Does salinity variation increase synergistic effects of triclosan and carbon nanotubes on Mytilus galloprovincialis? Responses on adult tissues and sperms

Lucia De Marchi, Rosa Freitas, Matteo Oliva, Alessia Cuccaro, Chiara Manzini, Federica Tardelli, Madalena Andrade, Marcelo Costa, Carla Leite, Andrea Morelli, Federica Chiellini, Carlo Pretti

PII: S0048-9697(20)32354-8

DOI: https://doi.org/10.1016/j.scitotenv.2020.138837

Reference: STOTEN 138837

To appear in: *Science of the Total Environment*

Received date: 10 March 2020

Revised date: 17 April 2020

Accepted date: 18 April 2020

Please cite this article as: L. De Marchi, R. Freitas, M. Oliva, et al., Does salinity variation increase synergistic effects of triclosan and carbon nanotubes on Mytilus galloprovincialis? Responses on adult tissues and sperms, *Science of the Total Environment* (2020), https://doi.org/10.1016/j.scitotenv.2020.138837

Does salinity variation increase synergistic effects of triclosan and carbon nanotubes on *Mytilus galloprovincialis*? Responses on adult tissues and sperms

Lucia De Marchi[1,2], Rosa Freitas[4*], Matteo Oliva[1,3], Alessia Cuccaro[1,4], Chiara Manzini[1], Federica Tardelli[1], Madalena Andrade[4], Marcelo Costa[4], Carla Leite[4], Andrea Morelli[5], Federica Chiellini[5], Carlo Pretti[1,3]

[1]Interuniversity Consortium of Marine Biology of Leghorn "G. Bacci", 57128, Livorno, Italy.

[2]Department of Biology, University of Pisa, Via Derna 1, 56126, Pisa, Italy

[3]Department of Veterinary Sciences, University of Pisa, San Piero a Grado, 56122, Pisa, Italy

[4] Centre for Environmental and Marine Studies (CESAM) & Department of Biology &, University of Aveiro 3810-193, Aveiro, Portugal

[5]Department of Chemistry and Industrial Chemistry, University of Pisa, Udr INSTM Pisa, 56126, Pisa, Italy

[*]Corresponding Author: Rosa Freitas, Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal; telef +351 234 370 782 (ext 22739) | mobile: +351 914525095; email: rosafreitas@ua.pt

ABSTRACT

The use of carbon nanotubes (CNTs) is rapidly increasing and several scientific studies have addressed their toxicological properties. However, only a very small number of publications have deal with the interaction between CNTs and other molecules. Triclosan (TCS) is an antibacterial agent used in personal care and household products. Commonly detected in aquatic ecosystems, there is a strong evidence that aquatic biota is sensitive to this compound. Aside from emergent pollutants, aquatic organisms are continuously subjected to abiotic variations including salinities. Therefore, the main goal of the present study was to better understand how physio-chemical interactions of CNTs with TCS under different salinity levels (37, 28 and 19) affect the mussel species *Mytilus galloprovincialis* through the evaluation of biochemical alterations on gametes (sperms) and adult tissues, providing more ecologically relevant information on organisms' responses. The results showed toxicological effects in terms of sperm metabolic activity and intracellular reactive oxygen species production as well as cellular damage and alteration of metabolic capacity at the adult's stage when exposed to both contaminants acting alone and in combination, under tested salinities. Moreover, when the mussels were exposed to the combination of both contaminants, they showed major toxic impacts on both assessed biological levels (adult tissues and sperms) especially under control salinity. This suggests that toxicity upon mixture exposure compared to single-substance exposure may impair mussels' populations, affecting reproduction success and growth.

Keywords: *Mytilus galloprovincialis***,** synergistic effects, carbon nanotubes, triclosan, sperm quality parameters, oxidative stress

1. INTRODUCTION

The industrial production of Carbon nanotubes (CNTs), one of the most important and widely used nanomaterials, as well as their applications are exponentially increasing (Freixa et al., 2018; Morozesk et al., 2018), together with the knowledge on their environmental toxicity (De Marchi et al., 2019a). As most recent literature shows, the predicted environmental concentrations (PECs) of CNTs were projected to be 0.05–5 mg/kg in biosolids (Keller and Lazareva, 2014) and 0.001-1000 μg/L in aquatic environment (Keller and Lazareva, 2014; Zhang et al., 2017), already showing toxic effects in exposed organisms depending on their physical and chemical characteristics (size, shape, surface area), diffusion capacity, aggregation/agglomeration properties in suspension, functionalization and their interactions with surrounding environments (Handy et al., 2012; He et al., 2014). Available literature already demonstrated the interaction between CNTs and cells which include cellular uptake, effects on cell signalling, membrane perturbations (De Marchi et al., 2018, 2019b), production of reactive oxygen species (ROS), and cell apoptosis (Zhao et al., 2012). Indirect non-specific toxic effects of CNTs have been also found in aquatic organisms, such as physical irritation and occlusion of surface tissues (e.g., gills) (Oberdörster et al., 2006), growth inhibition and genotoxicity (Mouchet et al., 2008). Nevertheless, CNTs toxicity may also result from the interaction with other contaminants that influence bioaccumulation of each co-exposed contaminant and the induced effects (Sun et al., 2009). The existing literature on the toxicity of CNTs co-existing with other contaminants is still limited but with a recent development (Deng et al., 2017). For example, Freitas et al. (2018) evaluated the impacts of Arsenic (As) (0.1 mg/L) and carboxylated Multi-Walled Carbon NanoTube (MWCNT-COOH) (0.1 mg/L) in the clam *Ruditapes philippinarum*, showing that although the accumulation of As was not affected by the presence of the CNTs, higher neurotoxicity was observed in clams exposed to the combination of both contaminants in comparison to the effects of As and CNTs individually. In another study conducted by Morozesk et al. (2018), which investigated the co-exposure of oxidized MWCNTs (ox-MWCNT) and cadmium (Cd) using a zebrafish liver cell line (ZFL), the authors demonstrated that

Cd presence in the medium did not interfere in protein corona composition of MWCNT but interfered in its colloidal stability and metal adsorption rate. Moreover, the ox-MWCNT increased Cd toxicity at low concentration probably by a synergistic effect, and induced apoptosis and necrosis in ZFL cells. Thus, the potential risks associated with the combined toxicity of contaminants and CNTs need to be evaluated.

Besides nanomaterials, Triclosan [5- chloro- 2- (2,4- dichlorophenoxy) phenol] (TCS) is one of the most common pollutant in the aquatic environment (Dhillon et al., 2015; Freitas et al., 2019). It is a non-ionic, broad spectrum antimicrobial used as an ingredient in disinfectants, soap, detergent, toothpaste, mouthwash, fabric, deodorant, shampoo and plastic additives, in addition to other personal care, veterinary, industrial and household products (Dann et al., 2010 The use of TCS is not highly regulated as it has a low acute toxicity and is generally accepted as well tolerated. Therefore, it is commonly detected in aquatic ecosystems in a range of µg/L (Dann et al., 2010; Olaniyan et al., 2016) showing that aquatic species are sensitive to TCS in this range of concentrations. Oliveira et al. (2009) evaluated the toxicity of TCS in zebrafish (*Danio rerio*) in embryos and adults' showing acute toxicity for embryo/larvae (96 h LC50 = 0.42 mg/L) and delayed hatching, evidence of embryo toxicity and altered biomarker responses, e.g. increased of cholinesterase (ChE), glutathione S-transferase (GSTs) and lactate dehydrogenase (LDH) levels. Moreover, TCS also presented acute toxicity in adult zebrafish (96 h LC50 = 0.34 mg/L). Canesi et al. (2007) demonstrated that in *M. galloprovincialis*, TCS acted on kinase-mediated cell signalling, lysosomal membranes and redox balance in different systems/organs in a low µM range concentrations.

Aside from emerging pollutants, aquatic organisms are also continuously exposed to environmental factors which influence the ecosystems biodiversity. For these, the Working Group on Biological Effects of Contaminants (WGBEC) proposed the use of hydrographic data (temperature, salinity, etc.) for the interpretation of biological effects in an integrated monitoring programme to assess ecosystem health (water, sediment, biota) (Hamer et al., 2008). In particular,

the seawater salinity changes have been considered form years one of the most important determinants (stressors) of organisms' distribution in rocky intertidal zones (Anestis et al., 2007). Permanent low salinities, frequency of salinity changes, as well as the changing rate of salinity have been demonstrated influencing the filtration and growth rates, maximum shell length, and early development and survival of mussel's species (Westerbom et al., 2002; Qiu et al., 2002). Considering that in intertidal zones and estuaries salinity can vary significantly from 4 to 38 (Hamer et al., 2008), the main goal of the present study was to better understand how interactions between CNTs and TCS under different salinity levels affects the mussel species *Mytilus galloprovincialis* through the evaluation of biochemical alterations on different biological levels (gametes and adult tissues), providing more ecologically relevant information on organisms' performance under different salinity fluctuations .

2. MATERIALS AND METHODS

2.1 Experimental set-up

Mytilus galloprovincialis were collected in the Gulf of La Spezia (Northwest Tyrrhenian coast, Italy, 44°03′50″N; 9°53′00″E). Organisms were transported to the laboratory and kept into 40 L natural seawater (NSW) (filtered at 0.45 µm: FNSW) under continuous aeration (oxygen saturation > 90%), at salinity 37.22 ± 1 and pH 8.1 ± 0.1 for two weeks' acclimation period. During this period, specimens were fed with AlgaMac Protein Plus, Aquafauna Bio-Marine every two-three days. After this period, specimens (3 *per* aquarium/ 9 *per* condition) were exposed to MWCNTs and TCS considering them as single or combined stressors, under three different salinities: I) 100% FNSW (salinity 37) used as controls; II) 75% FNSW (salinity 28) and III) 50% FNSW (salinity 19) (Hamer et al., 2008). For each salinity level, the following treatments were performed: CTL (control, 0.0 mg/L TCS and CNTs); TCS (1.0 µg/L); CNTs (0.10 mg/L); TCS+CNTs (1.0 µg/L TCS and 0.10 mg/L CNTs). The concentrations of both pollutants were chosen as environmental relevant concentration that have been found in aquatic system (Olaniyan et al., 2016; Zhang et al., 2017). The added contaminants were suspended in seawater and re-established weekly after complete water renewals to ensure the same exposure concentrations during the experiment. Moreover, both contaminants were homogenously dispersed using aeration tube one *per* aquarium, increasing both CNTs and TCS mass suspended in the water column. For each condition, 9 organisms were distributed into 3L aquaria (3 organisms *per* aquarium) for 28 days. Prior to experiment initiation and to avoid any osmotic stress, the salinity was progressively decreased (2 units) every 2 days until the testing values were reached, while the other parameters (pH, temperature and aeration conditions) in each aquarium were set up as in the acclimation period.

2.2 Materials description

2.2.1 Carbon nanotubes

CNTs were purchased from Times Nano: Chengdu Organic Chemicals Co. Ltd., Chinese Academy of Sciences (MWCNTs-COOH: TNMC1 series, http://www.timesnano.com) with manufacturer specifications of: diameter 2-5 nm; length 10-30 μm; carbon purity 98%; surface area 400m^2/g; amorphous carbon 8-10% and -COOH 3.86 wt%). The functionalized MWCNTs were produced by catalytic carbon vapor deposition (CCVD) process.

Characterization in the water media

A stock solution of 50 mg/L of MWCNTs-COOH was prepared in NSW (filtered at 0.45 μm), the same water used for the experiment. Dynamic light scattering (DLS), using a Delsa^{TM} NanoC Particle Size Analyser (Beckman Coulter) was realized for the analysis of the average size distribution of CNT suspensions in seawater at different exposure conditions and exposure times (T0: time zero, immediately after the dispersion of the materials in a water medium; T7: water samples collected after one week of exposure; T14: water samples collected after two weeks of exposure; T21: water samples collected after three weeks of exposure and T28: samples collected after four weeks of exposure). Measurements were obtained from 1 mL of suspension, being each analysis repeated 3 times. The hydrodynamic radius and polydispersity index (PDI) of the analyzed dispersions were determined using three replicates of each sample collected at each week of the experimental period and by applying the cumulant method. At the end of each measurement, undetected colloidal material was indicated as Invalid data (I.d.).

Accumulation in mussels' tissues

Each *M. galloprovincialis* specimen was sequentially submitted to probe sonication for 2 min and 30 sec (3x 45 sec) and freeze-drying lyophilization (-50°C, 0.060 mbar) to obtain a homogeneous dispersed solid material. In a 50 mL Erlenmeyer flask equipped with magnetic bar, 50 mL of ethanol was added to the pre-homogenized solid sample. Pre-homogenization of *M. galloprovincialis* samples was obtained by using an ultrasonic probe sonicator (Ikasonic U50

control, sonotrode: 0.50 nm, Max. Amplitude: 140 μm, Max. Acoustic power density: 125 W/cm) operating at 30 MHz frequency, Amplitude (100 %) and fixed pulse cycle (1 *per* second). The obtained dispersion was sequentially submitted to vigorous stirring for 4 h at room temperature and then left 2 h in static conditions to allow the separation of the solid from the supernatant fraction by gravity. The ethanolic extract (Surn) was isolated by the residual solid through accurate withdrawal of the liquid phase by means of a Pasteur pipette. The obtained extract was submitted to drying at reduced pressure to completely remove the solvent by evaporation. Both the dried unextracted and extracted residual solids were weighted and submitted to thermogravimetric analysis (TGA). TGA was performed on samples (5-10 mg) by using a TGA Q500 instrument (TA Instruments, Italy) in the temperature range 30 – 700 ˚C, at heating rate of 10 ˚C/min and under an air flow of 40 mL/min.

2.2.2 Triclosan

Characterization in the water media

The TCS concentration in water samples was obtained following the procedure reported by Cheng et al. (2011) and analyzed by Gas chromatography–mass spectrometry (GC–MS). All the extraction method was already described in Freitas et al. (2019).

Accumulation in the mussels' tissues

TCS was extracted from soft tissues as suggested by Schmidt and Snow (2016) by using a QuEChERS method and then processed according to the GC–MS method reported by Tohidi and Cai (2015). All the methodology was reported in Freitas et al. (2019).

2.3 Physiological and biochemical analyses

2.3.1 Mortality

Mortality was determined in *M. galloprovincialis* by subtracting the total number of dead individuals at the end of the exposure period (28 days) by the total number of individuals alive and

used at the beginning of the experiment, within each exposure conditions and expressed as percentage.

2.3.2 Sperm quality parameters

3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) and intracellular reactive oxygen species (ROS) were used to assess the sperm quality of the mussels after 28 days exposure period. From each experimental condition, sperm collection of samples was conducted following Mikhailov et al. (1997) through slightly cutting the mantles. The obtained sperm suspensions were collected in triplicate and kept on ice before biochemical analyses. When the mussels were exposed to TCS and salinity 28, no sperm suspension was obtained due to the immature gonads after 28 days of contamination.

Sperm viability and reactive oxygen species production

Sperm viability was evaluated using an adaptation of the MTT assay described by Van Meerloo et al. (2011). The aliquots were centrifuged at 5000 rpm at 4 ˚C for 2 min. The supernatant was discarded and the pellet was resuspended in 200 µL of FNSW. 150 µL of each sample was placed in a 96-well plate and 15 µL of MTT solution (5 mg/mL) was added. Then, the plate was incubated for 4 h at 20 ˚C. Before reading, 150 µL of ethanol was added to each well and the plate was left for 10 min incubation, allowing the dissolution of formazan crystals thoroughly. The absorbance was measured at two different wavelengths: 540 nm and 720 nm. The living cells percentage was determined with the use of the following formula:

$$\frac{\overline{\mathrm{OD}}\,\mathrm{TW} - \mathrm{B}}{\overline{\mathrm{OD}}\;\mathrm{CW} - \mathrm{B}}\; x\,100$$

where OD TW= optical density treated well; B= blank; OD CW= optical density control well.

The production of ROS was detected following Zielonka et al. (2008) methods modified by Gallo et al. (2018). The superoxide-specific fluorescent probe DHE based on aliquots of sperm

suspensions were incubated with 5mM DHE in the dark for 30 min at 20 ˚C. As positive control 30µM pyrogallol was used, incubated together with samples in the same conditions. The superoxide anion concentration was evaluated by setting the excitation at 350 nm and the emission at 590 nm. Results were expressed as arbitrary units of fluorescence intensity (a.u.).

2.3.3 Adults' tissues biochemical parameters

The individually whole body of frozen organisms (3 *per* aquarium) was pulverized and divided into 0.5 g fresh weight (FW) aliquots and used for biochemical analyses. Extractions were performed with specific buffers to determine: energy reserves content (glycogen (GLY) content), metabolic capacity (electron transport system (ETS) activity), oxidative status (lipid peroxidation (LPO) levels, reduced (GSH) and oxidized (GSSG) glutathione content), activity of antioxidant and biotransformation (catalase (CAT); glutathione S-transferases (GSTs) enzymes). 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) in a 1:2 proportion using TissueLyser II set at frequency 20 1/sec, during 1.30 sec and then centrifugated at 3000 *G* at 4 ° C, for 20 min. For LPO determination supernatants were extracted using 20 % (v/v) trichloroacetic acid (TCA) in a 1:2 proportion using TissueLyser II set at frequency 20 1/sec, during 1.30 sec and then centrifugated at 10000 *G* at 4 ° C, for 20 min. GSH and GSSG concentrations were determined in supernatants extracted with 0.6% sulfosalicylic acid in potassium phosphate buffer (0.1 M dipotassium phosphate; 0.1 M potassium dihydrogen phosphate; 5 mM EDTA; 0.1% (v/v) Triton X-100; pH 7.5) in a 1:2 proportion using TissueLyser II set at frequency 20 1/sec, during 1.30 sec and then centrifuged at 10000 *G* at 4 ˚C, for 20 min. For CAT, GSTs activities and GLY content, extraction was performed with potassium phosphate buffer (50 mM potassium dihydrogen phosphate; 50 mM dipotassium phosphate; 1 mM ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (w/v) polyvinylpyrroli- done (PVP); 1 mM dithiothreitol (DTT); pH 7.0) in a 1:2 proportion using TissueLyser II set at frequency 20 1/sec,

during 1.30 sec and then centrifugated at 10000 *G* at 4 ˚C, during 20 min. All supernatants were then reserved and stored at -80 ˚C or used immediately.

Metabolic capacity and energy reserve content

The activity of ETS was determined by the amount of formazan formed after adding p-IodoNitroTetrazolium following King and Packard (1975) and modifications performed by De Coen and Janssen (1997). The absorbance was read spectrophotometrically at 490 nm for 10 min in 25 sec intervals. The amount of formazan formed was calculated using the extinction coefficient (ε) = 15900 M^{-1} cm^{-1} and the results were expressed nmol of formazan formed per min *per* g FW.

The quantification of GLY content was done according to the sulphuric acid method (Dubois et al., 1956), using glucose standards (0-2 mg/mL). The absorbance was measured at 492 nm. Concentrations of GLY were expressed in mg *per* g of FW.

Oxidative status

The levels of LPO were measured by the quantification of thiobarbituric acid reactive substances (TBARS), according to Ohkawa et al. (1979) protocol. This methodology is based on the reaction of LPO by-products, namely malondialdehyde (MDA), with 2-thiobarbituric acid (TBA) forming TBARS. The amount of MDA was quantified spectrophotometrically and measured at 532 nm using ε = 1.56x105 M^{-1} cm^{-1}. Results were expressed as nmol of MDA equivalents *per* g FW.

GSH and GSSG contents were calculated following Rahman et al. (2014) protocols. The spectrophotometric reader assay method for GSH involves oxidation of GSH by the sulfhydryl reagent 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm. The spectrophotometric reader assay method for GSSG in cell extracts is based on the measurement of NADPH consumption by GR, a process that reduces GSSG present in the sample. The cell extracts are treated with 2-vinylpyridine, which covalently reacts with GSH (but not GSSG). The excess 2-vinylpyridine is neutralized with

triethanolamine. The absorbance was measurable at 412 nm. Reduced to oxidized glutathione ratio (GSH/GSSG) was calculated dividing GSH content by 2x the amount of GSSG.

Antioxidant and biotransformation enzyme activities

The activity of CAT was measured by the reaction of the enzyme with methanol in the presence of H_2O_2 (Johansson and Borg, 1988). The standard curve was determined using formaldehyde standards (0–150 µM). After 20 min in an orbital incubator at room temperature, the formaldehyde formation in the presence of Purpald was measured at 540 nm. The enzymatic activity was expressed in U per g FW. One U is defined as the amount of enzyme that generated the formation of 1.0 µmol formaldehyde *per* min.

The activity of GSTs activity was determined according to Habig et al. (1976). GSTs catalyse the conjugation of the substrate 1-chloro-2, 4-dinitrobenzene (CDNB) with glutathione, forming a thioether. Absorbance was measured at 340 nm and the activity of GSTs was determined using $\varepsilon = 9.6$ mM cm^{-1} for CDNB. Results were expressed in U per g of FW where U is defined as the number of enzymes that catalysis the formation of 1 µmol of dinitrophenyl thioether *per* min.

2.4 Statistical analysis

All the biochemical analyses of both sperms and adult tissues, were submitted to hypothesis testing using the PERMANOVA (permutational multivariate analysis of variance) + add-on in PRIMER v6 software. The pseudo-F p-values in the PERMANOVA main tests were evaluated in terms of significance. When the main test revealed statistically significant differences ($p \leq 0.05$), pairwise comparisons were performed. The t-statistic in the pair-wise comparisons was evaluated in terms of significance. Values lower than 0.05 were considered as significantly different.

The null hypotheses tested were: I) for each biomarker and for each salinity, no significant differences existed between contaminants; II) for each biomarker and for each contaminant, no significant differences existed between salinities.

3. RESULTS

3.1 Chemicals characterization

3.1.1 Carbon nanotubes

Characterization in the water media

DLS analysis was used to detect the presence of macro/micro/nano-sized particle aggregates of MWCNTs-COOH suspended in aqueous media containing *M. galloprovincialis* organisms at salinity 19, 28 and 37 either alone (CNTs) or in combination with TCS (CNTs+TCS). The mean size (nm) and the polydispersity index (PDI) of the analyzed suspensions are reported in Table 1. DLS and PDI analyses of experimental samples exposed to CNTs alone and in combination with TCS under different salinities among collection periods (T0, T7, T14, T21 and T28) were characterized by the presence of micro-sized aggregates whose hydrodynamic radius were directly correlated with the nominal concentrations of the samples. Furthermore, it was also possible to observe a time-dependent increase of the PDI in each condition due to the generation of large particles or aggregates in the investigated samples. Samples exposed to CNTs either alone or in combination with TCS at salinity 37 generally did not show aggregates with mean diameter values higher than those at salinity 28 and 19.

Accumulation in the mussels' tissues

A preliminary study was carried out to investigate the feasibility of using TGA by analyzing *M. galloprovincialis* exposed to the MWCNTs-COOH (CNT) and the organisms not exposed to the contaminants as reference (CTRL). The derivative of the TGA curves (DTG curves) were reported to better highlight, with respect to direct TGA curve, the temperatures at which thermal degradation of the samples occurs. The samples were analyzed under air atmosphere in order to detect the peak of degradation of MWCNTs-COOH, which inversely could not be identified under inert conditions due to the remarkable thermal stability of the materials. The DTG curves of the solids extracted (Surn) from organisms exposed to CNTs at salinity 37 (Figure 1B) evidenced the presence of a peak

of degradation between 510 °C and 600 °C, not detected in the relevant control (Figure 1A) possibly due to the presence of extracted MWCNTs-COOH.

Following the results obtained during the preliminary phase of investigation, evidence for detecting the presence of CNTs in the ethanolic extract of the analyzed samples, the developed extraction process was also carried out on marine organisms not exposed to the contaminants at salinity 28 and 19. Unexpectedly, the DTG curves revealed the presence of a degradation peak centered at 550°C in the control condition, due to a possible contamination from external sources. For these reasons, it was not possible to carry out further analysis.

Preliminary results obtained by TGA analysis highlighted the possibility to directly detect the presence of CNTs in marine organisms exposed to the referred contaminants due to the different thermal behavior of MWCNTs-COOH with respect to organic and inorganic materials. However, the contradictory results (possibly due to external contamination) obtained by the analysis of non-exposed organisms, emphasized the need to replicate the experiment under more controlled conditions.

3.1.2 Triclosan

Characterization in the water media

Results on the quantification of TCS in the water media alone and in combination with CNTs at the start of the experiment (T0) and after 7 days (T7), before water renewal and under different salinity levels were reported in table 2. Regardless of the different salinities, at T0, the TCS water concentrations were similar in the two conditions (TCS and CNTs+TCS), while after 7 days the concentration of TCS was lower compared to T0, especially in the samples collected under CNTs+TCS, suggesting a possible major uptake by the mussels exposed to those conditions.

Accumulation in the mussels' tissues

Bioaccumulation of TCS was assessed at the end of the exposure period (28 days) and under different salinity levels (Table 3). Due to the high mortality rate, no specimens exposed to the lowest salinity 19. were used for the accumulation analysis. Considering the organisms subjected to salinity 37 and 28, the results revealed the presence of TCS in mussels contaminated with both pollutants under both salinities. However, higher accumulation values were observed in organisms exposed to CNTs+TCS condition in comparison to TCS alone. Moreover, for each condition under the two salinity levels, the highest values were detected in mussels exposed to salinity 37 (TCS: 2.39±0.99 ng/g; CNTs+TCS: 3.07±0.98 ng/g) compared to salinity 28 (TCS: 0.76±0.14 ng/g; CNTs+TCS: 0.93±0.21 ng/g).

3.2 Physiological and biochemical analyses

3.2.1 Mortality

Uncontaminated organisms under salinity 37 and 28 demonstrated 100% survival after 28 days exposure. At the lowest tested salinity (19) mortality reached 56%, reason why organisms exposed to this salinity were not used in any of the analyses (both at the gametes and adults' stages).

No mortality was recorded when mussels were exposed to CNTs and TCS acting alone at salinity 37, while exposure to both contaminants under salinity 28 resulted in 11.1% mortality.

Exposures to CNTs+TCS under 37 and 28 resulted in 0 % and 22 % mortality, respectively.

3.2.2 Sperm quality parameters

Sperm viability and reactive oxygen species production

I) When the organisms under salinity 28 were exposed to CNTs and CNTs+TCS, the sperm viability significantly increased compared to control ones (Figure 2A). Considering the salinity control, significantly lower percentage of MTT was detected when the specimens were exposed to CNTs alone and in combination with TCS compared to the other treatments (Figure 2A).

II) When comparing organisms exposed to the same treatment, significant differences between salinities were observed among all conditions, showing higher (CTRL) and lower (CNTs and CNTs+TCS) percentage of MTT at salinity 37 compared to 28 (Figure 2A).

I) Considering the samples under salinity 28, significantly higher ROS production was observed in all exposed specimens compared to control ones, while under salinity 37 significantly higher ROS production was assessed only in samples exposed to CNTs compared to the other conditions (CTRL and TCS) (Figure 2B).

II) For each contaminant, significant differences between salinities were found in the sperm suspension collected from mussels contaminated with CNTs alone, showing higher ROS under low salinity compared to control salinity (Figure 2B).

3.2.3 Adults tissue biochemical parameters

Metabolic capacity and energy reserve content

I) Under salinity 28, significantly lower ETS activity was observed in contaminated organisms compared to control ones, while mussels submitted to salinity 37 showed significantly higher metabolic capacity when exposed to TCS alone and in combination with CNTs compared to the remaining conditions (Figure 3A).

II) For each contaminant, significantly higher ETS activity was detected in control specimens when submitted to salinity 28 compared to 37, while the opposite response was observed in mussels exposed to TCS (Figure 3A).

I) Significantly higher GLY content was observed in contaminated mussels compared to non-exposed ones, except for CNTs+TCS conditions at salinity 37 (Figure 3B).

II) For each contaminant, significant differences between salinities were identified only at CNTs+TCS condition, showing significantly lower GLY content under salinity 37 compared to salinity 28 (Figure 3B).

Oxidative status

I) Under salinity 28, significantly lower LPO levels were observed in contaminated organisms compared to control ones, with the lowest values in mussels exposed to CNTs alone. Mussels at control salinity showed significantly lower LPO levels when exposed to CNTs compared to mussels exposed to the remaining conditions (Figure 4A).

II) For each contaminant, significant differences between salinities were assessed under control condition as well as CNTs, showing in all cases lower LPO levels under salinity 28 (Figure 4A).

I) Under both salinity levels, no significant differences were observed in terms of GSH/GSSG in all conditions (Figure 4B).

II) For each contaminant, no significant differences were observed between salinities with the exception at CNTs condition, showing lower GSH/GSSG under salinity control compared to salinity 28 (Figure 4B).

Antioxidant and biotransformation enzyme activities

I) Under salinity 28, significantly higher CAT activity was detected when mussels were exposed to CTRL and TCS, while the lowest activity was observed in the specimens contaminated with CNTs+TCS compared to the other conditions. Considering the salinity 37, organisms showed significantly lower enzyme activity when exposed to CNTs+TCS in comparison to the remaining treatments (Figure 5A).

II) For each contaminant, significant differences between salinities were observed under TCS exposure alone, showing lower CAT activity under salinity control (Figure 5A).

I) Looking at the biotransformation enzymes in bivalves under low salinity, significantly lower activities were detected only when exposed to CNTs+TCS compared to the other treatments. Under salinity 37, GSTs activity was significantly higher under CNTs and TCS exposures, compared to organisms exposed to control and CNTs+TCS treatments (Figure 5B).

II) For each contaminant, significant differences between salinities were observed under all conditions with exception of CNTs+TCS, showing in all cases higher GSTs activity under low salinity in comparison to control salinity (Figure 5B).

4. DISCUSSION

The present study aimed at understanding how physio-chemical between CNTs and TCS under different salinity levels (from 19 to 37) affect the mussel *M. galloprovincialis* (both at gametes and adults' stages) through the evaluation of biochemical alterations.

In the present work, no biological responses were observed when the mussels were exposed to low salinity 19 due to the high mortality rate detected under this condition (≈ 60%). This result suggests a possible hypoosmotic stress even though this species is an euryhaline organism, able to tolerate a wide range of salinity fluctuations. Other studies already demonstrated the sensitivity of mussel's species to low salinity: Riisgård et al. (2012) showed that when *M. edulis* are exposed to 10, 15, 25 and 30 growth and salinity present a negative correlation, shown by a reduced shell growth rate and decreased growth rate In another study conducted with *M. galloprovincialis,* Hamer et al. (2008) investigated the progressive acclimatisation of the species to decreasing seawater salinities (37, 28, 18.5 and 11) and its effects on several biochemical markers and biotests. The authors showed that oxygen consumption rate was a concentration-dependent process and increased considerably up to 65% in mussels under salinity 18.5 compared to control one. Looking the results obtained in the present work under salinity 28, the mussels showed ≈11% of mortality when exposed to both contaminates acting alone and in combination confirming again that reduced salinity may affect negatively the mussels by a shortening in survival time as already demonstrated by Eertman et al. (1993).

Survival and growth of mussel populations will be largely dependent on the ability of these organisms to tolerate strong fluctuations in environmental conditions. These have to be taken considered as indicators of water quality and organisms' health in biomonitoring studies. Moreover, population survival depends on organisms' resilience to additional pollution stress as pollutants can greatly affect individual's sensitivity to abiotic factors.

It has already been demonstrated that successful fertilisation for any species depends on the production of high-quality sperm and fertilisation capacity (Lewis et al., 2012). All these aspects of

fertilisation have the potential to be disrupted by environmental perturbances or exposure to contaminants (Lewis et al., 2012 Since there is scarce knowledge on the consequences of contaminants in male fertility in aquatic invertebrates, the present work assessed sperm impairments due to the presence of TCS, CNTs and salinity changes in terms of oxidative stress and sperm viability.

The mitochondrial status is an important sperm quality parameter strictly related to viability and motility. In a study conducted by Fitzpatrick et al. (2008), the mitochondrial function of the mussels *Mytilus trossulus* seemed to be affected by copper exposure (0.32, 1.0, 3.2, 10.0, 32.0, and 100.0 μg/L), showing a decrease in sperm viability above the 10 μg/L concentration, suggesting disruption of the mitochondrial respiration chain and interruption of the ATP synthesis that caused sperm apoptosis. The present results were in agreement with this study, showing a decrease of sperm viability percentage (expressed as succinate dehydrogenase activity (MTT)), in individuals exposed to CNTs alone (47.03±4.02 %MTT) and in combination with TCS (54.12±2.56 %MTT) under control salinity 37. On the other hand, when the mussels were submitted to salinity 28, no MTT activity variations were observed (≈100 %MTT). Invertebrates evolved mechanisms to survive environmental challenges by modulating their metabolic pathways (Chainy et al., 2016). Thus, decrease in salinity might activate a defence mechanism to cope with environmental pollutants such as CNTs and TCS, resulting in no sperm survival variation in contaminated specimens compared to control ones. These results suggest that the variation of the salinity could not affect the sensitivity of the mussels at the gametes stage when exposed to different xenobiotics. In addition, findings show that the actual pollution scenarios can be more harmful in terms of reproduction success with the possibility to impair mussel populations.

Oxyradical damage to lipids, proteins and DNA of organisms, due to the generation of reactive oxygen species (ROS), has been recognized as a negative cause affecting sperm functioning (Alahmar, 2019). The results obtained in the present study revealed that when mussels were subjected to both salinities (28 and 37), ROS production increased in sperm suspensions in all

exposed organisms compared to their relative control, and most severe effects were observed under CNTs individual exposure (sal. 28: 46563.16±3279.32 fluorescence intensity; sal. 37: 32327.5±819.06 fluorescence intensity). Although information on the toxic effect of MWCNTs or, in general, of CNTs on male reproductive system is limited, results from studies conducted on mammals revealed that MWCNTs are able to influence the male reproductive function and lead to oxidative stress, reducing gonadal epithelium thickness (Vasyukova et al., 2015). This hypothesis thus supports the results obtained in the present study.

Sublethal effects of contaminants can be detected both at the gamete and at the adult stage. While, the investigation of possible impacts of pollutants on life-history evolution across different life stages remains unexplored. For this reason, in the present study metabolic capacity and energy reserves impairments as well as oxidative status alterations were investigated at adults' stage. Results generally showed higher toxic impacts when the mussels were exposed to the combination of CNTs and TCS under salinity control. When considering TCS in tissues of mussels exposed to this compound alone and in combination with CNTs, results showed the highest quantity TCS in organisms exposed to CNTs+TCS condition under salinity 37. This indicates a possible additive or synergistic mechanism which resulted into greater accumulation of TCS when in the presence of CNTs.

Moreover, these results show that under low salinity, organisms exposed to contaminants limit their accumulation possibly by strongly reducing their filtration rate. These results are corroborated by a significant decrease on mussel's metabolic capacity (measured by ETS activity) when exposed to salinity 28 (≈20±4 nmol/min/g FW) regardless the pollutant in comparison to non-contaminated mussels at the same salinity (44.24±4.30 nmol/min/g FW). Furthermore, mussels under control salinity increased their ETS when exposed to CNTs+TCS compared to non-contaminated mussels at the same salinity, which could explain higher concentration of both pollutants at this condition. Such results highlight that the variation of abiotic factors (such as

salinity levels) can modify the behaviour of the contaminants as well as their interaction, probably generating a synergistic effect, which induced higher toxic impact under this condition due to a perturbation of redox state. Moreover, under control salinity (37) and in the presence of both pollutants, *M. galloprovincialis* decreased the GLY content (3.95±1.38 mg/g FW), showing that sublethal stress led to increase energy expenditure during basal metabolism to cope with stress induced by the combination of these two compounds. Such results may indicate a synergistic effect caused by both pollutants together. De Marchi et al. (2017) also demonstrated that when the clams *R. philippinarum* were exposed to pristine and carboxylated MWCNTs, they decreased the GLY and PROT content which may indicate that clams were using GLY and PROT to fuel up their defence mechanisms against CNTs toxicity. Under 28 *M. galloprovincialis* exposed to all conditions presented a decrease of ETS activity with an associated reduced expenditure of energy reserves expressed by an increase of GLY content. This impairment of the function of ETS activity could be caused by salinity variation and not by the type of contaminants (as the metabolic and energy responses were similar under all conditions). Such results confirm that despite estuarine bivalves are often exposed to short-term (tidal) and long-term (rain periods) that causes changes in salinity (Verdelhos et al., 2015), they can exhibit physiological and morphological abnormalities when submitted to low salinity levels (Munari et al., 2011), indicating that salinity may alter the metabolic rate of the organisms.

Concerning the decrease of CAT activity in mussels exposed to CNTs+TCS at salinity control (27.11±2.62 U/g FW), we may hypothesise that the combination of both pollutants is the most stressful condition to mussels, inhibiting their antioxidant capacity. The inhibition of CAT may not only result from increased reactive oxygen species (ROS) production due to the presence of both pollutants but also due to an increase ETS observed under this condition, as the mitochondrial system also generates ROS during cells respiration. In fact, ETS activity has been recognized as one of the major cellular generators of ROS, which include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl free radical (-OH) (Liu et al., 2002). Higher ROS may produce an imbalance

between endogenous and exogenous ROS and subsequently a decrease of antioxidant defences (Valavanidis et al., 2006).

On the contrary, results regarding the antioxidant system obtained under salinity 28 (≈35.5±2.2 U/g FW) were not in line with the metabolic capacity pattern and further investigations are required.

The multicomponent enzymes involved in the detoxification of different xenobiotics, GSTs play an important role in protecting tissues from oxidative stress (Fournier et al., 1992). They have already been used as biomarkers for cellular damage as these enzymes exhibit many of the required characteristics, i.e. specific localization, high cytosolic concentration and relatively short half-life (Pérez et al., 2004). The behaviour of GSTs activity was different between salinities as well as between conditions. Under salinity control, this enzyme was activated only when exposed to TCS and CNTs acting alone (0.11±0.011 and 0.11±0.015 U/g FW respectively), while at low salinity GSTs were inhibited when mussels were exposed to CNTs+TCS (0.08±0.01 U/g FW). These results evidence once again the high stress level generated by the combination of both pollutants that limited the increase of GSTs activity at this condition. In invertebrates these enzymes can be induced by certain xenobiotics such as polycyclic aromatic hydrocarbons (PAH), polycyclic biphenyls (PCBs) (Pinkus et al., 1993; Williams et al., 1998) NPs and TCS (Canesi et al., 2007; Ciacci et al., 2012; Garaud et al., 2014; Minetto et al., 2014; Cid et al., 2015; Goodchild et al., 2016; Park et al., 2017; De Marchi et al., 2018; 2019b), increasing as a function of the concentration of these xenobiotics in seawater (Stien et al., 1998; Boutet et al., 2004). This suggests that these contaminants may represent a substrate for phase II enzymes (Canesi et al., 2007) independently if the contamination can be generated by the action of single compound or by the combination of more.

Looking to oxidative status, no LPO was detected in all exposed individuals, regardless the salinity tested. Similarly, independently of the salinity and pollutant, mussels redox balance was not

changed compared to control conditions. These results show that under decreased salinity in the presence or absence of pollutants, mussels were able to avoid cellular damage probably thanks to their capacity to reduce their metabolism and avoid accumulation of pollutants. Lower LPO levels may also result from decreased ETS activity that generated lower amount of ROS during cellular respiration. In agreement with such findings, Britto et al. (2019) also revealed a lowering of aerobic metabolism that reduce the electron flow, resulting into a less pro-oxidant condition (measured by a decrease of LPO levels) in clams exposed to Cu and graphene oxide (GO) + Cu treatments.

Under control salinity, although higher concentrations of pollutants were found in mussel's soft tissues, organisms were able to prevent cellular damage by increasing their biotransformation capacity. Similar findings were observed by Freitas et al. (2019), showing that *M. galloprovincialis* exposed to TCS and Diclofenac (DIC) at 1 μg/L each, were able to prevent cellular damage (expressed as decrease of LPO levels) by activating their GSTs in all exposed individuals.

CONCLUSION

In conclusion, the present findings demonstrated that mussels exposed to CNTs and TCS - acting alone or in combination - were higlhy affected by the compounds' toxicity in both cellular lines especially at salinity control. This brings to evidence that actual pollution scenarios may impair mussels populations, affecting reproduction success and growth. Moreover, major metabolic impaiment and oxidative stress were observed at CNTs+TCS condition especially at the adult stage, assuming that this co-exposure resulted into changes in the toxicological pathways, increasing the impacts induced in the organisms and highlighting the potential environmental risk of these contaminants.

ACKNOWLEDGMENTS

This work was also funded by national funds (OE), through FCT – Fundação para a Ciência e a Tecnologia, I.P., in the scope of the framework contract foreseen in the numbers 4, 5 and 6 of the

article 23, of the Decree-Law 57/2016, of August 29, changed by Law 57/2017, of July 19. Thanks are due for the financial support to CESAM (UIDB/50017/2020+UIDP/50017/2020), to FCT/MEC through national funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. This work was also financially supported by the project BISPECIAl: BIvalveS under Polluted Environment and ClImate chAnge (POCI-01-0145-FEDER-028425) funded by FEDER, through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI), and by national funds (OE), through FCT/MCTES. The manuscript has been edited by an English-speaking native, Marta Musso (914468, Swansea University (Swansea, Wales, United Kingdom)).

References

Alahmar, A. T. (2019). Role of oxidative stress in male infertility: An updated review. *Journal of Human Reproductive Sciences*, 12(1), 4.

Anestis, A., Lazou, A., Portner, H.O., Michaelidis, B., 2007. Behavioral, metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. Am. J. Physiol. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 293, 911–921.

Boutet, I., Tanguy, A. & Moraga, D. (2004). Response of the Pacific oyster *Crassostrea gigas* to hydrocarbon contamination under experimental conditions. *Gene*, 329, 147-157.

Britto, R. S., Nascimento, J. P., Serode, T., Santos, A. P., Soares, A. M. V. M, Figueira, E., Furtado, C., Lima-Ventura, J., Monserrat J. M. & Freitas, R. (2019). The effects of co-exposure of graphene oxide and copper under different pH conditions in Manila clam *Ruditapes philippinarum*. *Environmental Science and Pollution Research*, 1-12.

Canesi, L., Ciacci, C., Lorusso, L. C., Betti, M., Gallo, G., Pojana, G. & Marcomini, A. (2007). Effects of Triclosan on *Mytilus galloprovincialis* hemocyte function and digestive gland enzyme activities: possible

modes of action on non-target organisms. C*omparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 145(3), 464-472.

Chainy, G. B. N., Paital, B. & Dandapat, J. (2016). An overview of seasonal changes in oxidative stress and antioxidant defence parameters in some invertebrate and vertebrate species. *Scientifica*, 2016.

Cheng, C.- Y., Wang, Y.- C., Ding, W.- H. (2011). Determination of Triclosan in aqueous samples using solid-phase extraction followed by on-line derivatization gas chromatography-mass spectrometry. *Analytical Sciences*, 27, 197–202

Ciacci, C., Canonico, B., Bilaniĉovă, D., Fabbri, R., Cortese, K., Gallo, G., Marcomini, A., Pojana, G. & Canesi L. (2012). Immunomodulation by different types of N-oxides in the hemocytes of the marine bivalve *Mytilus galloprovincialis*. *PLoS ONE*, 7(5), 1–11.

Cid, A., Picado, A., Correia, J. B., Chaves, R., Silva, H., Caldeira, J., de Matos, A. P. A. & Diniz, M. S. (2015). Oxidative stress and histological changes following exposure to diamond nanoparticles in the freshwater Asian clam *Corbicula fluminea* (Müller, 1774). *Journal of Hazardous Materials*, 284, 27–34.

Cunha, I., García, L. M. & Guilhermino, L. (2005). Sea-urchin (*Paracentrotus lividus*) glutathione S-transferases and cholinesterase activities as biomarkers of environmental contamination. *Journal of Environmental Monitoring*, 7(4), 288-294.

Dann, A. B. & Hontela, A. (2010). Triclosan: environmental exposure, toxicity and mechanisms of action. *Journal of Applied Toxicology*, 31(4), 285–311.

De Marchi, L., Coppola, F., Soares, A. M., Pretti, C., Monserrat, J. M., della Torre, C. & Freitas, R. (2019a). Engineered nanomaterials: From their properties and applications, to their toxicity towards marine bivalves in a changing environment. *Environmental research*, 108683.

De Marchi, L., Neto, V., Pretti, C., Chiellini, F., Morelli, A., Soares, A. M. V. M., Figueira, E. & Freitas, R. (2019b). The influence of Climate Change on the fate and behavior of different carbon nanotubes materials and implication to estuarine invertebrates. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 219, 103-115

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini, F., Morelli, A., Soares, A. M. V. M. & Freitas, R. (2018). Effects of multi-walled carbon nanotube materials on *Ruditapes philippinarum* under climate change: the case of salinity shifts. *Aquatic Toxicology*, 199, 199–211.

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini, F., Morelli, A., Soares, A.M.V.M. & Freitas, R. (2017). Toxic effects of multi-walled carbon nanotubes on bivalves: comparison between functionalized and nonfunctionalized nanoparticles. *Science of the Total Environment*, 622, 1532-1542.

DeLorenzo, M. E. & Fleming, J. (2008). Individual and mixture effects of selected pharmaceuticals and personal care products on the marine phytoplankton species *Dunaliella tertiolecta*. *Archives of Environmental Contamination and Toxicology*. 54(2), 203–210

Deng, R., Lin, D., Zhu, L., Majumdar, S., White, J. C., Gardea-Torresdey, J. L. & Xing, B. (2017). Nanoparticle interactions with co-existing contaminants: joint toxicity, bioaccumulation and risk. *Nanotoxicology*, 11(5), 591–612.

Dhillon, G. S., Kaur, S., Pulicharla, R., Brar, S. K., Cledón, M., Verma, M. & Surampalli, R. Y. (2015). Triclosan: current status, occurrence, environmental risks and bioaccumulation potential. *International Journal of Environmental Research and Public Health*, 12(5), 5657-5684.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. T. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28(3), 350-356.

Eertman, R.H.M., Wagenvoort, A.J., Hummel, H. & Smaal, A.C., 1993. Survival in air" of the blue mussel *Mytilus edulis* L. as a sensitive response to pollution-induced environmental stress. *Journal of Experimental Marine Biology and Ecology*, 170, 179–195.

Fitzpatrick, J. L., Nadella, S., Bucking, C., Balshine, S. & Wood, C. M. (2008). The relative sensitivity of sperm, eggs and embryos to copper in the blue mussel (*Mytilus trossulus*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 147(4), 441–449.

Fournier, D., Bride, J. M., Poirie, M., Berge, J. B. & Plapp, F. W. (1992). Insect glutathione S-transferases. Biochemical characteristics of the major forms from houseflies susceptible and resistant to insecticides. *Journal of Biological Chemistry*, 267(3), 1840-1845.

Freitas, R., Coppola, F., De Marchi, L., Codela, V., Pretti, C., Chiellini, F., Morelli, A., Polese, G., Soares, A.M.V.M. & Figueira, E. (2018). The influence of arsenic on the toxicity of carbon nanoparticles in bivalves. *Journal of Hazardous Materials*, 358, 484-493.

Freitas, R., Coppola, F., Costa, S., Pretti, C., Intorre, L., Meucci, V., Soares, A.M.V.M. & Solé, M. (2019). The influence of temperature on the effects induced by Triclosan and Diclofenac in mussels. *Science of The Total Environment*, 663, 992-999.

Freixa, A., Acuña, V., Sanchís, J., Farré, M., Barceló, D. & Sabater, S. (2018). Ecotoxicological effects of carbon based nanomaterials in aquatic organisms. *Science of the Total Environment*, 619–620, 328–337.

Garaud, M., Trapp, J., Devin, S., Cossu-Leguille, C., Pain-Devin, S., Felten, V. & Giamberini, L. (2015). Multibiomarker assessment of cerium dioxide nanoparticle ($nCeO_2$) sublethal effects on two freshwater invertebrates, *Dreissena polymorpha* and *Gammarus roeseli*. *Aquatic Toxicology*, 158, 63-74.

Goodchild, C. G., Frederich, M. & Zeeman, S. I. (2016). Is altered behavior linked to cellular energy regulation in a freshwater mussel (*Elliptio complanata*) exposed to triclosan? *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 179, 150-157.

Habig, W.H., Pabst, M.J. & Jakoby, W.B. (1976). Glutathione S-transferase AA from rat liver. *Archives of Biochemistry and Biophysics*, 175 (2), 710–716.

Hamer, B., Jakšić, Ž., Pavičić-Hamer, D., Perić, L., Medaković, D., Ivanković, D., Pavičić, J., Zilberberg, C., Schröder, H. C., Müller, W. E. G., Smodlaka, N., Batela, R. & Smodlaka, N. (2008). Effect of hypoosmotic stress by low salinity acclimation of Mediterranean mussels *Mytilus galloprovincialis* on biological parameters used for pollution assessment. *Aquatic Toxicology*, 89(3), 137-151.

Handy, R. D., Van Den Brink, N., Chappell, M., Mühling, M., Behra, R., Dušinská, M., Simpson, P., Ahtiainen, J., Jha, A. N., Seiter, J., Bednar, A., Kennedy, A., Fernandes, T. F. & Riediker, M. (2012). Practical considerations for conducting ecotoxicity test methods with manufactured nanomaterials: what have we learnt so far? *Ecotoxicology*, 21 (4), 933–972.

He, X., Aker, W. G., Leszczynski, J. & Hwang, H. (2014). Using a holistic approach to assess the impact of engineered nanomaterials inducing toxicity in aquatic systems Journal of Food and Drug Analysis, 22, 128–146.

Johansson, L. H. & Håkan Borg, L. A. (1988). A spectrophotometric method for determination of catalase activity in small tissue samples. *Analytical Biochemistry*, 174 (1), 331–336.

Keller, A. A., Lazareva, A. (2014). Predicted releases of engineered nanomaterials: from global to regional to local Environmental Science & Technology Letters, 1 (1), 65–70.

King, F. D. & Packard, T.T. (1975). Respiration and the activity of the respiratory electron transport system in marine zooplankton. *Limnology and Oceanography*, 20, 849–854.

Lewis, C. & Ford, A. T. (2012). Infertility in male aquatic invertebrates: A review. *Aquatic Toxicology*, 120-121, 79–89.

Liu, Y., Fiskum, G. & Schubert, D. (2002). Generation of reactive oxygen species by the mitochondrial electron transport chain. *Journal of Neurochemistry*, 80(5), 780-787.

Mikhailov, A. T., Torrado, M., Korochkin, L. I., Kopantzeva, M. A. & Méndez, J. (1997). Male-predominant carboxylesterase expression in the reproductive system of molluscs and insects: immunochemical and biochemical similarity between *Mytilus* male associated polypeptide (MAP) and *Drosophila* sex-specific esterase S. *Comparative Biochemistry and Physiology Part B: Biochemistry & Molecular Biology*, 118(1), 197-208.

Minetto, D., Libralato, G. & Ghirardini, A.V. (2014). Ecotoxicity of engineered TiO_2 nanoparticles to saltwater organisms: an overview. *Environment International*, 66, 18-27.

Morozesk, M., Franqui, L. S., Mansano, A. S., Martinez, D. S. T. & Fernandes, M. N. (2018). Interactions of oxidized multiwalled carbon nanotube with cadmium on zebrafish cell line: The influence of two co-exposure protocols on in vitro toxicity tests. *Aquatic Toxicology*, 200, 136-147.

Mouchet, F., Landois, P., Sarremejean, E., Bernard, G., Puech, P., Pinelli, E., Flahaut, E. & Gauthier, L. (2008). Characterisation and in vivo ecotoxicity evaluation of double-wall carbon nanotubes in larvae of the amphibian *Xenopus laevis*. *Aquatic Toxicology*, 87, 127.

Munari, M., Matozzo, V. & Marin, M.G. (2011). Combined effects of temperature and salinity on functional responses of haemocytes and survival in air of the clam *Ruditapes philippinarum. Fish & Shellfish Immunology*, 30(4), 1024-1030.

Oberdörster, E., Zhu, S., Blickley, T.M., McClellan-Green, P. & Haasch, M.L. (2006). Ecotoxicology of carbon-based engineered nanoparticles: Effects of fullerene (C_{60}) on aquatic organisms. *Carbon*, 44, 1112.

Ohkawa, H., Ohishi, N. & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, 95, 351–358.

Olaniyan, L. W. B., Mkwetshana, N. & Okoh, A. I. (2016). Triclosan in water, implications for human and environmental health. *Springerplus*, 5(1), 1639.

Oliveira, R., Domingues, I., Grisolia, C. K. & Soares, A. M. (2009). Effects of triclosan on zebrafish early-life stages and adults. *Environmental Science and Pollution Research*, 16(6), 679-688.

Park, J. C., Han, J., Lee, M. C., Seo, J. S. & Lee, J. S. (2017). Effects of triclosan (TCS) on fecundity, the antioxidant system, and oxidative stress-mediated gene expression in the copepod *Tigriopus japonicus*. *Aquatic Toxicology*, 189, 16-24.

Pérez, E., Blasco, J. & Solè, M. (2004). Biomarker responses to pollution in two invertebrate species: *Scrobicularia plana* and *Nereis diversicolor* from the Cádiz bay (SW Spain). *Marine Environmental Research*, 58 (2–5), 275–79.

Pinkus, R., Bergelson, S. & Daniel, V. (1993). Phenobarbital induction of AP-1 binding activity mediates activation of glutathione S-transferase and quinone reductase gene expression. *Biochemical Journal*, 290(3), 637-640.

Qiu, J. W., Tremblay, R. & Bourget, E. (2002). Ontogenetic changes in hyposaline tolerance in the mussels *Mytilus edulis* and *M. trossulus*: implications for distribution. *Marine Ecology Progress Series*, 228, 143-152.

Rahman, S., Kim, K. H., Saha, S. K., Swaraz, A. M. & Paul, D. K. (2014). Review of remediation techniques for arsenic (As) contamination: a novel approach utilizing bio-organisms. *Journal of Environmental Management*, 134, 175–185.

Riisgård, H. U., Bøttiger L. & Pleissner, D. (2012). Effect of salinity on growth of mussels, *Mytilus edulis*, with special reference to Great Belt (Denmark). *Open Journal of Marine Science*, 2, 167-176.

Schmidt, M.L. & Snow, N.H. (2016). Making the case for QuEChERS-gas chromatography of drugs. *TrAC Trends in Analytical Chemistry*, 75, 49–56.

Stien, X., Percic, P., Gnassia-Barelli, M., Roméo, M. & Lafaurie, M. (1998). Evaluation of biomarkers in caged fishes and mussels to assess the quality of waters in a bay of the NW Mediterranean Sea. *Environmental Pollution*, 99(3), 339-345.

Sun, H., Zhang, X., Zhang, Z., Chen, Y. & Crittenden, J. C. (2009). Influence of titanium dioxide nanoparticles on speciation and bioavailability of arsenite. *Environmental Pollution*, 157, 1165–1170.

Tohidi, F. & Cai, Z. (2015). GC/MS analysis of triclosan and its degradation by-products in wastewater and sludge samples from different treatments. *Environmental Science and Pollution Research*, 22(15), 11387-11400.

Valavanidis, A., Vlahogianni, T., Dassenakis, M. & Scoullos, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety*, 64(2), 178–189.

Van Meerloo, J., Kaspers, G. J. L., Cloos, J. (2011). Cell sensitivity assays: the MTT assay. In Cancer cell culture (pp. 237–245). Springer.

Vasyukova, I., Gusev, A. & Tkachev, A. (2015). Reproductive toxicity of carbon nanomaterials: a review. In *IOP Conference Series: Materials Science and Engineering* (Vol. 98, No. 1, p. 012001). IOP Publishing.

Verdelhos, T., Marques, J.C. & Anastácio, P. (2015). The impact of estuarine salinity changes on the bivalves *Scrobicularia plana* and *Cerastoderma edule*, illustrated by behavioral and mortality responses on a laboratory assay. *Ecological Indicators*, 52, 96–104.

Williams, D. E., Lech, J. J. & Buhler, D. R. (1998). Xenobiotics and xenoestrogens in fish: modulation of cytochrome P450 and carcinogenesis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 399(2), 179-192.

Westerbom, M., Kilpi, M. & Mustonen, O. (2002) Blue mussels, *Mytilus edulis*, at the edge of the range: population structure, growth and biomass along a salinity gradient in the north-eastern Baltic Sea. Marine Biology, 140(991), 999.

Zhang, X., Zhou, Q., Zou, W. & Hu, X. (2017). Molecular mechanisms of toxicity induced by graphene oxide at predicted environmental concentrations. *Environmental Science & Technology*, 51(14), 7861-7871.

Zhao, X. & Liu, R. (2012). Recent progress and perspectives on the toxicity of carbon nanotubes at organism, organ, cell, and biomacromolecule levels. *Environment International*, 40, 244-255.

Zielonka, J., Vasquez-Vivar, J. & Kalyanaraman, B. (2008). Detection of 2-hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine. *Nature Protocols*, 3(1), 8.

Figure captions

Figure 1. A. TGA/DTG analysis of ethanolic extract (37_CTRL_Surn) obtained from *M. galloprovincialis* exposed to salinity 37 in absence of CNTs (37_CTRL). **B.** TGA/DTG analysis of ethanolic extract (37_CNT_A_Surn) obtained from *M. galloprovincialis* exposed to CNTs at salinity 37 (37_CNT_A).

Figure 2. A. Sperm viability (MTT) mean values; **B.** Reactive oxygen species (ROS) levels (mean ± standard deviation) in *M. galloprovincialis* spermatozoids exposed to different conditions (control, 0.0 mg/L TCS and CNTs); TCS (1.0 µg/L); CNTs (0.10 mg/L); CNTs+TCS (1.0 mg/L TCS and 0.10 mg/L CNTs). Significant differences ($p \leq 0.05$) between salinities for each exposure condition were represented with different letters: lowercase and regular letters for salinity 28; uppercase and regular letters for salinity 37. Significant differences ($p \leq 0.05$) between exposure concentrations for each salinity were represented with asterisks (*).

Figure 3. A. Electron transport system (ETS) activity; **B**. Glycogen (GLY) content (mean ± standard deviation) in *M. galloprovincialis* exposed to different conditions (control, 0.0 mg/L TCS and CNTs); TCS (1.0 µg/L); CNTs (0.10 mg/L); CNTs+TCS (1.0 mg/L TCS and 0.10 mg/L CNTs). Significant differences ($p \leq 0.05$) between salinities for each exposure condition were represented with different letters: lowercase and regular letters for salinity 28; uppercase and regular letters for salinity 37. Significant differences ($p \leq 0.05$) between exposure concentrations for each salinity were represented with asterisks (*).

Figure 4. A. Lipid peroxidation (LPO) levels; **B.** GSH/GSSG (mean ± standard deviation), in *M. galloprovincialis* exposed to different conditions (control, 0.0 mg/L TCS and CNTs); TCS (1.0 µg/L); CNTs (0.10 mg/L); CNTs+TCS (1.0 mg/L TCS and 0.10 mg/L CNTs). Significant differences ($p \leq 0.05$) between salinities for each exposure condition were represented with different letters: lowercase and regular letters for salinity 28; uppercase and regular letters for salinity 37. Significant differences ($p \leq 0.05$) between exposure concentrations for each salinity were represented with asterisks (*).

Figure 5. A. Catalase (CAT) activity; **B.** Glutathione S-Transferases (GSTs) activity (mean ± standard deviation), in *M. galloprovincialis* exposed to different conditions (control, 0.0 mg/L TCS and CNTs); TCS (1.0 µg/L); CNTs (0.10 mg/L); CNTs+TCS (1.0 mg/L TCS and 0.10 mg/L CNTs). Significant differences ($p \leq 0.05$) between salinities for each exposure condition were represented with different letters: lowercase and regular letters for salinity 28; uppercase and regular letters for salinity 37. Significant differences ($p \leq 0.05$) between exposure concentrations for each salinity were represented with asterisks (*).

Author contribution statements

Rosa Freitas and Carlo Pretti conceived and planned the experiments

Carlo Pretti and Rosa Freitas supervised the project

Lucia De Marchi, Matteo Oliva, Alessia Cuccaro, Rosa Freitas and Carlo Pretti conceived of the presented idea.

Lucia De Marchi, Rosa Freitas, Matteo Oliva, Alessia Cuccaro, Chiara Manzini, Federica Tardelli, Madalena Andrade, Marcelo Costa, Carla Leite, Andrea Morelli, Federica Chiellini, Carlo Pretti carried out the experiment.

Lucia De Marchi, Rosa Freitas, Matteo Oliva, Alessia Cuccaro Federica Chiellini and Carlo Pretti contributed to the interpretation of the results.

Lucia De Marchi, Rosa Freitas, Matteo Oliva, Alessia Cuccaro, Chiara Manzini and Federica Tardelli developed the theory and performed the computations.

Lucia De Marchi wrote the manuscript with support from Matteo Oliva, Alessia Cuccaro, Rosa Freitas, Federica Chiellini and Carlo Pretti.

Chiara Manzini, Federica Tardelli Madalena Andrade, Marcelo Costa, Carla Leite, Andrea Morelli, Federica Chiellini and Carlo Pretti verified the analytical methods.

All authors discussed the results and contributed to the final manuscript.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Table 1. Size (nm) and Polydispersity Index (PDI) of COOH-MWCNTs suspensions seawater (salinity 19, 28 and 37) alone (CNTs) and in combination with Triclosan (CNTs+TCS) among different collection period (T7; T14; T21 andT28).

	CNTs						**CNTs+TCS**					
	Sal. 19		**Sal. 28**		**Sal. 37**		**Sal. 19**		**Sal. 28**		**Sal. 37**	
	DLS (nm)	**PDI**	**DLS (nm)**	**PDI**	**DLS (nm)**	**PDI**	**DLS (nm)**	**PDI**	**DLS (nm)**	**PDI**	**DLS (nm)**	**PDI**
T7	2649.5	1.096	2134.7	0.716	2518.7	1.102	1478.5	0.572	2613.3	1.224	1445.7	0.547
T14	3595.5	1.486	1622.6	0.789	4822.0	1.873	2594.7	0.712	3702.8	1.601	2471.3	1.202
T21	3270.1	1.507	2010.9	0.832	1787.7	0.785	1363.4	0.302	3043.9	1.212	2153.7	0.925
T28	4947.2	1.801	3643.5	1.450	1488.9	0.520	2300.0	0.905	I.d.	-	2867.1	1.307

I.d.: "Invalid data" (not detected colloidal material into the analysed sample at the end of 120 acquisitions)

Table 2. Quantification (µg/L) of Triclosan in suspensions seawater (salinity 19, 28 and 37) alone (TCS) and in combination with Carbon nanotubes (CNTs+TCS) at the start of the experiment (T0) and after one week of exposure (T7).

	T0				**T7**			
	CTRL	**CNTs**	**TCS**	**CNTs+TCS**	**CTRL**	**CNTs**	**TCS**	**CNTs+TCS**
Sal. 19	<LOD	-	0.54±0.11	0.67±0.14	<LOD	-	0.11±0.04	0.02±0.009
Sal. 28	<LOD	-	0.59±0.15	0.82±0.12	<LOD	-	0.12±0.06	<LOD
Sal. 37	<LOD	-	0.66±0.12	0.50±0.16	<LOD	-	0.09±0.03	0.01±0.008

LOD: 0.008 µg/L (DW)

Table 3. Quantification (ng/g) of Triclosan in tissue (DW) (salinity 28 and 37) alone (TCS) and in combination with Carbon nanotubes (CNTs+TCS) at the end of the exposure period (T28).

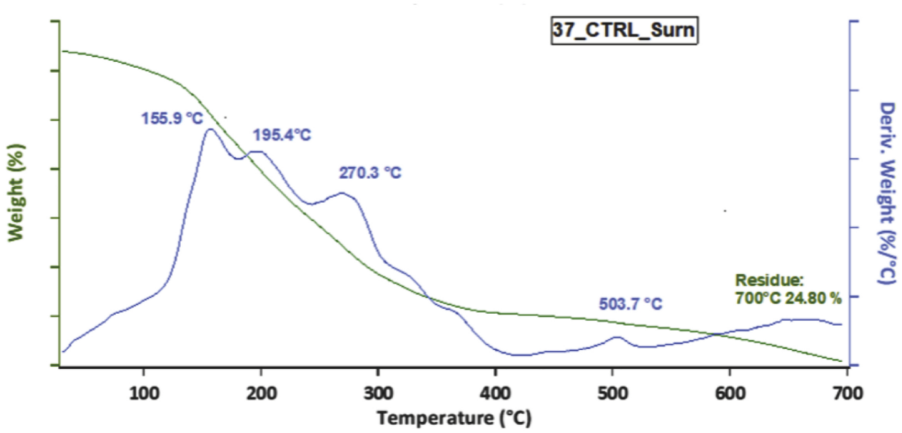
	T28			
	CTRL	**CNTs**	**TCS**	**CNTs+TCS**
Sal. 28	<LOD	<LOD	0.76±0.14	0.93±0.21
Sal. 37	<LOD	<LOD	2.39±0.99	3.07±0.98

LOD: 0.13 ng/g (DW)

Highlights

- Evaluation of CNTs and TCS' effects on *M. galloprovincialis* tissues and sperms
- The combination of both contaminants showed major toxic impacts
- Both sperms and adult tissues under salinity control were affected by CNTs+TCS

A


37_CTRL_Surn
155.9 °C
195.4°C
270.3 °C
503.7 °C
Residue:
700°C 24.80 %
Weight (%)
Deriv. Weight (%/°C)
100
200
300
400
500
600
700
Temperature (°C)

B

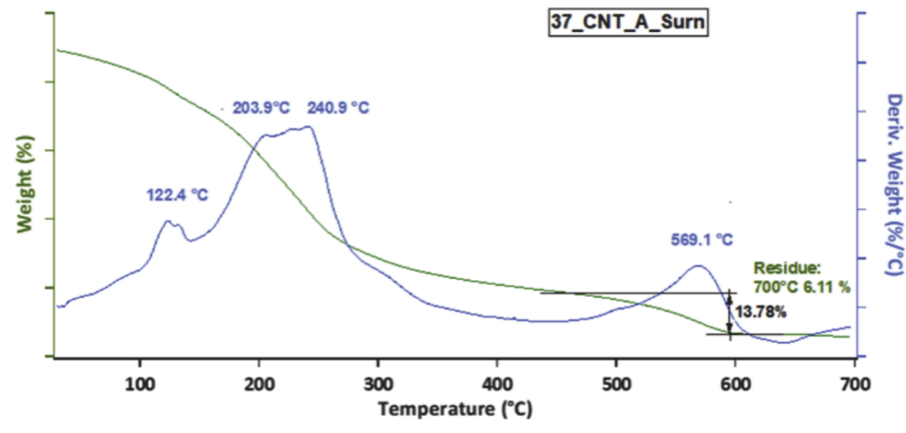
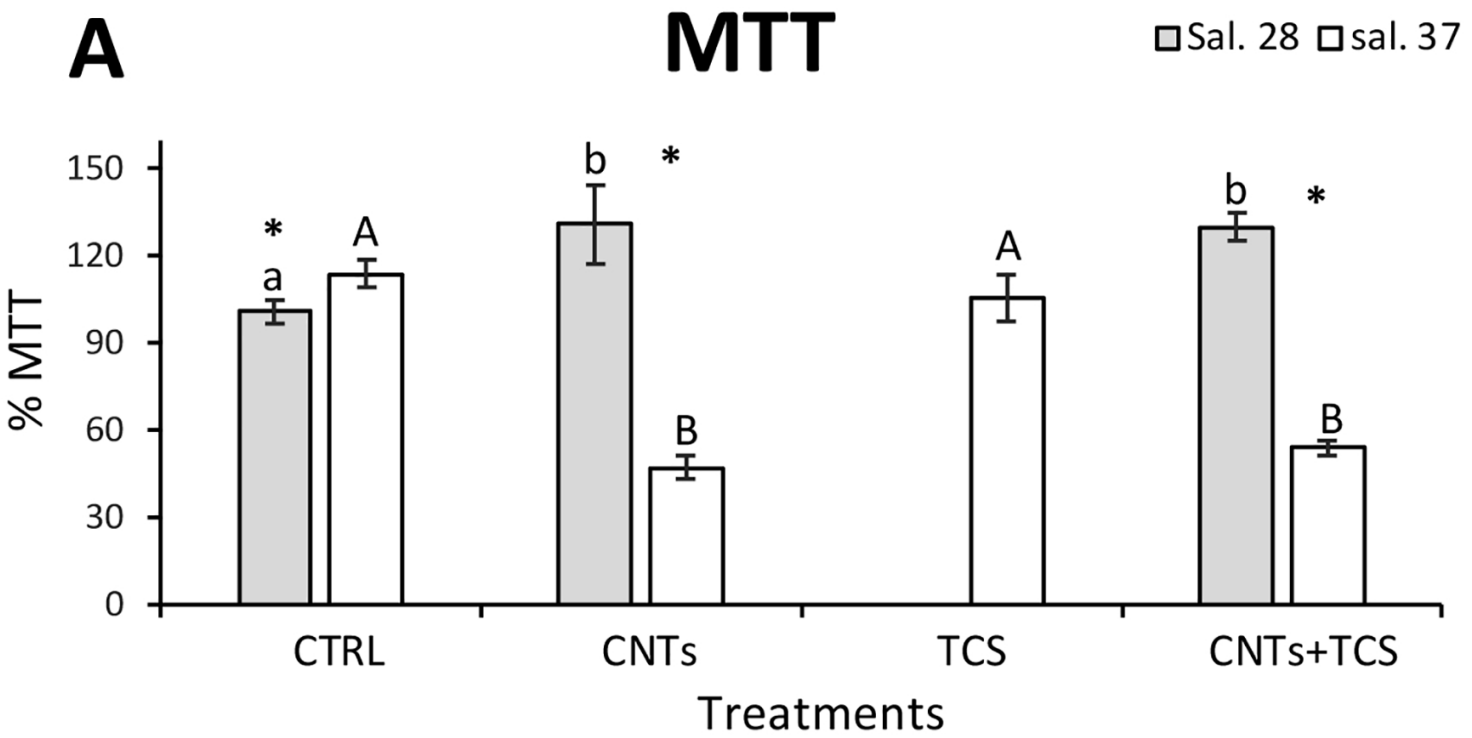

37_CNT_A_Surn
203.9°C
240.9 °C
122.4 °C
569.1 °C
Residue:
700°C 6.11 %
13.78%
Weight (%)
Deriv. Weight (%/°C)
100
200
300
400
500
600
700
Temperature (°C)

Figure 1


A
MTT
Sal. 28
sal. 37
% MTT
150
120
90
60
30
0
a
A
b
B
A
b
B
*
*
*
CTRL
CNTs
TCS
CNTs+TCS
Treatments

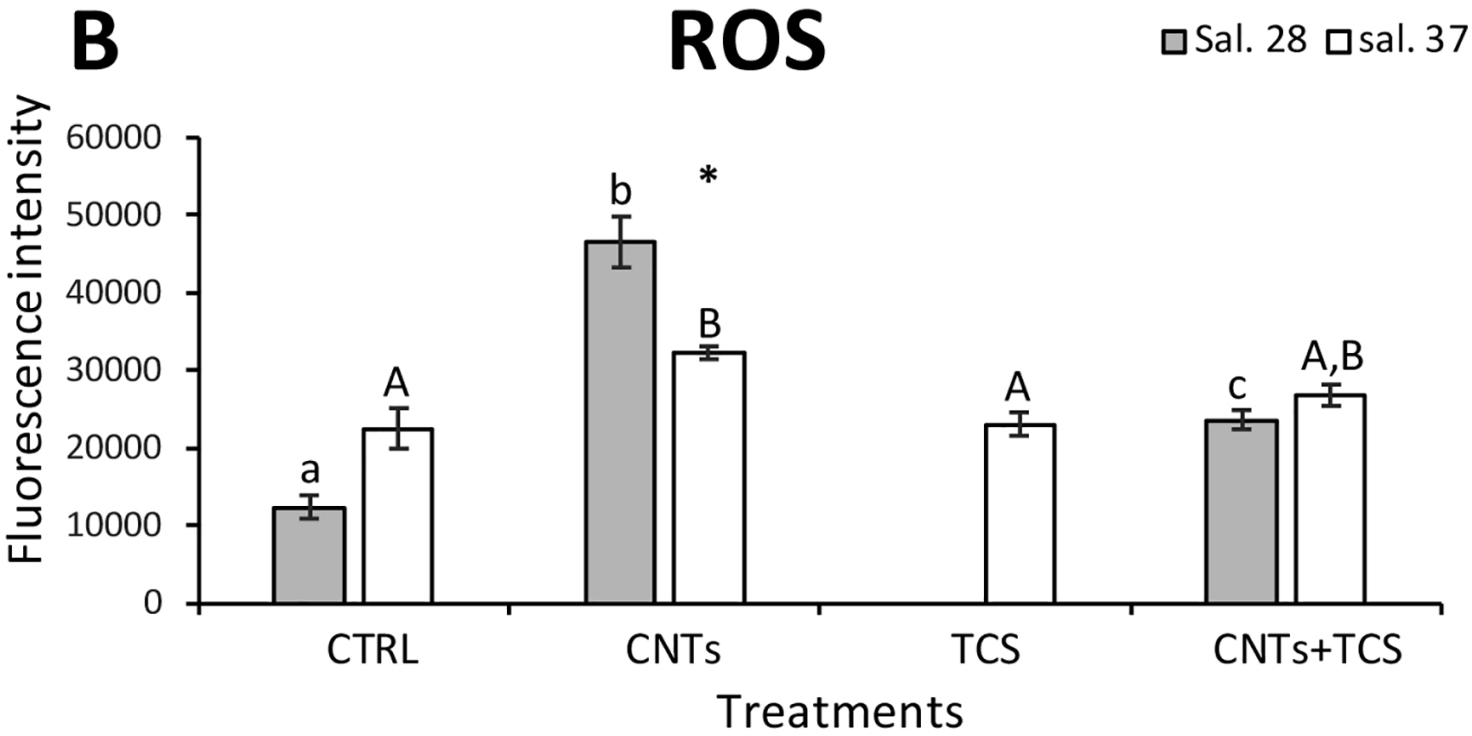
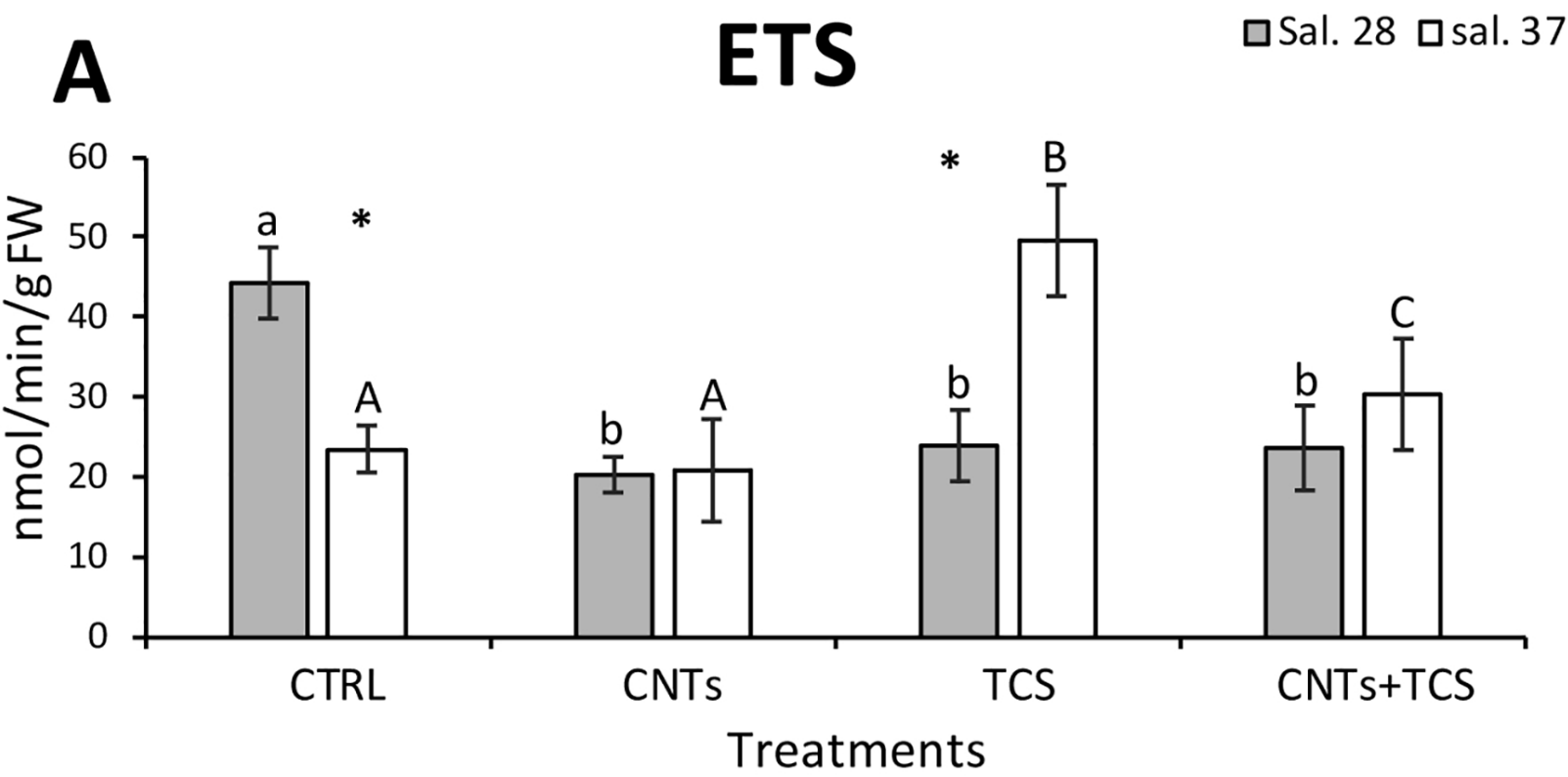

B
ROS
Sal. 28
sal. 37
Fluorescence intensity
60000
50000
40000
30000
20000
10000
0
a
A
b
B
A
c
A,B
*
CTRL
CNTs
TCS
CNTs+TCS
Treatments

Figure 2


A
ETS
Sal. 28
sal. 37
nmol/min/g FW
60
50
40
30
20
10
0
a
*
A
b
A
*
B
b
b
C
CTRL
CNTs
TCS
CNTs+TCS
Treatments

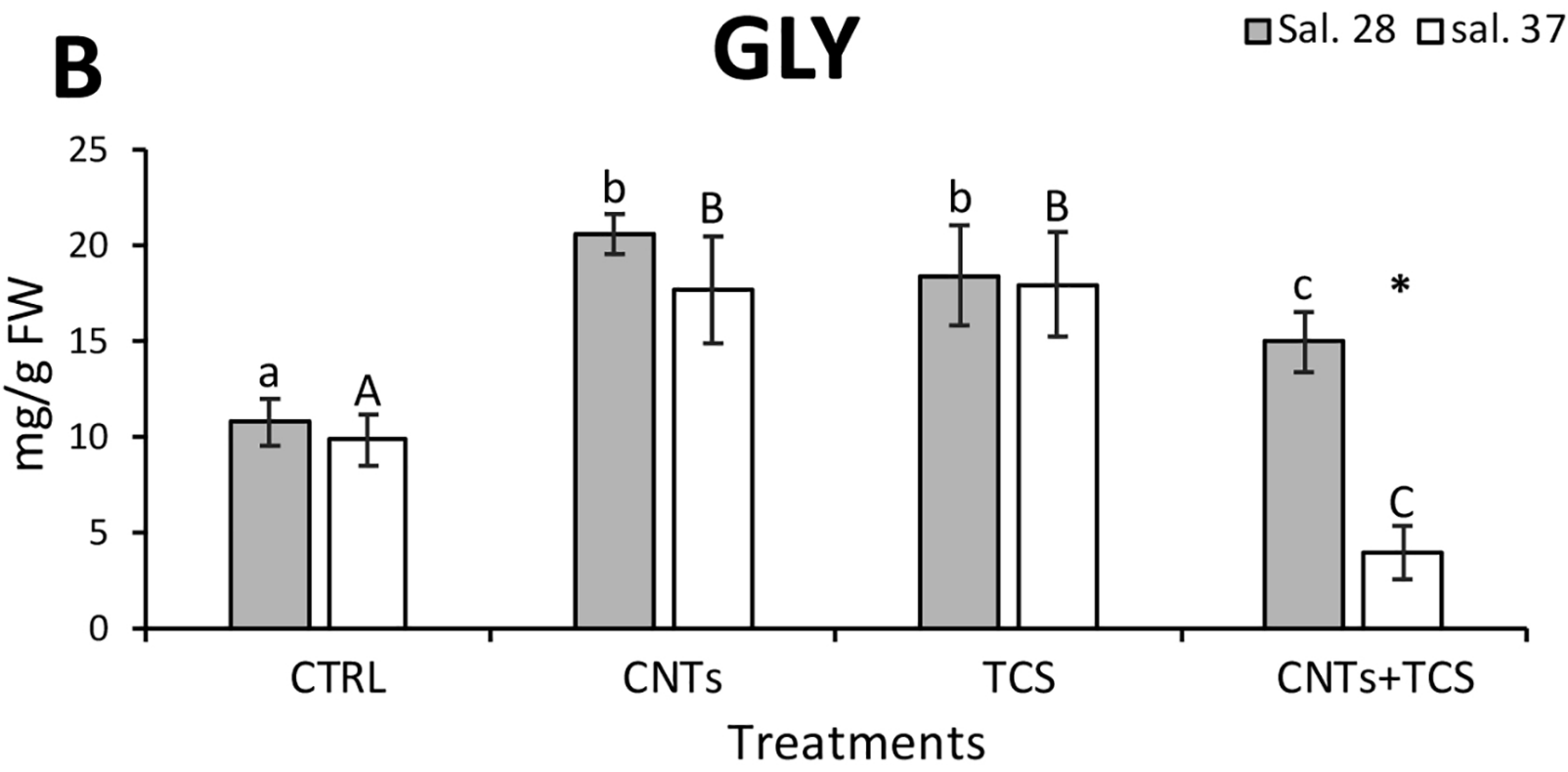

B
GLY
Sal. 28
sal. 37
mg/g FW
25
20
15
10
5
0
a
A
b
B
b
B
c
*
C
CTRL
CNTs
TCS
CNTs+TCS
Treatments

Figure 3

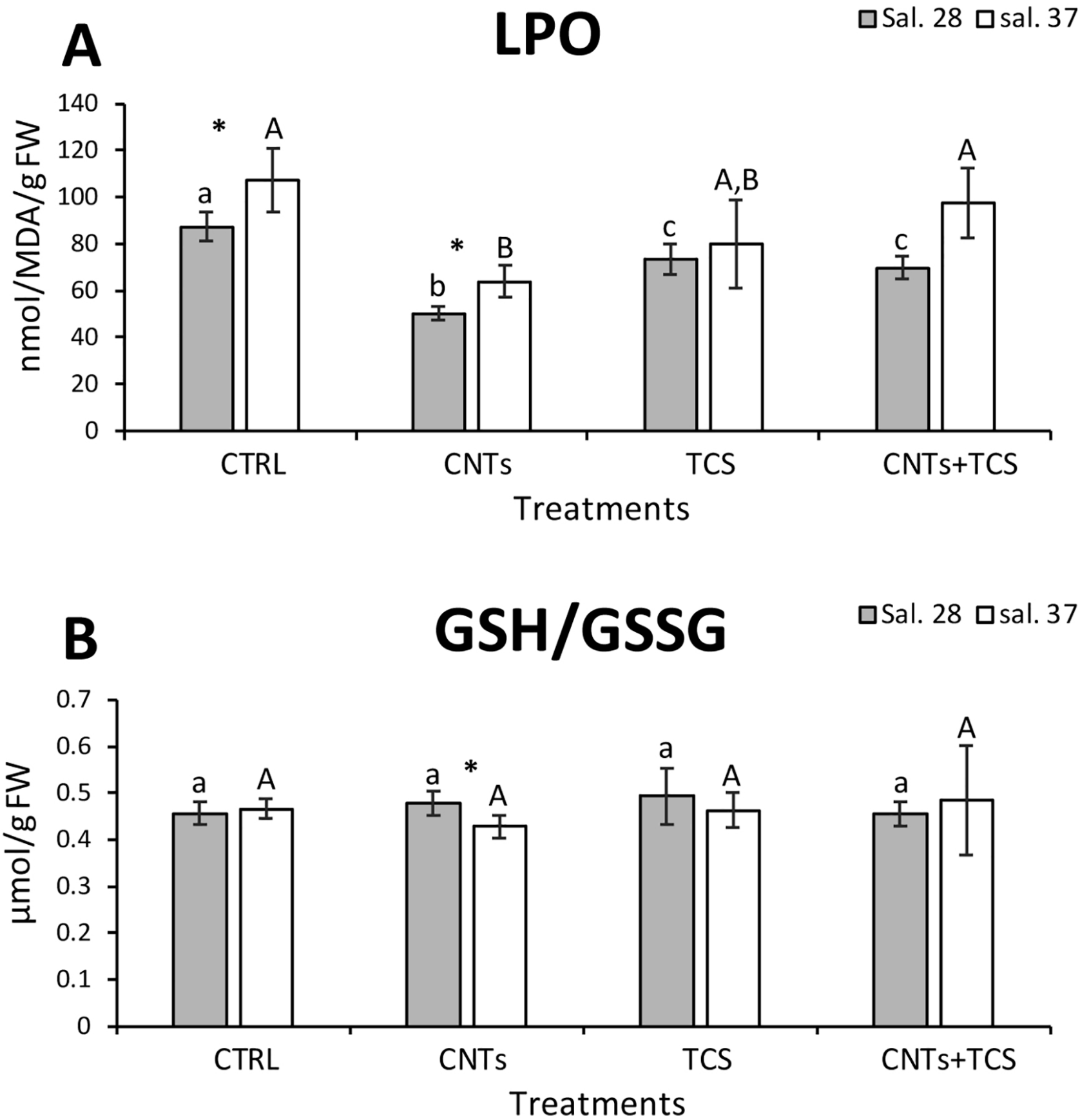
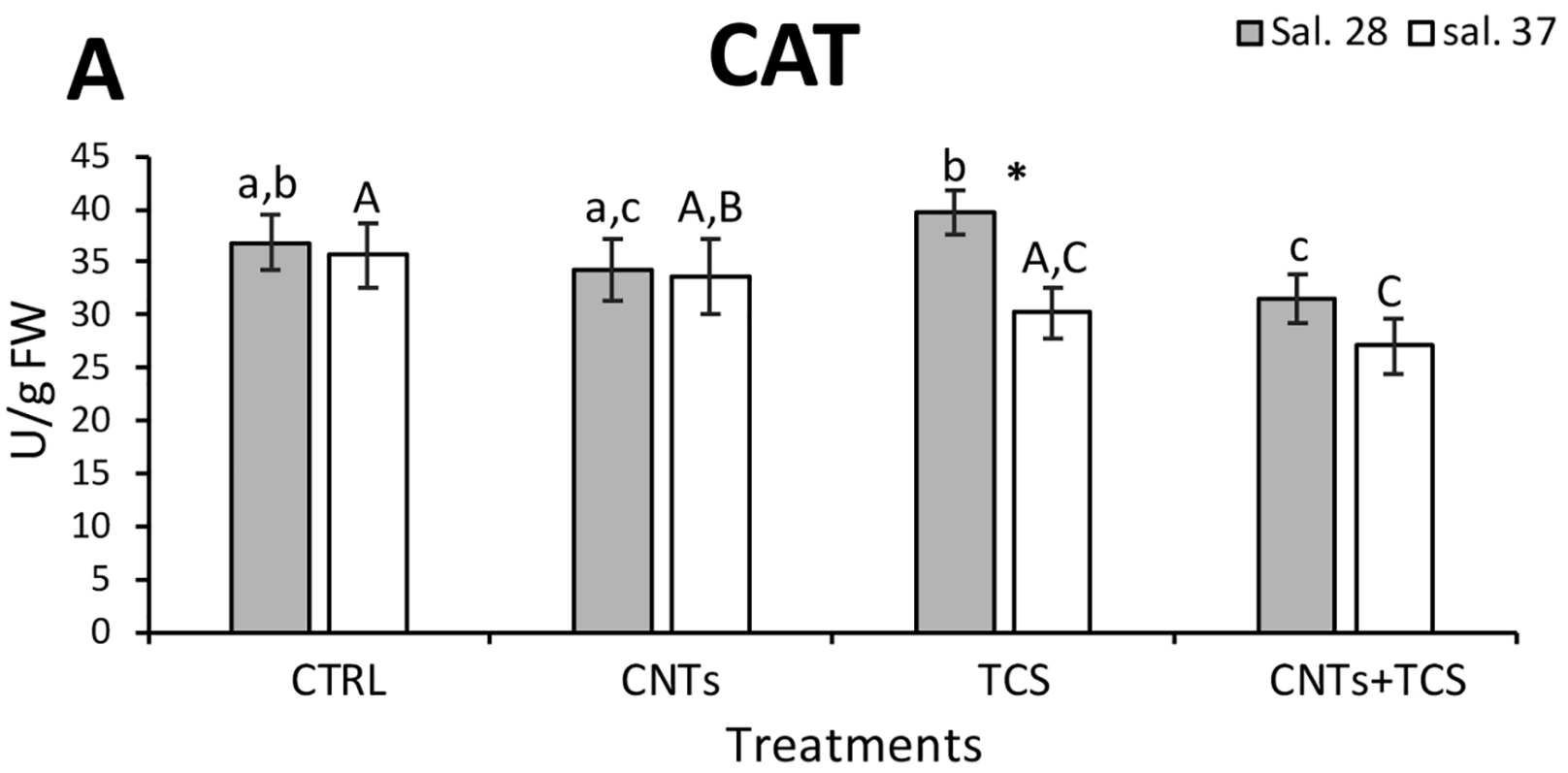

A
LPO
Sal. 28
sal. 37
nmol/MDA/g FW
140
120
100
80
60
40
20
0
*
A
a
*
B
b
A,B
c
A
c
CTRL
CNTs
TCS
CNTs+TCS
Treatments
B
GSH/GSSG
Sal. 28
sal. 37
µmol/g FW
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
A
a
*
A
a
A
a
A
CTRL
CNTs
TCS
CNTs+TCS
Treatments

Figure 4


A
CAT
Sal. 28
sal. 37
U/g FW
45
40
35
30
25
20
15
10
5
0
a,b
A
a,c
A,B
b
*
A,C
c
C
CTRL
CNTs
TCS
CNTs+TCS
Treatments

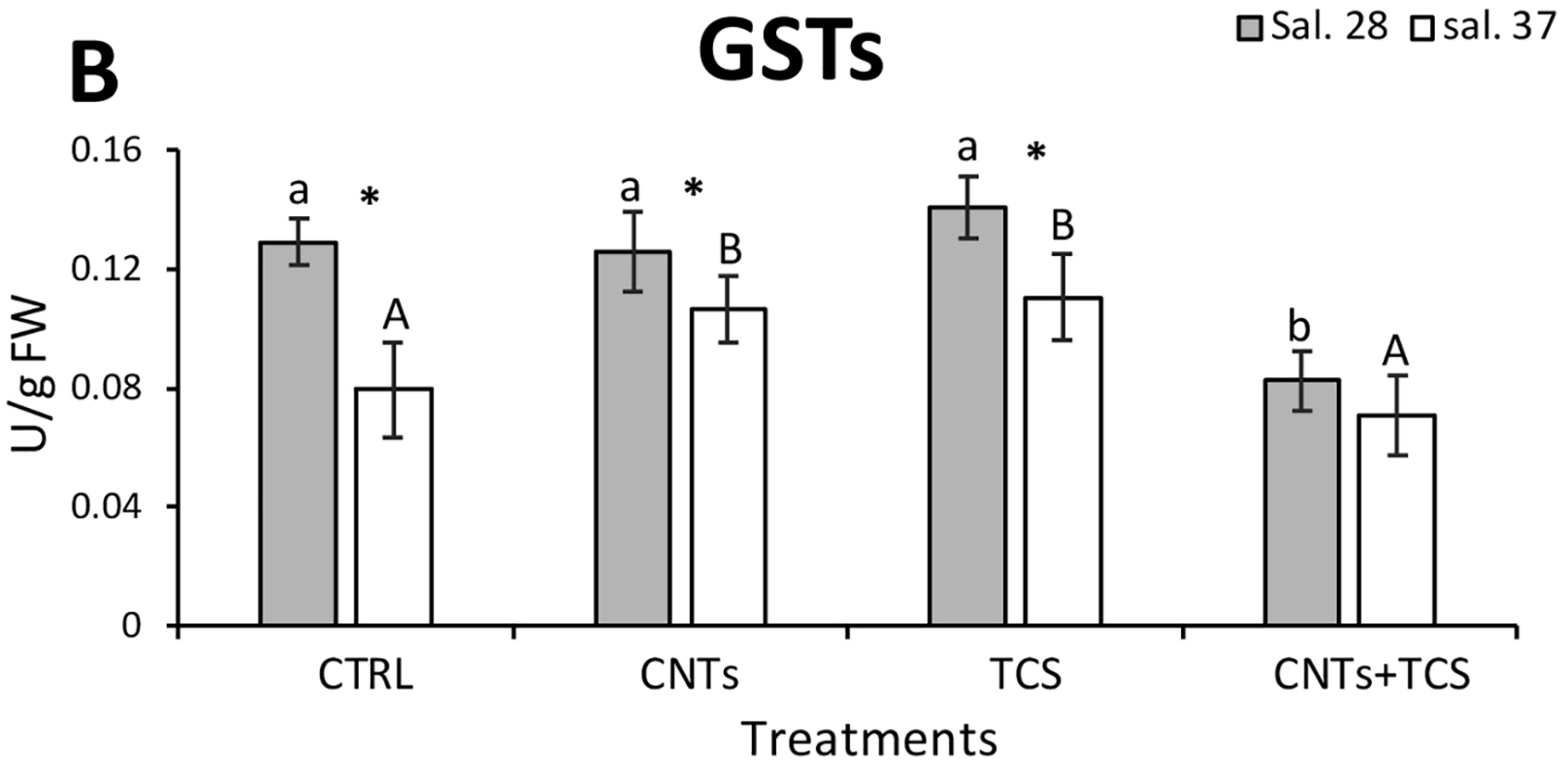

B
GSTs
Sal. 28
sal. 37
U/g FW
0.16
0.12
0.08
0.04
0
a
*
A
a
*
B
a
*
B
b
A
CTRL
CNTs
TCS
CNTs+TCS
Treatments

Figure 5