtain and more research is needed in order to develop standardized methods enabling accurate characterization of MPs exposure.

### 3.2 Analysis of microplastics in oyster's tissues and biodeposits

No particles of PE or PP were observed in the digestive gland, gills and other tissues of oysters exposed for 10 days to the mixture of PE and PP whatever the tested concentrations. In biodeposits, MP were identified with a mean of 0.059 MPs/mg of biodeposits for oysters exposed to 100 µg/L, 0.006 MPs/mg for 10 µg/L and 0.003 MP/mg for 0.008 µg/L (Table 1). No MPs were observed in oysters' tissue or biodeposits at the end of the depuration.

As both type of particles (PE and PP) were identified in biodeposits of oysters exposed to the 3 concentrations of MPs tested, it may be suggested that organisms filtered MPs but eliminated the particles rapidly in faeces and/or pseudofaeces. Similar behaviour was detected in the same species (*C. gigas)* exposed to PS MPs (2 - 6 µm, 23 µg/L) (Sussarellu 2016) while in mussels (*Mytilus* spp and *Perna Perna*), recent studies have shown a possible accumulation

of MPs in tissues with a significant decrease during depuration (Birnstiel et al., 2019; Revel et al., 2019).

### 3.3 Reproductive status and tissue alteration

Histological examination showed 4 gametogenetic stages: course of spawning (IIIB; cycle n), late spawning/post spawning (IIID; cycle n), sexual resting (0), early beginning of gonial mitosis (I; cycle n+1). Above all, no significant differences in gametogenesis stages were observed between the oysters exposed to various conditions ($Chi^2$ test, p=0.55) and therefore reproductive status should not have any influence on biomarker interpretation. Control and exposed oysters generally showed few tissue alterations indicating a "relatively good health status" of the organisms. The average of tissue alteration index ranged from 0 (destructuration of gills; hemocytic aggregates; necrosis in digestive gland at various conditions) to 3.8 ± 0.2 (SEM) (destructuration of the mantle and palps at 100 µg of MPs/L) (Figure 2). For diffuse hemocytic infiltrations in the digestive tract and in the connective tissue, the level increased with the concentration of MPs but not significantly (Kruskal-Wallis test, p=0.086 and p=0.085, respectively) (Fig. 2). For the other type of tissue alterations, no significant differences were found between control and MP-exposed organisms (Kruskal- Wallis tests, p>0.05). Tissue alteration has been previously investigated in bivalves and one study showed no evident histopathological damage in whole-body sections of Mediterranean mussel *M. galloprovincialis* exposed to microsphere of PS (10-1000 MP/mL) which was linked to the smooth edges of microbeads used according to Gonçalves et al. (2019). In oyster *C. gigas*, no cellular inflammatory features were observed in organisms after 2 months of exposure to PS (Sussarellu et al., 2016). In comparison, a study conducted in *M. edulis* have shown an increase in ceroids and hemocyte infiltrations after 7 days of exposure to PS at 32 µg of microspheres/L (Paul-Pont et al., 2016). The reasons why very different

tissue responses can be found may lay in the lack of homogeneity in protocols used, diversity of MP shapes, and the lack of standardised techniques for quantification of MPs and reported concentration from the field (Phuong et al., 2016). The later may influence the calculation of "environmental concentration" used in laboratories and makes it difficult to compare the results between studies.

### 3.4 Physiological impact

During the exposure period, 10% of mortality was measured. Mean CI of oysters was not significantly modified between organisms from T0 (16.7 ± 3) and the control at T10 (16.6 ± 1.5) and between the control and other exposure concentrations (0.008 µg/L: 14.7 ± 1.3, 10 µg/L: 15.2 ± 1.3 and 100 µg/ L: 16.3 ± 2.3) at T10. CI values were close to previously published data on *C. gigas* for good physiological conditions (Seguin et al., 2016).

MPs did not induce any significant modification of the CR of oysters either during the exposure or the depuration period (Fig. 1). Comparing these results to literature is tricky as in previous studies (e.g. Wegner et al. 2012; Xu et al., 2016; Sussarellu et al., 2016; Oliveira et al., 2018) experimental conditions differ in MP particle nature, size, shape and/or concentration, all variables known to especially affect particle capture and selection by suspension-feeding bivalves (see the review by Ward and Shumway, 2004). However, in a previous study, we also observed that clearance rates of *Mytilus edulis* exposed to the same MP contamination (fragments of MP), were not modified compared to a control without MP particles (Revel et al. 2019). By contrast with some others studies that showed a decrease in filtration activity of bivalves exposed to MP particles (e.g. Xu et al., 2016; Oliveira et al., 2018), we can hypothesize that, in the conditions tested, the two species we studied deal with MP contamination not in regulating clearance rate to avoid their capture but through their depuration by egestion in biodeposits.

### 3.5 Oxidative stress

In gills and the digestive gland, no significant differences in CAT, SOD and GST activities were observed in organisms exposed to MPs compared to the control organisms. Only a tendency to an increase in CAT and SOD can be observed for digestive glands oysters exposed to 10 µg/L, and in CAT and GST activities for gills oysters exposed to 10 and 100 µg/L could be observed (Fig. 3).

Gene expression level of antioxidant enzymes Gpx, GST, SOD and CAT was not significantly modified in gills nor in the digestive gland (Fig. 4), and no differences in ROS content was observed between control and oysters exposed to 0.008, 10 or 100 µg of MPs/L (Fig. 5).

In this study, the exposure to the mixture of PE and PP MPs did not significantly modify the antioxidant enzyme activities in targeted organs which could be linked to the exposure, close to environmental conditions. However, we observed few tendencies and the lack of significant differences could be linked to the setup of the experiment and the number of oysters that could be exposed. Since we used environmental concentrations of MP without any dispersant, with daily water renewal, we chose to expose organisms to a reduced volume of water with 3 aeration systems to enable dispersion of MP. In this set-up, only a reduce number of oysters could then be exposed in aquariums and biomarkers were measured on 5 individuals instead of a pool of several (usually 3 to 5 pools of oysters) which is often used to limit the individual variability. Previous studies reported an increase in SOD and an inhibition of CAT activity in mussel *M. edulis* and clams *S. plana* exposed to PS MPs respectively at 32 µg of microspheres/L (2 and 6 µm) (Paul-Pont et al., 2016) or 1 mg/L (20 µm) (Ribeiro et al., 2017). To the opposite, CAT induction was measured in the zebra mussel *Dreissena polymorpha* after 6 days of exposure to two sizes of PS microbeads (1 µm: 5 x $10^5$ MPs/L and

10 µm: 5 x $10^5$ MPs/L) (Magni et al. 2018). In our study, the ROS contents in oysters exposed to MPs were not significantly different from the controls. Other studies reported increase in ROS production but only after exposure to very high concentrations of MPs. Increase in ROS production in the sheepshead minnow exposed for 4 days to PE microspheres of spherical (150 - 180 µm) or fragmented form (300 - 355 µm; 50 - 250 mg/L) was reported in Choi et al. (2018).

Very recently, authors started evaluating the Multixenobiotic resistance system (MXR) response to MPs. Franzelitti et al. (2019) have shown a different modulation of MXR according to MP size or particle concentration in mussel *M. galloprovincialis* suggesting that modulation of MXR activity could be a defensive response following MP ingestion. However, exposure was relatively short (4 days) and chronic studies should be conducted to better understand the impact of MPs on MRX in oysters.

### 3.6 Immune marker and DNA damage

Very low activity of AcP was measured in oysters with 0.0059 ± 0.0011 U/mg of proteins in control organisms and 0.0036 ± 0.0007, 0.0036 ± 0.0014 and 0.0041 ± 0.0007 U/mg of proteins in organisms exposed to the MP mixture at 0.008, 10 and 100 µg/L respectively. Previous studies have recognized hydrolase AcP activity as an appropriate marker to evaluate immune system impairments in bivalves from polluted environments (Wootton et al., 2003). Our results showed no alteration in AcP activity in oysters exposed to MPs. By contrast, previous work have shown the induction of AcP in bivalves exposed to nanoparticles or herbicides (Buffet et al., 2014; Matozzo et al. 2018).

Genotoxic effects of MPs, analysed by the comet assay and expressed as % of tail DNA, are shown in Figure 5. No statistically significant increase in DNA damage from oysters exposed to MPs was observed which is consistent with the fact that we did not

observe any increase in ROS level. The comet assay is a rapid and sensitive test enabling the detection of DNA strand breaks and has been developed for various aquatic organisms including bivalves and gastropods (Frenzilli et al., 2009). Increase in DNA damage following MP exposure was previously observed in bivalves but for exposure concentrations up to two orders of magnitude higher than those tested in the present study: nano-PS at 0.05 - 50 mg/L (Brandts et al., 2018), PE at 1.5 g/L (Avio et al., 2015) in mussel *M. galloprovincialis,* and PS at 1 mg/L in clam *S. plana* (Ribeiro et al., 2017).

## 4. Conclusion

In the present study, oysters *C. gigas* exposed to a mixture of PP and PE MPs with various shapes and wide size range (<400 µm) for 10 days showed no accumulation in tissues but MPs were observed in biodeposits. These results confirmed an uptake of MPs from the water column. No significant modification of the clearance rate, tissue integrity, oxidative stress, immune parameter and DNA damage was observed. Thus, our results suggest that PE and PP fragments (<400µm) represent a limited threat towards the Pacific oyster *C. gigas* despite ingestion but longer-term studies should be conducted to investigate potential chronic effect of MPs on oysters.

Although there is evidence that plastic particles can induce oxidative stress, generate inflammatory, genotoxic and neurotoxic effects, there is still a poor understanding of MP toxicity and even sometimes contradictory results. Moreover, despite the increasing number of studies on the presence and toxicity of MPs to marine fauna, the assessment of MP impact remains a challenge since in aquatic ecosystems animals are exposed to a variety of polymers with different compositions, shapes and sizes. There is a lack of standardised protocols and units (particles/L, particles/m², etc.) used for MPs concentration which makes it difficult to compare results between the studies. In laboratory experiments, there is also various

parameters that need to be considered such as the choice of MPs (microbeads versus fibres or fragments), the exposure concentrations and the dispersion technique (use of solvents or air pump) which differs greatly between studies. MPs can also be associated with additives (phthalates, bisphenol A, flame retardant, etc.) or environmental contaminants such as persistent organic pollutants which can also induce toxic effects.

This study shows no effect of PP and PE MPs in oysters considering different endpoints from molecular to tissue levels. Mechanisms of uptake by gills, digestive system and eventually translocation of the ingested particles into the circulation system and tissues should other be investigated.

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**Figures and tables caption**

**Table 1**. Average concentration and size range of PE and PP microplastics recovered from biodeposits (faeces and pseudofaeces) of oysters *Crassostrea gigas* after 10 days of exposure to PE and PP.

**Fig. 1**. Algal reduction measured with a cell counting chamber (Malassez) for different times of incubation in metabolic chambers filled each with an individual oyster *Crassostrea gigas* after 10 days of exposure to 0 MPs/L, or to PE and PP microplastics at 0.008, 10 and 100 µg MPs/L (mean ± SD, N=4).

**Fig. 2**. Levels (average index + SEM, N=5) of tissue alterations in *Crassostrea gigas* observed by histology at T0 and after 10 days in control oysters ("0") and in oysters exposed to PE and PP microplastics at 0.008, 10 and 100 µg MPs L-1. A: Hemocytic infiltrations in the digestive tract; B: Diffuse hemocytic infiltrations in the connective tissue; C: Hemocyte aggregations in the connective tissue; D: Mantle and palps destructuration (poor condition).

**Fig. 3**. Enzymatic activity of GST, SOD and CAT in gills and digestive gland of oysters *Crassostrea gigas* at T0 and after 10 days in control oysters (0 MPs/L) and in oysters exposed to PE and PP microplastics at 0.008, 10 and 100 µg MPs/L (mean ± SD, N=5).

**Fig. 4**. Relative gene expression for GPx, GST, CAT and SOD in gills and digestive gland of oysters *Crassostrea gigas* at T0 and after 10 days in control oysters (0 MPs/L) and in oysters exposed to PE and PP microplastics at 0.008, 10 and 100 µg MPs/L (mean ± SD, N=5).

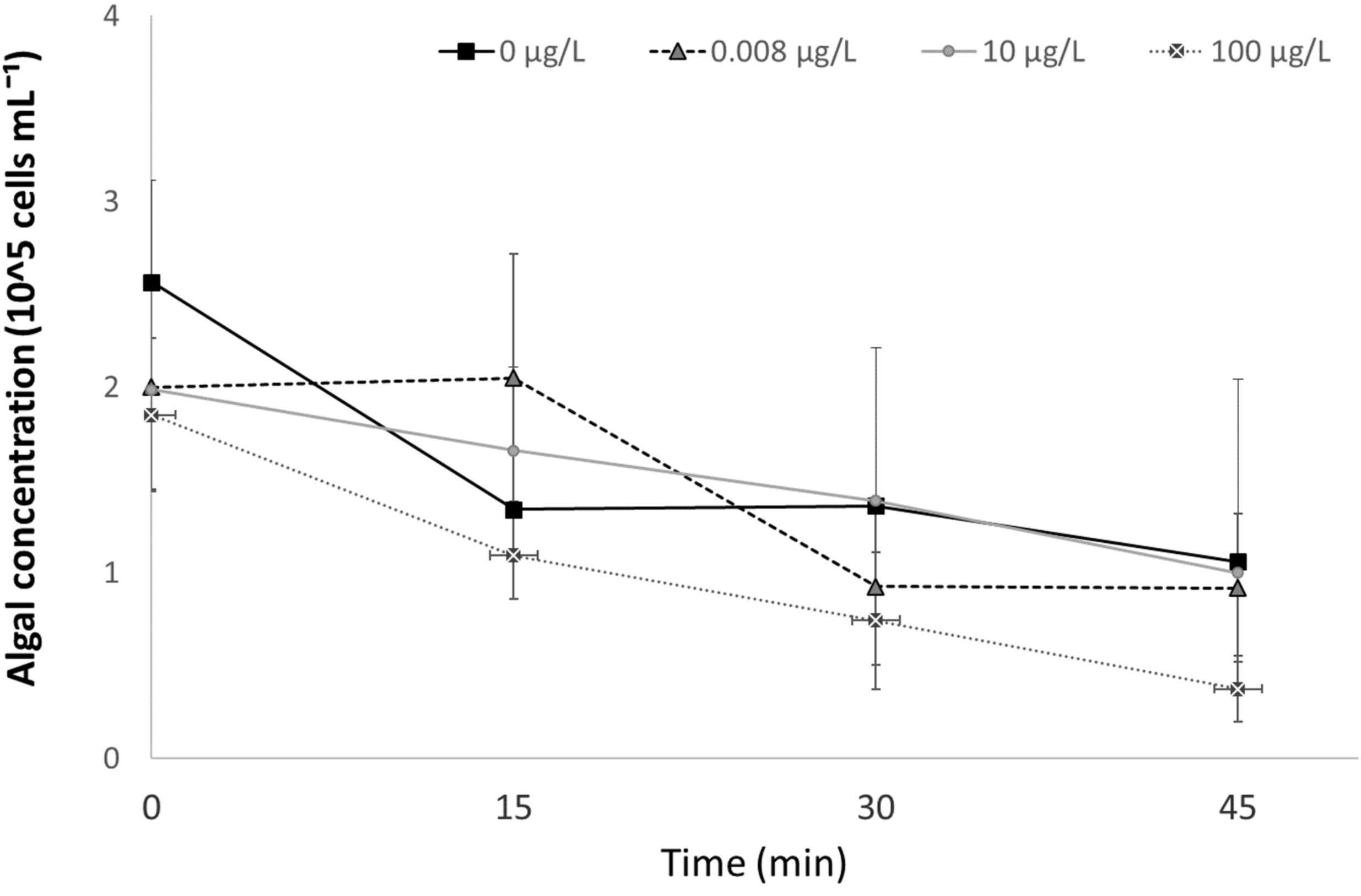
**Fig. 5**. ROS production (A) and DNA integrity (B) of oysters *Crassostrea gigas* after 10 days in control oysters (0 MPs/L) and in oysters exposed to PE and PP microplastics

at 0.008, 10 and 100 µg MPs/L (mean + SD, N=3 to 4 for ROS; n=5 for DNA integrity).

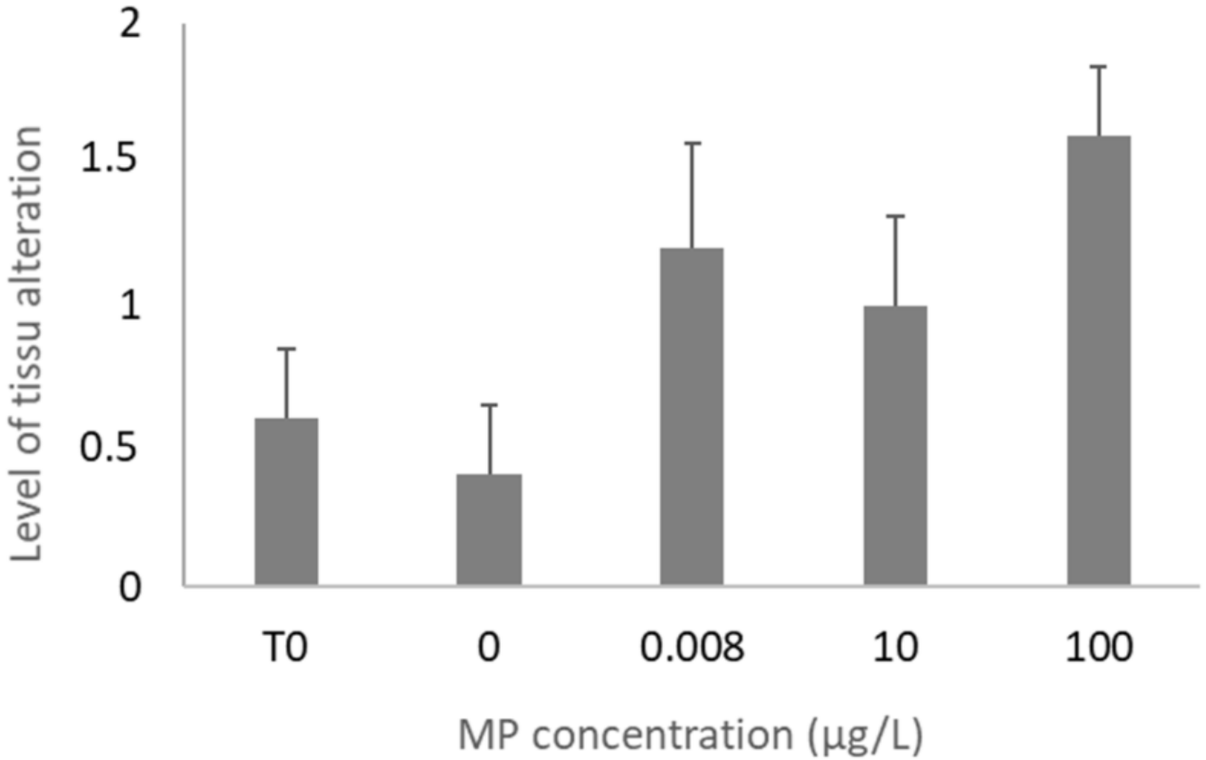
## Supplementary material

**1:** Detailed sampling and analysis conducted on oysters. CR = clearance rate; Histo = histopathology; AcP = acid phosphatase (immune marker); OS = oxidative stress; CI = condition index; MPs content = analysis of microplastics in tissues.

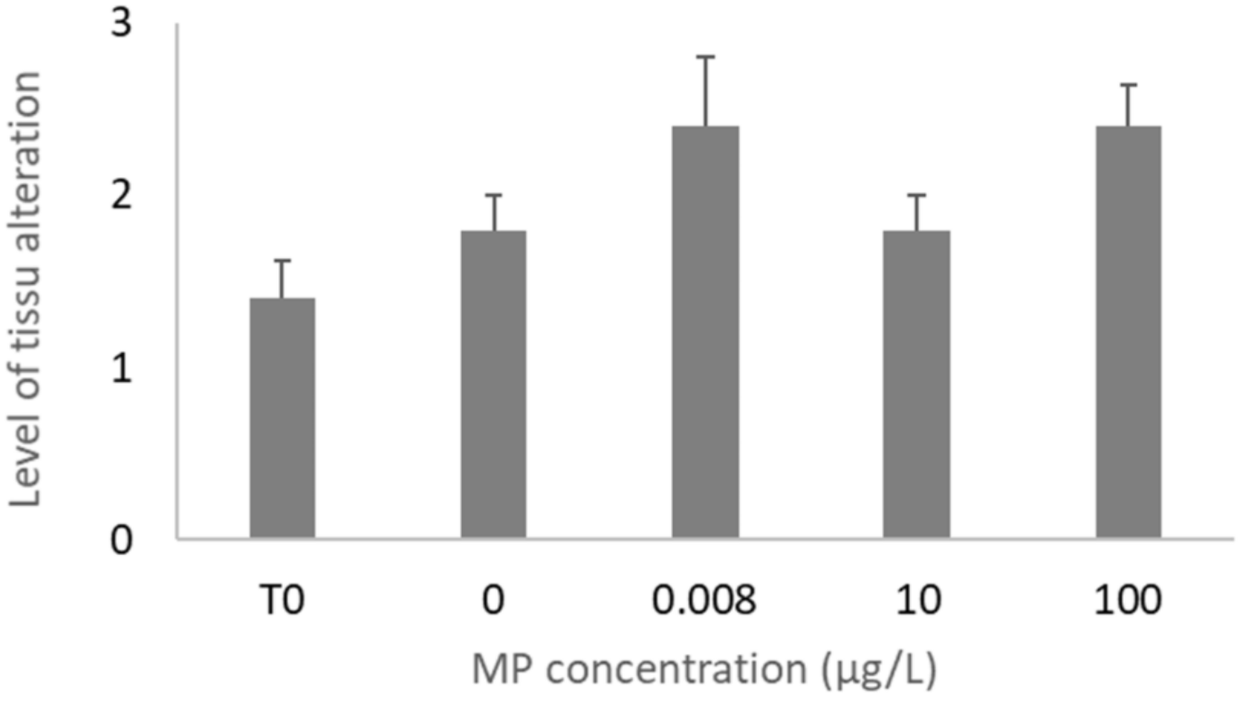
**2:** Forward and reverse prime sequences for Real-Time PCR analysis and GeneBank accession numbers.


0 µg/L
0.008 µg/L
10 µg/L
100 µg/L
Algal concentration (10^5 cells mL⁻¹)
4
3
2
1
0
0
15
30
45
Time (min)

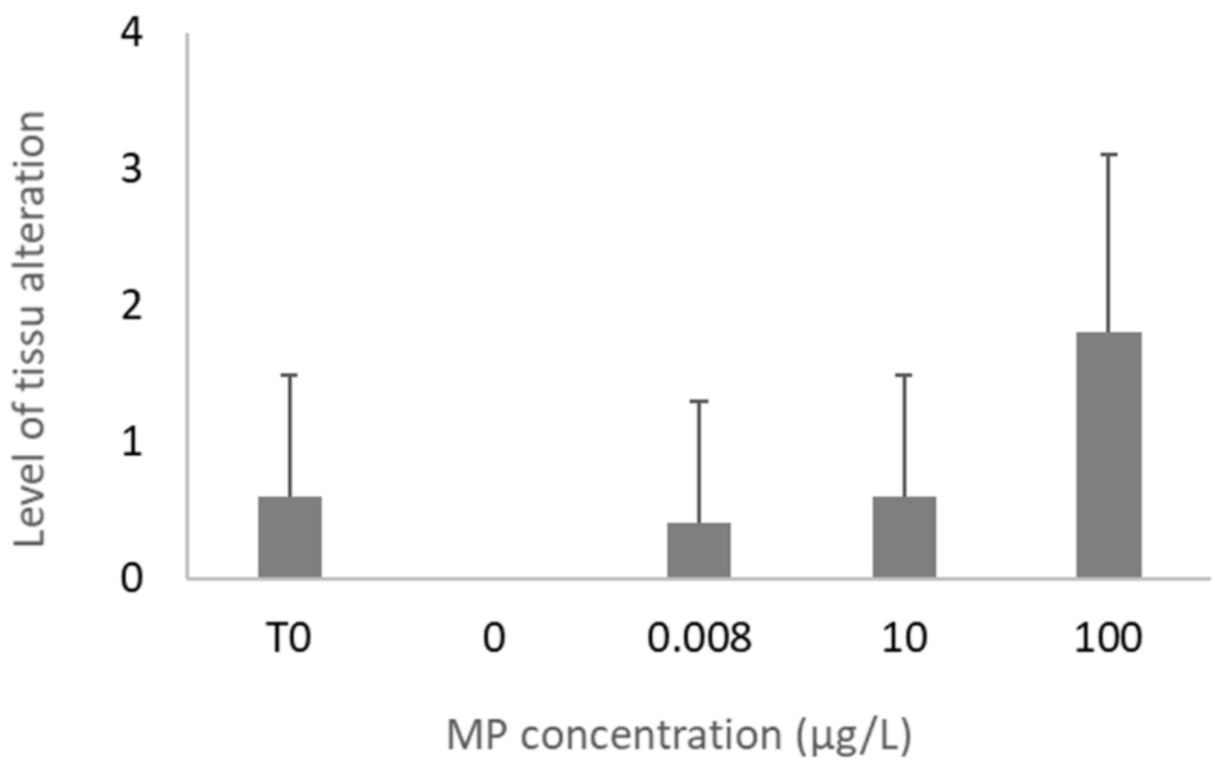
A


Level of tissu alteration
2
1.5
1
0.5
0
T0
0
0.008
10
100
MP concentration (µg/L)

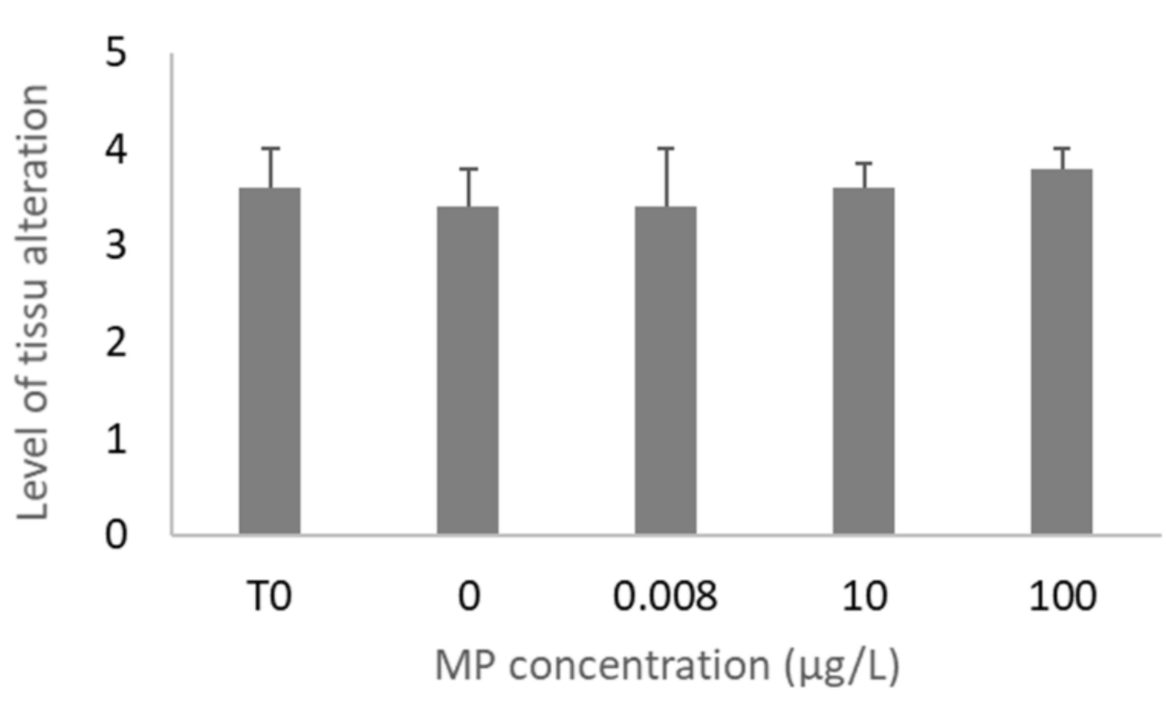
B


Level of tissu alteration
3
2
1
0
T0
0
0.008
10
100
MP concentration (µg/L)

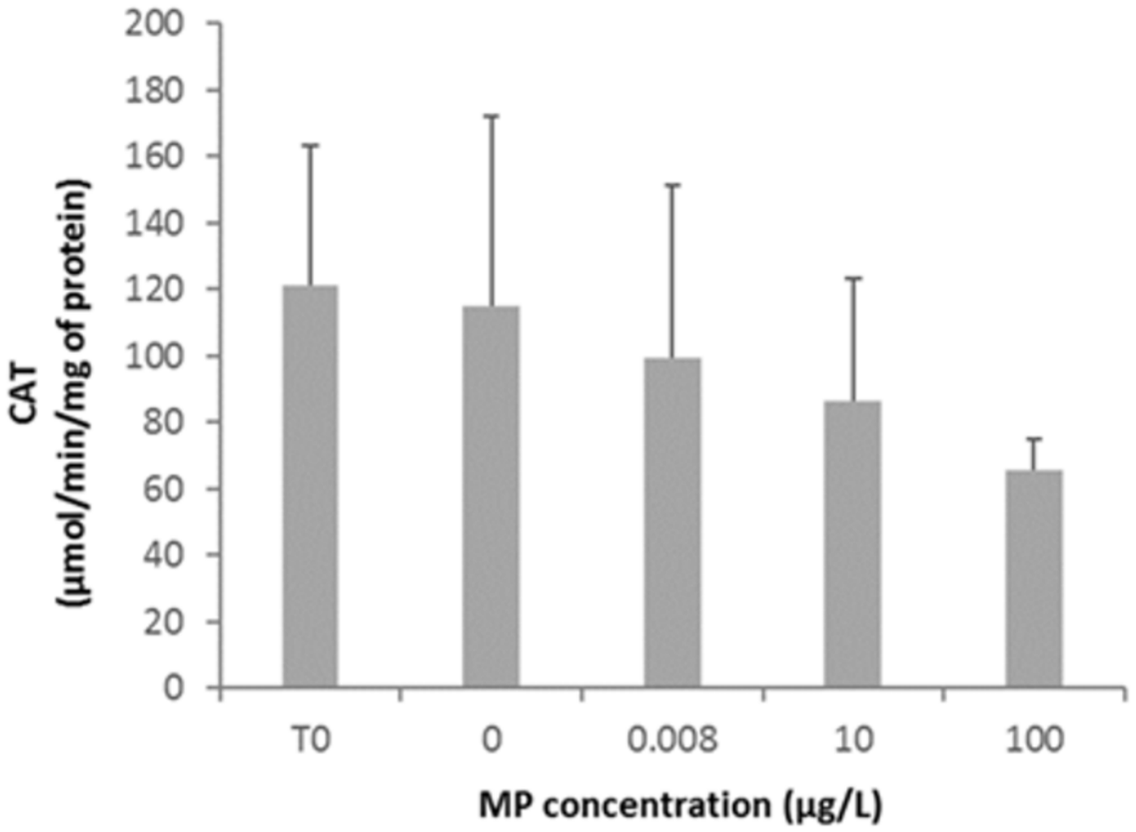
C


Level of tissu alteration
4
3
2
1
0
T0
0
0.008
10
100
MP concentration (µg/L)

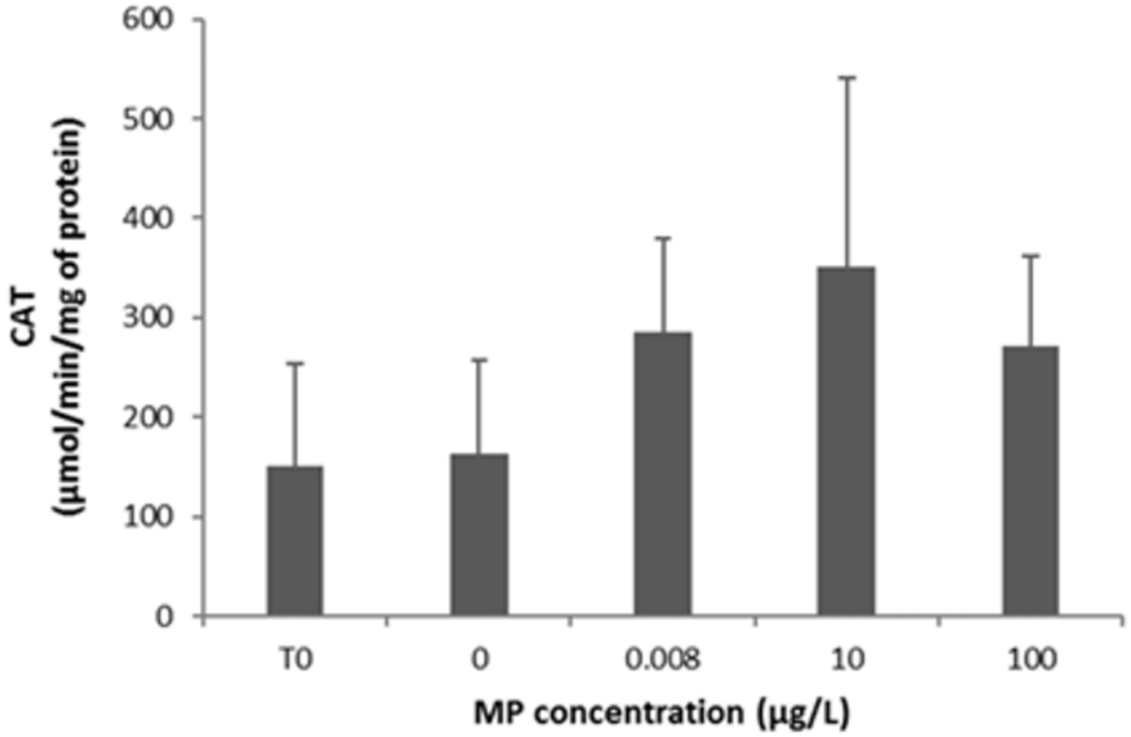
D

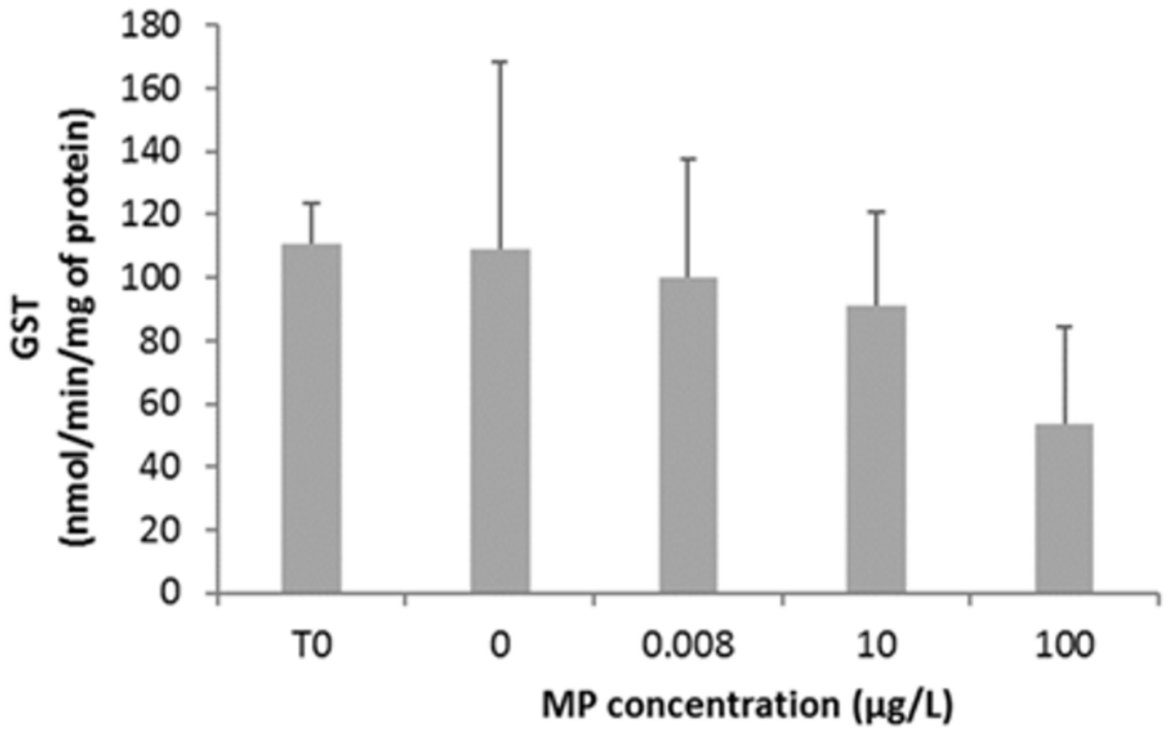

Level of tissu alteration
5
4
3
2
1
0
T0
0
0.008
10
100
MP concentration (µg/L)

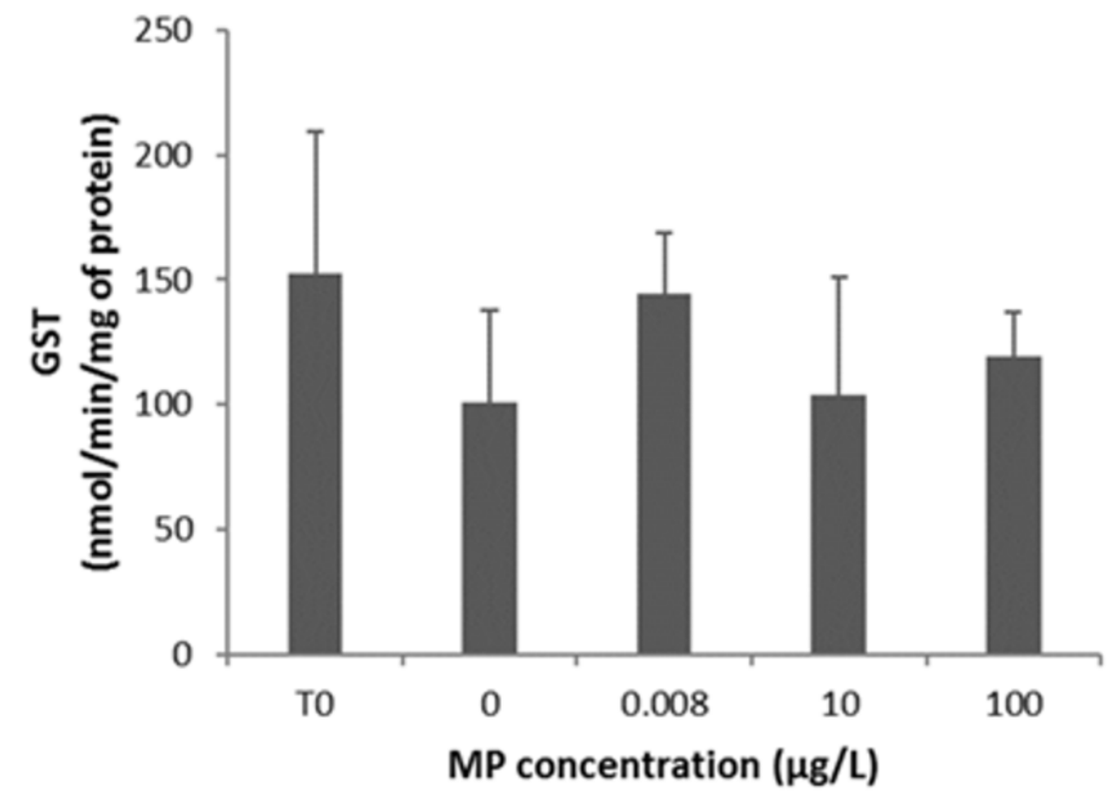
Gills

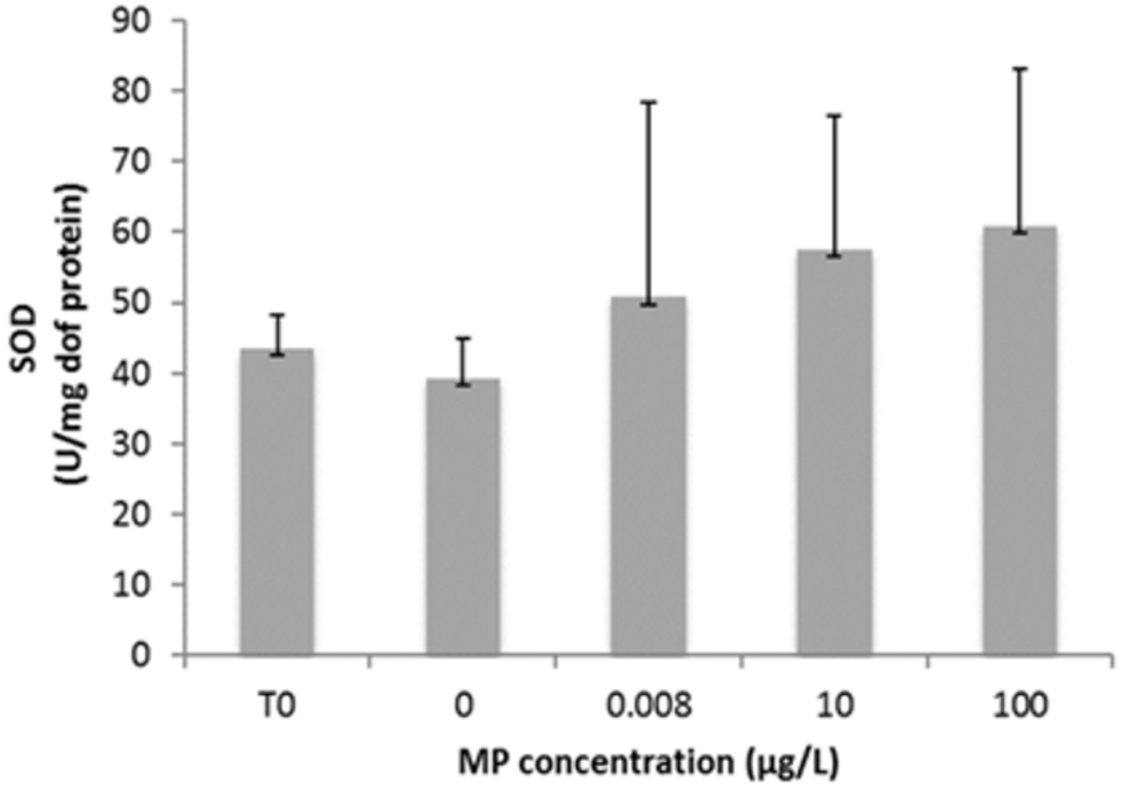


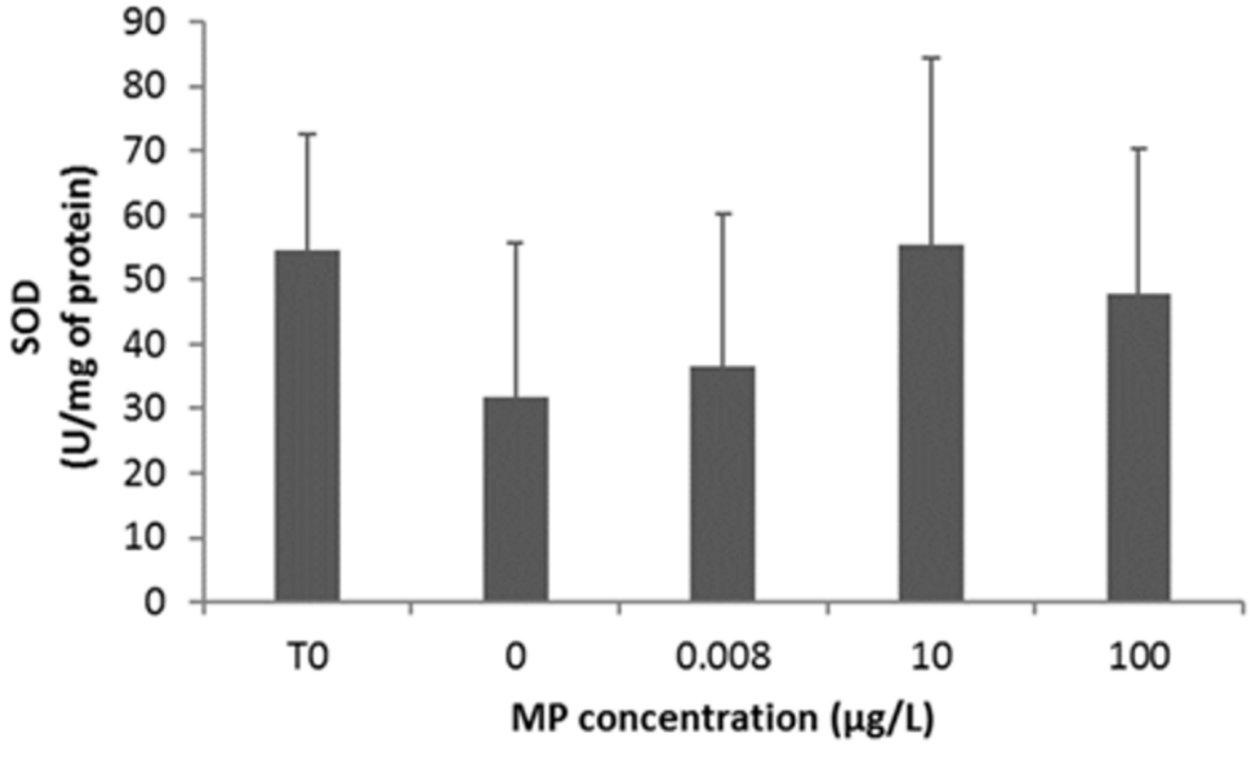

Digestive gland

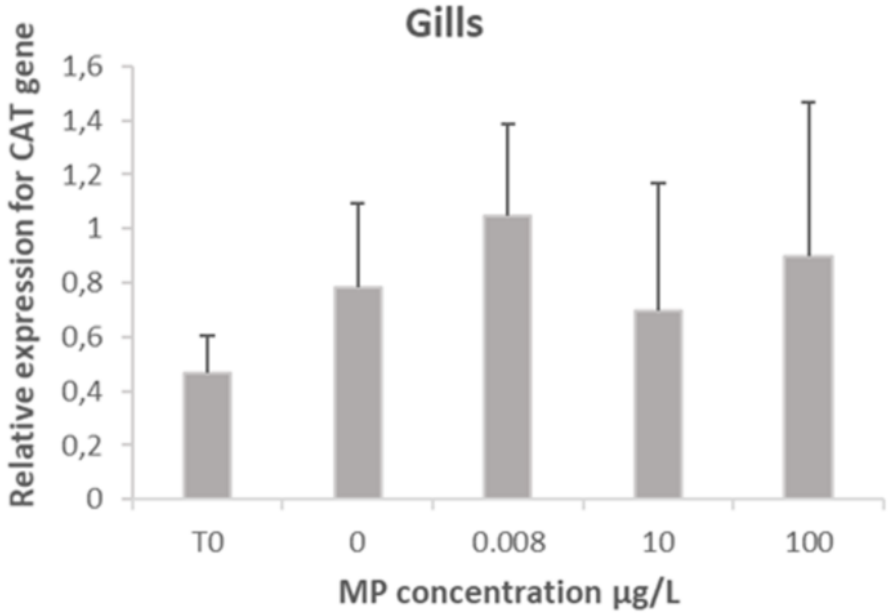


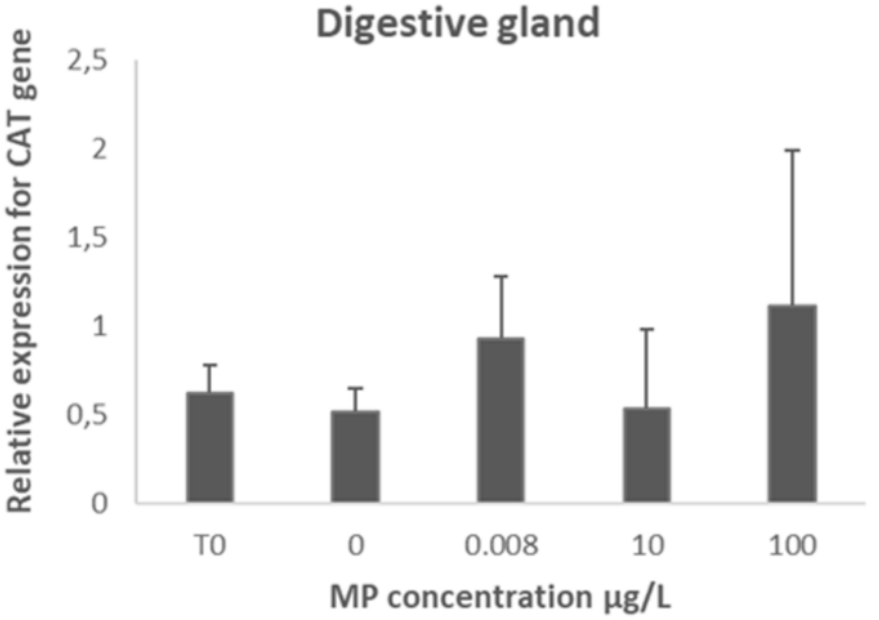



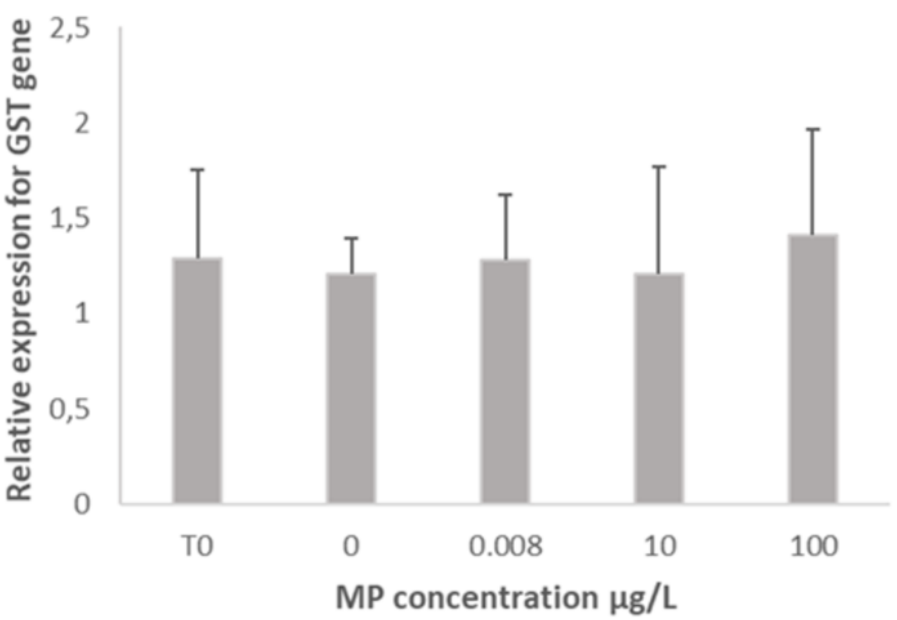



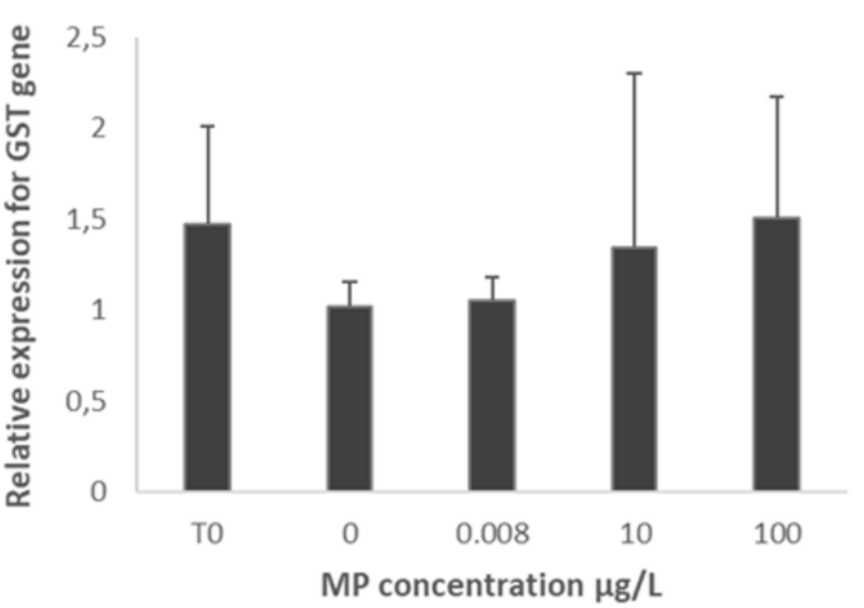



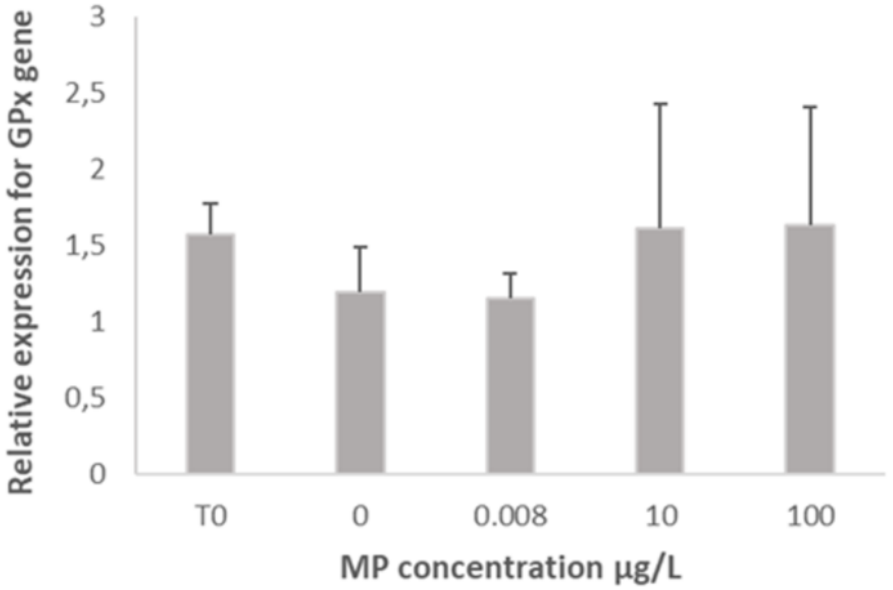



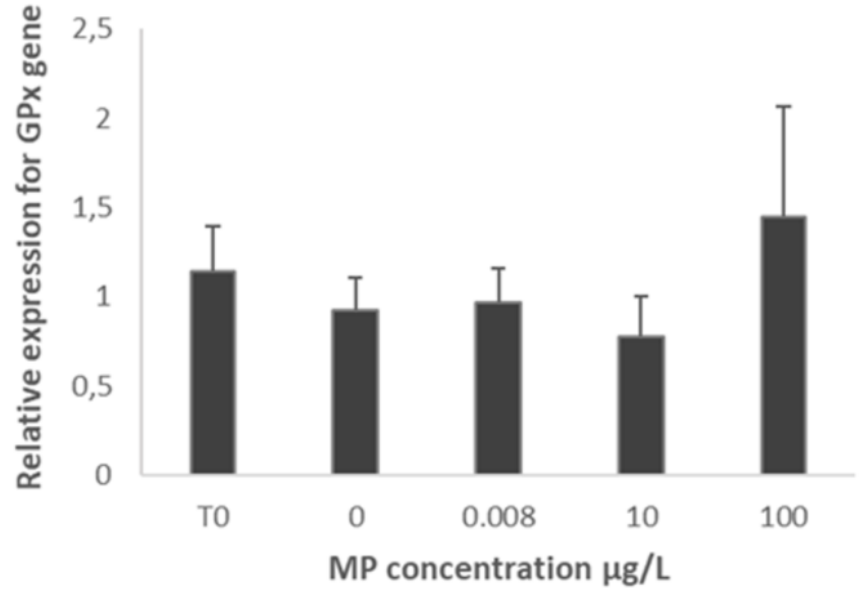

Gills
Relative expression for CAT gene
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
T0
0
0.008
10
100
MP concentration µg/L

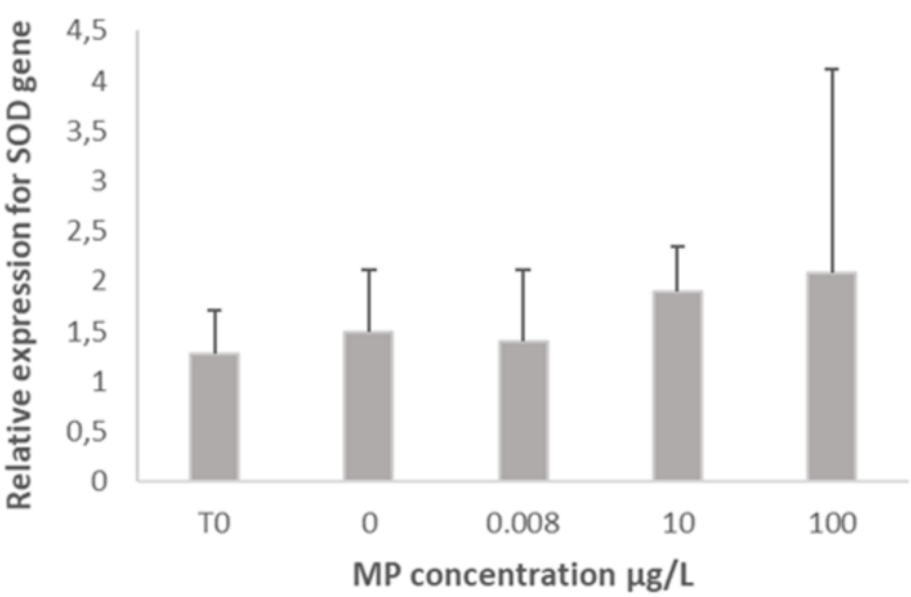

Digestive gland
Relative expression for CAT gene
2,5
2
1,5
1
0,5
0
T0
0
0.008
10
100
MP concentration µg/L

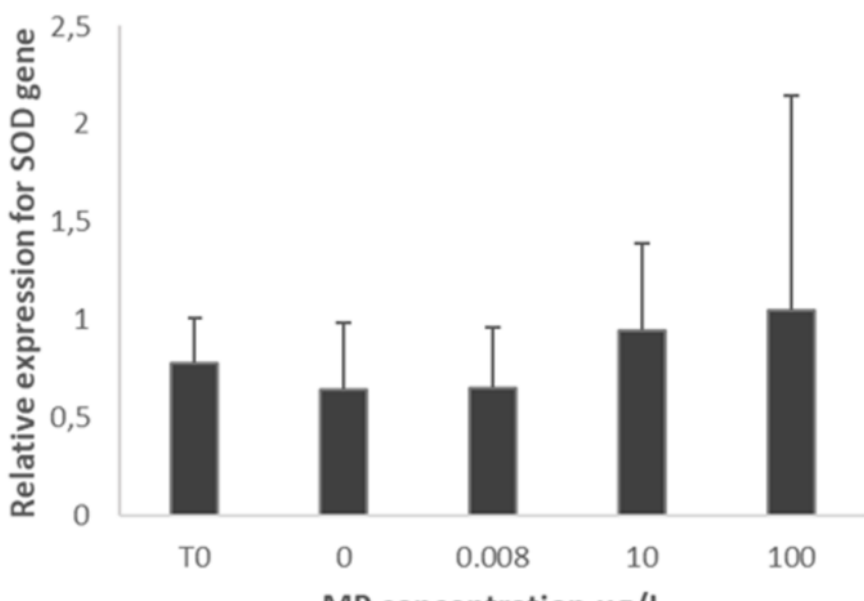

Relative expression for GST gene
2,5
2
1,5
1
0,5
0
T0
0
0.008
10
100
MP concentration µg/L


Relative expression for GST gene
2,5
2
1,5
1
0,5
0
T0
0
0.008
10
100
MP concentration µg/L

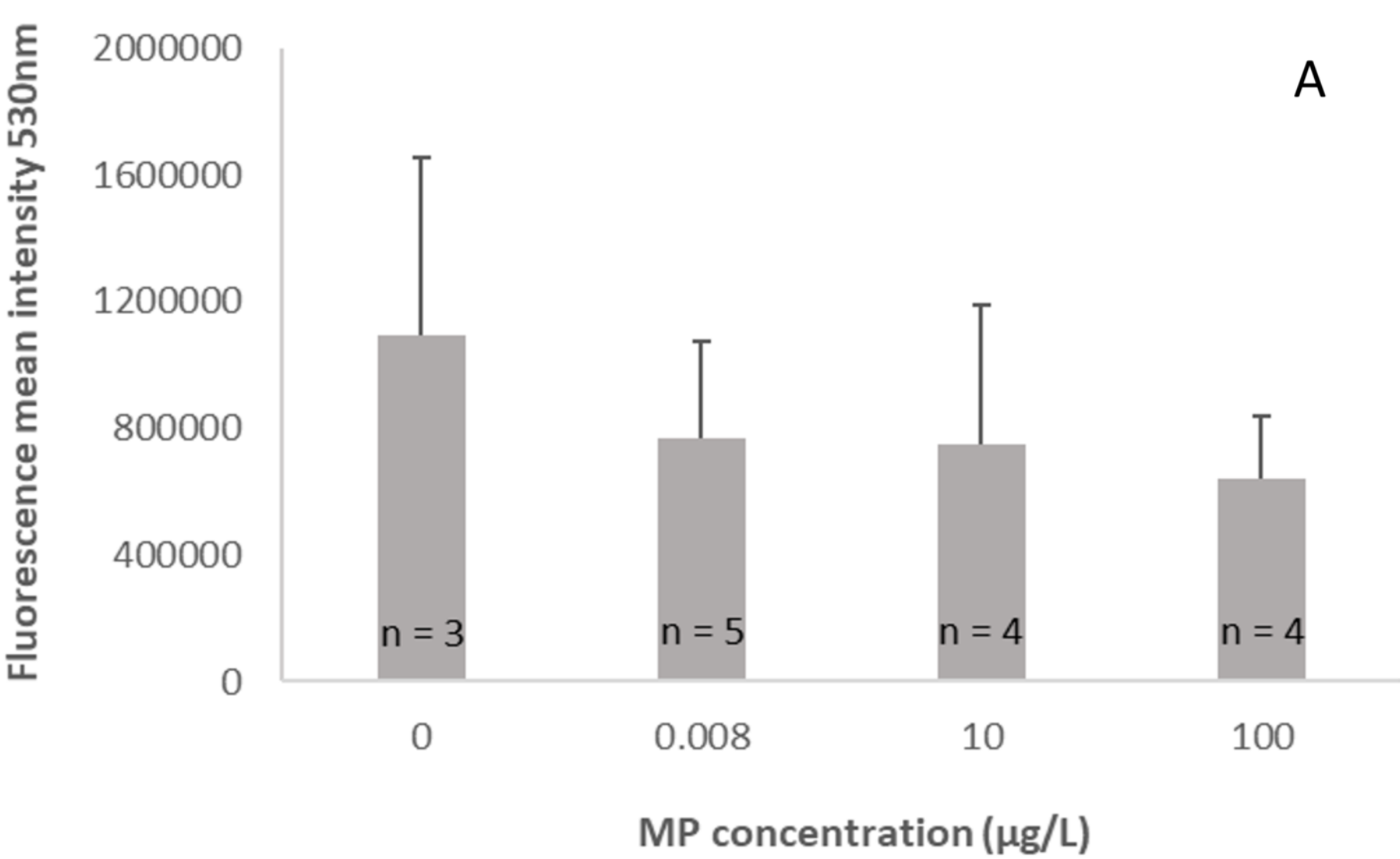

Relative expression for GPx gene
3
2,5
2
1,5
1
0,5
0
T0
0
0.008
10
100
MP concentration µg/L


Relative expression for GPx gene
2,5
2
1,5
1
0,5
0
T0
0
0.008
10
100
MP concentration µg/L

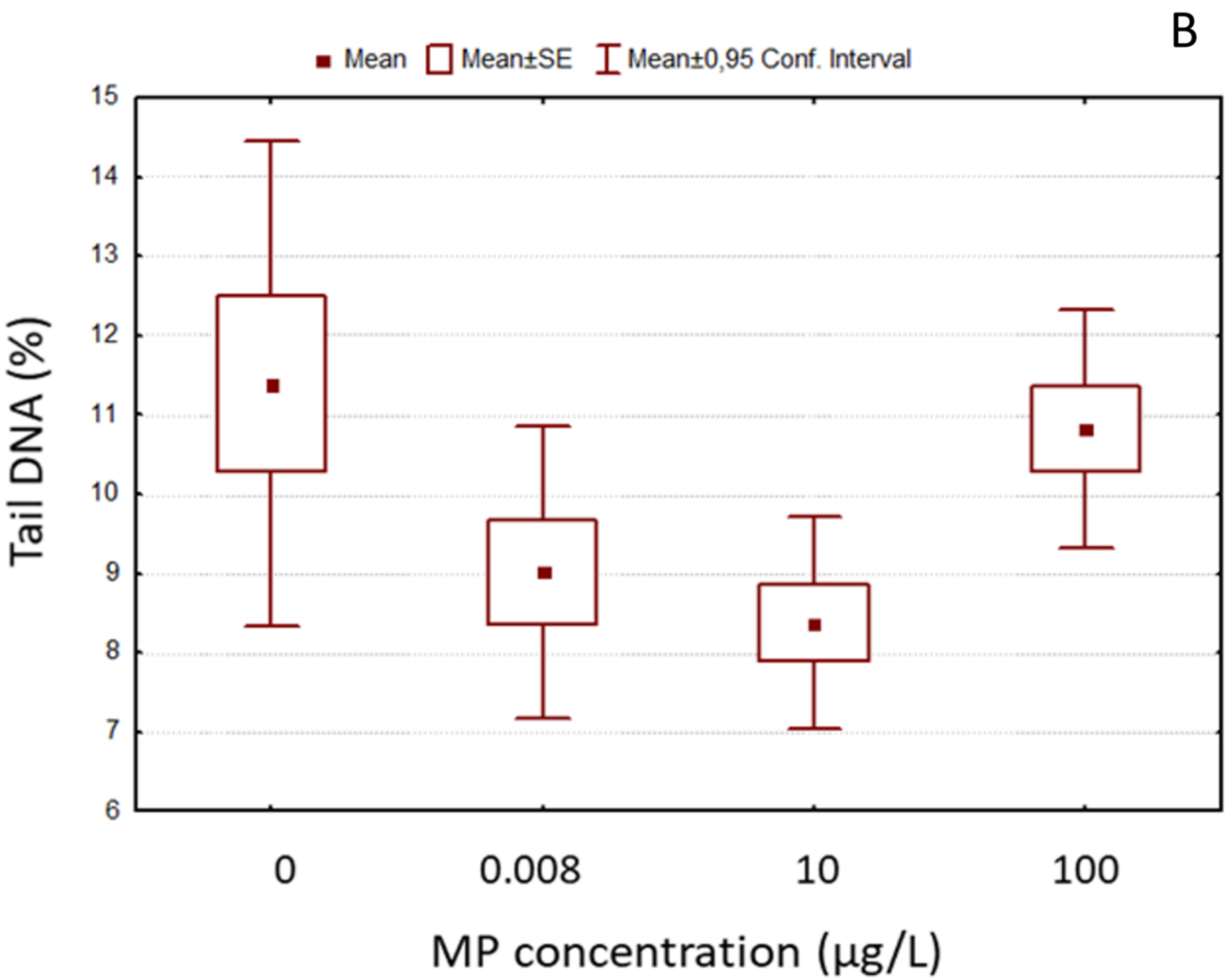

Relative expression for SOD gene
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
T0
0
0.008
10
100
MP concentration µg/L


Relative expression for SOD gene
2,5
2
1,5
1
0,5
0
T0
0
0.008
10
100
MP concentration µg/L

A


Fluorescence mean intensity 530nm
2000000
1600000
1200000
800000
400000
0
n = 3
n = 5
n = 4
n = 4
0
0.008
10
100
MP concentration (µg/L)

B


Mean
Mean±SE
Mean±0,95 Conf. Interval
Tail DNA (%)
15
14
13
12
11
10
9
8
7
6
0
0.008
10
100
MP concentration (µg/L)

| Treatment | Number of PE and PP particles | Average concentration | Size range in µm |
|---|---|---|---|
| Control (0µg/L) | 0 | - | - |
| 0.008 µg/L | 2 PP | 0.003 MPs/mg | 40 - 240 |
| 10 µg/L | 1 PE 3 PP | 0.006 MPs/mg | 120 - 240 |
| 100 µg/L | 27 PE, 12 PP | 0.056 MPs/mg | 40 - 320 |