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# Unrevealing the interactive effects of climate change and oil contamination on lab-simulated estuarine benthic communities

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**Abstract**

There is growing concern that modifications to the global environment such as ocean acidification and increased ultraviolet radiation may interact with anthropogenic pollutants to adversely affect the future marine environment. Despite this, little is known about the nature of the potential risks posed by such interactions. Here, we performed a multifactorial microcosm experiment to assess the impact of ocean acidification, ultraviolet radiation B (UV-B) and oil hydrocarbon contamination on sediment chemistry, the microbial community (composition and function) and biochemical marker response of selected indicator species.

We found that increased ocean acidification and oil contamination in the absence of UV-B will significantly alter bacterial composition by, among other changes, greatly reducing the relative abundance of *Desulfobacterales*, known to be important oil hydrocarbon degraders. Along with changes in bacterial composition, we identified concomitant shifts in the composition of aromatic hydrocarbons in the sediment and an increase in oxidative stress effects on our indicator species. Interestingly, our study identifies UV-B as a critical component in the interaction between these factors, since its presence alleviates harmful effects caused by the combination of reduced pH and oil pollution. The model system used here shows that the interactive effect of reduced pH and oil contamination can adversely affect the structure and functioning of sediment benthic communities, with the potential to exacerbate the toxicity of oil hydrocarbons in marine ecosystems.

**Introduction**

Climate change and anthropogenic pollution are altering the oceans with profound consequences to marine ecosystems at regional and global scales (Doney, 2010). The oceans remove a quarter of the $CO_2$ emitted to the atmosphere by anthropogenic activities (Sabine *et al.*, 2004) and therefore play a fundamental role in regulating global warming. However, the uptake of excess

$CO_2$ by the oceans is also changing the chemical equilibrium of seawater and lowering pH at an unprecedented rate (Hönisch *et al.*, 2012).

Increasing concentrations of greenhouse gases are also expected to interact with ozone and alter its spatial distribution and exchanges between the stratosphere and troposphere, with potential effects on ultraviolet B radiation levels (UV-B) that reach the Earth (Bais *et al.*, 2011). In addition to changes to the Earth´s climate system, and despite existing rules and regulations, human activities continue to affect coastal and open-ocean environments causing a continuous influx of pollutants into these ecosystems (Doney, 2010). Oil hydrocarbons have been pointed out as one of the most widespread oceanic pollutants. Up to 1.3 million tonnes of oil are released annually into marine environments, of which approximately 47% is derived from natural seepages (NAS, 2003). While the number of spills from tanker accidents has declined in recent decades (Jernelöv, 2010), damaged pipelines are increasingly becoming important sources of pollution. With oil exploration moving to deeper waters, the risks of major blowouts, such as the Deepwater Horizon incident are increasing (Jernelöv, 2010). Deepwater Horizon alone released an estimated $4.4\times10^6\pm20\%$ barrels of oil ($7.0\times10^5$ $m^3$) (Crone & Tolstoy, 2010) causing severe environmental (ecological and socio-economical) damage.

While the direct effects of climate change and pollution on marine organisms and ecosystems are being increasingly documented (Doney *et al.*, 2012, Kroeker *et al.*, 2013, Shahidul Islam & Tanaka, 2004), the potential interactions between these factors are still largely unknown. Presently, there is a lack of fundamental knowledge about the synergistic effects of ocean acidification, UVR (mainly UV-B) and anthropogenic pollutants (such as oil hydrocarbons) on the functioning of coastal ecosystems and the processes of environmental recovery. We hypothesised that coastal communities and their ecological functions will be modified by the interaction between these factors. To test this, we assessed the interactive effects of parameters associated with climate change (ocean acidification and UV-B) and anthropogenic pollution (oil contamination) on estuarine benthic communities in a Experimental Life Support System (ELSS) designed

specifically for this purpose (Coelho *et al.*, 2013a). We focused our analysis on the sediment compartment, namely on the structure and function of the bacterial community, on oxidative stress and neurological biochemical markers of representative macrobenthic intertidal species and on abiotic stressors. Assessing the impact of such interactions on bacterial communities and the biogeochemical processes they mediate is fundamental to determining how ecosystems under multiple stressors will respond in the near future (Reid, 2011). Bacterial activity is of particular importance when considering the interactive effects between climate change and anthropogenic pollutants on open ecosystems; bacterial communities are often identified as the main drivers of crude-oil remediation in several environments, including intertidal sediment (McGenity, 2014). Likewise, the benthic estuarine invertebrates selected for the present study, namely the epibenthic gastropod *Peringia ulvae* (synonym: *Hydrobia ulvae*) and the benthic polychaete *Hediste diversicolor*, are major components of biomass production in many estuarine ecosystems (Krell *et al.*, 2011, Moreira *et al.*, 2006) and important links in estuarine food webs. They are also a primary food source for other invertebrates, birds and commercial fish species (Cardoso *et al.*, 2005, Cardoso *et al.*, 2002, Dolbeth *et al.*, 2008). The ecological relevance and the wide geographical distribution of these species led to their proposal as good indicators of estuarine environmental quality (Conde *et al.*, 2013, Moreira *et al.*, 2006). In the present study, we followed a multibiomarker approach using the above mentioned benthic invertebrate species; this approach has been recommended as a tool for monitoring pollution by the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR) and the International Council for the Exploration of the Sea (ICES) (Solé *et al.*, 2009). To the best of our knowledge, the integrated response of benthic microbes and macrofauna exposed to pollutants under simulated climate change conditions has never been addressed before.

## Materials and Methods

*Sample collection and ELSS manipulation*

Sediment cores (approximately 13 cm high) were collected in the Ria de Aveiro, a shallow coastal lagoon, which comprises the Vouga River estuary (Portugal), (40°37'32"N.8°44'10"W) and transferred directly into individual microcosms on the 24$^{th}$ of May 2011 (Figure S1). The microcosms were subsequently taken to a marine experimental life support system (ELSS) that sustained the microcosms during 57 days. The ELSS was previously validated to study climate change scenarios on benthic marine communities as described in Coelho et al. (2013a). Briefly, the ELSS is a framework that can maintain individual microcosms (glass tanks 25 cm high, 28 cm length and 12.4 cm width, each with a maximum functional water volume of approximately 3 L) under highly controlled conditions, while enabling the simulation of fundamental marine dynamics including the diurnal light cycle, tidal simulation, water temperature and water pH (Figure S1).

A fully factorial experiment was designed using 32 independent microcosms with three factors: UV-B radiation, water pH and oil contamination. Each factor had two levels: UV-B (with and without), pH (normal and reduced) and oil (with and without). Each factor and the combination between factors were replicated into four random independent microcosms and arranged in a randomized split-plot design with UV-B as the whole plot and pH and oil as the sub-plot levels. The design enabled us to distinguish between 8 treatments as follows.1. Cont (no UV-B, normal pH and no oil); 2. OnpH (no UV-B, reduced pH, no oil); 3. OnUV (UV-B, normal pH and no oil); 4. UVpH (UV-B, reduced pH, no oil); 5. OnOi (no UV-B, normal pH, oil); 6. UVOi (UV-B, normal pH, oil); 7. pHOi (no UV-B, reduced pH, oil) and finally, 8. UVTot (UV-B, reduced pH, oil).

In the first phase of this experiment the sediment was stabilized during 21 days. During this phase the light and synthetic water were gradually adjusted to the environmental test conditions (supporting information). The second phase started on day 22 with the simulation of an oil spill by pouring crude oil (0.5% v/v) into randomly selected treatments during five consecutive high tides. The system was operated with Synthetic saltwater, prepared by mixing freshwater, purified by a four stage reverse osmosis unit (Aqua-win RO-6080) with a commercially available salt mixture (Tropic Marin Pro Reef salt – Tropic Marine, Germany). The ELSS simulated a uniform semi-diurnal tidal regime (approximately 1 min to fill and to empty), creating two high tides and two low tides daily. Water pH was manipulated by bubbling $CO_2$ into water (Gattuso & Lavigne, 2009) reservoirs prior to addition into the microcosms. The acidified water was supplied to the microcosms during each high tide. During each tidal cycle about 50% of the water volume (1.5 l) of each microcosm was renewed (flow-trough non-recirculated). The pH was monitored in the acidified and control microcosm with a calibrated pH meter (orion star portable meter, Thermo Fisher Scientific Inc, USA). The average pH was 7.62±0.11 in acidified microcosms (measured at the end of each low tide, before the addition of new acidified water) and 7.94 ±0.10 in the control microcosms (Figure S2). This reduction falls within the 0.3-0.4 pH reduction modeled for global sea surface for the year 2100 (Caldeira & Wickett, 2003). Salinity (32.6±1.5) and temperature (19±1.5 ºC) were adjusted to simulate the conditions recorded at the sampling location and kept constant as previously described (Coelho *et al.*, 2013a).

A 14-hour diurnal light cycle (average day length in summer months at Portuguese latitudes) was simulated with light intensity varying from 50% to 100% of the total fluorescent tube intensity (Table S1). Two UV-B conditions were implemented in the microcosm ensemble: microcosms irradiated with UV-B and microcosms where the UV-B was filtered out. In the microcosms with UV-B, a constant UV-B irradiance of 1426.36±93.32 $mW/m^2$, during 4 hours per day around noontime, was used. This dose is similar to the typical DNA-damage UV dose observed at Lisbon, in June. Given that in the water column shorter wavelengths (UV-B) are reduced more

effectively than longer wavelengths (UV-A) (Tedetti & Sempéré, 2006) and that UV-A plays an important biological role (Bargagli, 2005), a similar amount of UV-A integrated irradiance of 2875.91±264.62 mW $m^{-2}$ was supplemented to the microcosms. A detailed description of the seawater experimental manipulation and the UV-B supplementation (including light spectra – Figure S3) is available as supporting information.

*Water inorganic nutrient analysis*

Water samples for dissolved inorganic nutrient (nitrate $NO_3^-$; sulphate $SO_4^{2-}$; ammonium $NH_4^+$ and o-phosphate $PO_4^{3-}$) determination were collected from each microcosm at the end of the experiment (57 days). Water aliquots were immediately filtered (Whatman GF/C glass-fibre filter) and stored frozen at -20 ºC until analysis. $NO_3^-$ determination followed the 8039 method described in the Hach Spectrophotometer (DR 2000) standard analytical procedure (Hach, USA, DR2000, 44863-00). The determination of $NH_4^+$ and $PO_4^{3-}$ concentrations were carried out following standard spectrophotometric methods described in Limnologisk (Metodik, 1992). The determination of $SO_4^{2-}$ followed the 8051 method described in the Hach Spectrophotometer (DR 2000) standard analytical procedures (Hach, USA, DR2000, 44863-00). The analytical quality control was ensured by duplicate samples and by the analysis of blanks between samples. We checked for deviations from normality in nutrient concentrations with the shapiro.test () function and tested for homogenous variance with the bartlett.test() function in R (http://www.r-project.org/; Accessed January 2013). We tested for significant differences among treatments in normally distributed nutrient concentrations ($NO_3^-$, $SO_4^{2-}$ and $NH_4^+$) with a one-way ANOVA using the aov() function in R and tested for significant differences in non-normal distributed nutrient concentrations ($PO_4$) using the adonis() function in the vegan package (Oksanen *et al.*, 2012). In the aov() function, the concentration of dissolved inorganic nutrients was the response variable and treatment was the independent variable. We also

included the luminaires as the error term. In the adonis() analysis, the Euclidean distance matrix of nutrient concentration was the response variable with treatment as independent variable. The number of permutations was set at 999. The argument strata was included to allow randomizations only within each luminaire (Oksanen *et al.*, 2012).

*Active bacterial community and sediment chemistry analysis*

*Sediment sampling*

Sediment samples were collected at the end of the experiment (57 days of operation). For the active bacterial community analysis, four composite samples consisting of four sediment mini-cores (ca. 1 cm of top sediment with a 1 cm diameter) were collected in each of the 32 microcosms (8 tested treatments with 4 independent replicates). For the aromatic and aliphatic hydrocarbon analysis, approximately 5 grams of sediment were collected using mini-cores (ca. 3 cm in length and 1.5 cm in diameter) from each oil contaminated microcosm (4 independent microcosm replicates from the OnOi, UVOi, pHOi and UVTot groups) and from 4 control microcosms (Cont). Finally, for the non-target exploratory analysis of sediment volatile compounds, approximately 6 grams of sediment were collected using mini-cores (ca. 3.5 cm of top sediment with a 1.5 cm diameter) in each of the 32 microcosms.

*Active bacterial community and predicted metagenome analysis*

RNA extraction and cDNA synthesis were conducted as previously described (Coelho *et al.*, 2013b). A barcoded pyrosequencing approach was used for bacterial community analysis. Fragments of the 16S ribosomal RNA (rRNA) gene were sequenced for each sample with primers V3 Forward (5'-ACTCCTACGGGAGGCAG-3') and V4 Reverse (5'-

TACNVRRGTHTCTAATYC-3') using the 454 Genome Sequencer FLX Titanium (Life Sciences Roche Diagnostics Ltd, West Sussex, UK). Only sequences containing exact matches to primer sequences and barcode tags were used for further analysis.

Raw sequencing reads were quality trimmed according to published recommendations (Kuczynski *et al.*, 2011) and the QIIME software package (http://qiime.org; Accessed 4 May 2012) was used to analyze the results of the runs. Sequences can be downloaded from the NCBI ShortRead Archive (study accession PRJNA197342). The PICRUSt (http://picrust.github.com/picrust/Accessed September 2013) software package was used to predict the metagenome functional content from the 16S rRNA dataset. A detailed description of the QIIME and the PICRUSt pipeline is available as supporting information.

Total rarefied operational taxonomic units (OTUs, comprising sequences sharing ≥97% similarity) per sample was estimated with a self-written function (Gomes *et al.*, 2010) in R. Rarefied OTU richness was selected for a minimum sampling size of 1800 sequences. The Shapiro.test() function was used to test for normality and the bartlett.test() function was used to test the assumption of homogenous variances. The Shapiro test revealed that OTU richness did not deviate significantly from normality (Shapiro-Wilk normality test, $P = 0.167$) and the Bartlett test showed homogenous variances between treatments (Bartlett test: chi-squared = 12.1, df = 7, $P = 0.096$). A one-way ANOVA using the aov() function in R was then used to test if rarefied richness differed significantly among treatments. Variation in OTU composition among treatments was tested for significance using the adonis() function in vegan (Oksanen *et al.*, 2012). In the adonis() analysis, the Bray-Curtis distance matrix of OTU composition was the response variable with treatment as independent variable. The number of permutations was set at 999. The argument strata was included to allow randomizations only within each luminaire (Oksanen *et al.*, 2012). Variation in composition among treatments was assessed with Principal Coordinates Analysis (PCO) using the cmdscale() function in R and the Bray–Curtis distance matrix as input.

Variation in OTU and KO relative abundances were analyzed with a mixed-model approach, treating Oil, UV-B and reduced pH as fixed effects and the luminaires as the random effect. Since we wanted to examine the interaction between Oil, UV-B and pH we included an interaction term. The mixed-model was fitted using the glmer() function in the lme4 package (version 0.999999-0) in R with family argument set to binomial (Bates *et al.*, 2011). Overdispersion was accounted for by including individual level variability as a random effect (Bates *et al.*, 2011). The significance on the main effects and interaction terms was tested using z -statistics. With this approach we accounted for pseudo-replication (i.e., oil contamination, reduced water pH and UV-B treatment split within four luminaires).

*Aromatic and aliphatic hydrocarbon analysis*

Sediment aliphatic and aromatic hydrocarbons were determined using a soxhlet extraction followed by a GC-MS analysis (Yan *et al.*, 2009). The determination of the aliphatic and aromatic fractions was performed on a Network GC system 6890/5973 (Agilent Technologies, USA) mass spectrometer with a 30 mm x 0.25 mm x 0.25 μm film thickness VF-5MS fused silica capillary column in the selective ion mode (SIM) for aromatic hydrocarbons or in scanning mode for aliphatic hydrocarbons. Concentration of the 16 US EPA priority polycyclic aromatic hydrocarbons (PAHs) were assessed using an internal standard mixture (Supelco) peaks area method. A full description of the aromatic and 20 most abundant aliphatic oil compounds analysis is available as supporting information. We checked for significant deviations from normality with the shapiro.test() function in R. The Shapiro test revealed that hydrocarbon concentrations deviated significantly from normality, even after logarithmic and square-root transformation. We, therefore, tested for significant differences among treatments using a Kruskal-Wallis test with the Krustal.test() function in R.

*Non-target exploratory analysis of sediment volatile compounds*

Volatile compounds were analyzed in the sediment by solid phase microextraction followed by comprehensive two-dimensional gas chromatography-time of flight mass spectrometry (SPME/GC × GC-ToFMS)(Silva *et al.*, 2010). The SPME holder for manual sampling and fibre were purchased from Supelco (Aldrich, Bellefonte, PA, USA). A total of 32 chromatograms (4 replicates x 8 treatments) were generated and processed with Chroma-TOF® (LECO) software (baseline correction, deconvolution, peak picking and integration) at signal-to-noise threshold of 100. The DTIC (Deconvoluted Total Ion Current) GC × GC area data were used as an approach to estimate the relative content of each compound in the sediment. Compound alignment was performed with Guineu software (http://code.google.com/p/guineu/Accessed 14 January 2013). Guineu software aligns the data based on compounds retention times, spectral information and identification (Castillo *et al.*, 2011).

First, the compounds from all the samples were aligned. A square matrix containing the areas of the compounds aligned at least in 8 different samples was used for subsequent analysis. Variation in composition among treatments was assessed with PCO analysis. The PCO was generated using the cmdscale() function in the R base package and wascores() function in vegan. Prior to the PCO, the raw data was $\log_{10}(x+1)$ transformed and used to produce a distance matrix with the Euclidean distance with the vegdist() function in vegan (Oksanen *et al.*, 2012). We used the procrustes() function in vegan to assess congruence among metabolomic and barcode pyrosequencing data (active bacterial community) PCO ordinations. Default values were used for the arguments in the procrustes() analysis. In addition to the procrustes() function, the protest() function in vegan was used to estimate the significance of the procrustes statistic. The number of permutations in the protest() function was set to 999.

Next, in accordance with the trend observed for multivariate statistical analysis, only compounds from samples where pH was reduced (with and without oil) were aligned. A square matrix

containing the areas of the compounds aligned in at least 4 different samples was used in subsequent analysis. Variation in composition among treatments was assessed with a PCO analysis as previously described. Peak apex plots were then constructed combining $^{1}t_{R}$ and $^{2}t_{R}$ values of compounds that cluster near OnOi and pHOi. Peak apex plots indicate the position of the maximum modulated peak of GC × GC analysis, in the 2D chromatographic space. Compounds structurally related appear on a similar space on the peak apex (2D structured chromatogram) (Marriott *et al.*, 2004).

*Stress biochemical markers of benthic macroinvertebrates*

*Organism collection and handling*

The benthic invertebrate species *Peringia ulvae* and *Hediste diversicolor* were collected, at intertidal reference sites in June 2011. Details on the sampling location and procedures for organism collection, transport and maintenance are outlined in the supporting information. *P. ulvae* and *H. diversicolor* were introduced into the microcosms (20 and 7 organisms/replicate, respectively) after the initial period of stabilization (21 days). At the end of the experiment organisms were retrieved from the sediment, counted and immediately frozen in liquid nitrogen and stored at -80ºC until further analysis. Pools of 20 individuals for the gastropod species and of 2 - 3 individuals for the polychaetes from each microcosm replicate were weighed (fresh weight) and homogenised in ice-cold phosphate buffer (50 mM, pH=7.0 with 0.1% Triton X-100) (1:5 m/v). Homogenates were centrifuged at 10000g for 10 minutes at 4ºC and supernatants were stored at -80ºC until the enzymatic analysis.

*Invertebrate's biochemical parameters*

For both invertebrate species, enzyme activities (CAT-catalase, SOD-superoxide dismutase, GST-glutationeS-transferase, AChE-acetylcholinesterase) and lipid peroxidation (LPO) were measured in triplicate; due to biomass limitations no SOD measurement was made for *P. ulvae*. Enzyme activities were expressed in nmol of hydrolyzed substrate per minute per mg of protein, except for CAT and SOD, which were expressed in μmol of hydrolyzed substrate per minute per mg of protein and SOD units per gram of protein. The total content in protein of each aliquot was determined spectrophotometrically according to Bradford (1976) using bovine γ-globulin as standard. CAT, SOD, GST and AChE activity and LPO were measured spectrophotometrically at 240, 505, 340, 414, and 535 nm, respectively, following exactly the same procedures described in Coelho et al (2013).

Among the biochemical markers of exposure analysed, CAT and SOD are important antioxidant enzymes in the combat against reactive oxygen species (ROS), generated as a by-product of cellular metabolism or in response to different stress agents. Thus, both antioxidant enzymes are activated in response to an increase in ROS. SOD is responsible for the transformation of the superoxide anion radical in hydrogen peroxide, while CAT neutralizes hydrogen peroxide in water and oxygen. GST catalyses the conjugation of glutathione (a non enzymatic antioxidant) with lipophilic compounds (such as PAHs) and it also plays an important role in protection against oxidative stress (Matozzo *et al.*, 2013). When the defense capacity of the cell is overwhelmed, oxidative stress occurs leading to the peroxidation of membrane lipids (biomarker of effect) and the impairment of several intracellular molecules (Bocchetti & Regoli, 2006). AChE is an important enzyme from cholynergic synapses, and is responsible for the hydrolysis of the acetylcholine neurotransmitter, cleaning the synaptic cleft and allowing the transmission of nervous impulses between nervous cells and neuromuscular junctions. The inhibition of this enzyme has been reported as an indicator of exposure to neurotoxic organic compounds mainly

organophosphorous and carbamates (Sarkar *et al.*, 2006) but also metals and PAHs (Guilhermino *et al.*, 1998).

We tested the variation among factors for each biomarker using the adonis() function. In the adonis() analysis, the Euclidean distance matrix of enzyme activity was the response variable with oil, pH and UV-B as independent variables. Since we wanted to examine the interaction between oil, UV-B and pH we included an interaction term. The number of permutations was set at 999; all other arguments used the default values set in the function. The argument strata was included to allow randomizations only within each luminaire (Oksanen *et al.*, 2012).

## Results

### *Water nutrients, aromatic and aliphatic oil hydrocarbons*

$NO^-_3$ and $SO_4^{-2}$ (but not $PO_4^{3-}$ and $NH_4^+$) differed significantly among treatments (Figure S4). The most noteworthy effect was an increase in $SO_4^{-2}$ concentration in oil treatments.

The chromatography analysis of the 16 US EPA priority PAHs and aliphatic hydrocarbons of sediment collected from oil contaminated and control treatments were highly variable and did not reveal significant differences (Figure S5 and S6).

### *Active bacterial community*

To examine changes in OTU richness, rarefaction curves were generated for all treatments (Figure S7). Controlling for sampling size (n= 1800 sequences), rarefied OTU richness varied from 490±68.52 in OnOi, to 557±48.68 in OnUV (Figure S8). No significant differences in OTU richness were detected between the different treatments (Anova, $F_{7,24} = 1.29$, $R^2 = 0.868$, P = 0.54). In contrast, there was a highly significant variation in OTU composition among

treatments (Adonis analysis: $F_{7,31} = 1.563$, $R^2 = 0.313$, $P<0.001$) (Figure 1). The first PCO axis separated the control samples (with and without oil) and the samples retrieved from pHOi; the second axis was primarily related to variation between OnUV (that cluster near the control) and pHOi (Figure 1). UVTot also diverged from other treatments, although less pronouncedly than the sole combination of reduced pH (OnpH) and oil (OnOi) contamination.

Using the RDP classifier tool with a confidence threshold of 80%, 128313 out of 129282 (99.25%) qualified sequences were assigned to known phyla. Our results showed that *Proteobacteria* was the most abundant phylum varying from 95.92±1.35% in OnOi to 88.55±5.20% in the pHOi treatment. We therefore tested the independent and interactive effects of oil, pH and UV-B treatments on the relative abundance of the three most representative proteobacterial orders and unclassified OTUs at the order level (Figure 2). Together, these four treatments varied from 87.21±2.39% in Cont to 76.12±9.91% in pHOi treatments (the relative abundance of all *Proteobacteria* orders is available as supporting information Figure S9).

The most notable effect detected was a reduction in the relative abundance of *Desulfobacterales* (*Deltaproteobacteria*) due to an interaction between oil and reduced pH in the absence of UV-B (Wald Z test, $z = -3.046$, $P = 0.002$). This reduction is particularly noteworthy when considering oil contaminated treatments in the absence of UV-B. In this case, oil contaminated microcosms showed a reduction in *Desulfobacterales* relative abundance from 50.71±6.96%, under normal pH, to 19.56±7.36% under reduced pH.

However, in the presence of UV-B, the combination of reduced pH and oil did not appear to affect the relative abundance of this order. This result suggests that the presence of UV-B attenuates the interaction between pH and oil. Likewise, the *Methylococcales* order (*Gammaproteobacteria*) showed a reduction in relative abundance due to an interaction between oil and reduced pH (Wald Z test, $z = -2.167$, $P = 0.032$) in the absence of UV-B; registering lowest abundance under this circumstance (8.45±3.92%). However, a significant interaction of oil, reduced pH and UV-B (Wald Z test, $z = 2.156$, $P = 0.031$) appears to overcome the effect of oil

and reduced pH without UV-B. The relative abundance of *Methylococcales* was highest when all treatments were present (18.59±2.56%) (UVTot). A significant interaction between oil and reduced pH was also detected in *Chromatiales* and unclassified OTUs (order level) (Wald Z test, z = 3.719, P = 0.0002 and Wald Z test, z=1.980, P = 0.047). In this case, both groups registered their highest relative abundance under the combination of oil and reduced pH (24.04±8.26% and 24.05±7.30%).

Besides the changes in the three most abundant orders and unclassified OTUs (order level), significant changes were also detected in low abundant *Alteromonadales* and *Desulforomonadales* orders (Figure S9). In *Alteromonadales*, a significant interaction was detected between oil and reduced pH in the absence of UV-B (Wald Z test, z = 3.249, P = 0.001) and between oil, reduced pH and UV-B (Wald Z test, z = -2.494, P = 0.013). The relative abundance of this order registered its highest abundance in pHOi treatment (5.17±0.57%); while in UVTot the abundance was within the same magnitude than the other treatments (0.74±0.34%). Regarding *Desulforomonadales*, we detected a strong effect of oil (Wald Z test, z = -3.431, P < 0.001) and a significant interaction between oil and UV-B (Wald Z test, z = 4.656, P < 0.001). This order registered its higher relative abundance in UVOi treatment (1.22±0.59%).

We used PICRUSt to predict metagenome functional content based on the Kyoto encyclopedia of genes and genomes (KEGG) classification (Kanehisa & Goto, 2000). We focused on the relative abundance of KEGG orthologs (KO) assigned to the "xenobiotics biodegradation and metabolism" category. The independent and interactive effects of oil, reduced pH and UV-B on the relative abundance of the six most representative degradation pathways were analysed (Figure 3). The relative abundance of all KEGG categories and of all "xenobiotics biodegradation and metabolism" pathways is available as supporting information (Fig. S11). In line with the taxonomic analysis, the most significant effects were detected between oil and reduced pH in the absence of UV-B. A significant interaction between oil and reduced pH was found for the benzoate (Wald Z test, z = -3.14, P = 0.002), caprolactam (Wald Z test, z = -3.40,

P = 0.002) and ethylbenzene (Wald Z test, z = -4.53, P < 0.001) degradation pathways. The relative abundance of KO orthologs assigned to these pathways decreased with oil contamination under reduced pH conditions. On the other hand, a significant interaction between oil and reduced pH appears to reduce KOs assigned to toluene degradation (Wald Z test, z = 3.2, P = 0.001) and the metabolism of xenobiotics by cytochrome P450 (Wald the Z test, z = 3, P = 0.0027). The relative abundance of the pathways remained stable in all the treatments with UV-B.

*Non-target exploratory analysis of sediment volatile compounds*

The non-target exploratory analysis into the sediment volatile compounds revealed significant differences between treatments (Adonis analysis: $F_{7,31} = 2.094$, $R^2 = 0.379$, $P < 0.001$). A principal coordinates analysis (PCO) was applied to visualize the dissimilarities between all the treatments (Figure S12). The primary axis of variation was between treatments with oil and treatments without oil; the secondary axis of variation was between treatments with and without UV-B. Interestingly, pHOi clustered together with UVOi and UVTot; suggesting a similar volatile compound composition between these treatments. In order to determine if there was any congruence between the trends observed from the active bacterial community and from the volatile compound data, we compared the PCO ordinations obtained with both datasets using procrustes analysis (Figure S13). This procedure revealed significant congruence between microbiome and volatile compound profiling (Procrustes correlation, R= 0.461, P=0.001). However, the relatively large residual distances among the same treatments revealed that both datasets did not follow exactly the same pattern. Indeed, the active bacterial community and the corresponding predicted metagenome revealed a more drastic effect of pHOi on estuarine sediment functioning than did the non-target volatile compound analysis. Nevertheless, pHOi did reveal marked differences regarding their sediment volatile compound composition when

compared to OnOi. Considering these results, we further focused our analysis on the differences between OnOi and pHOi. Oil contaminated samples contained distinct compounds depending on whether they were housed under normal or reduced pH conditions (Figure S14). Interestingly, a higher number of compounds structurally related to the higher molecular weight fraction (between $C_{14}$ and $C_{20}$) detected, containing mainly aromatic and polycyclic aromatic hydrocarbons were associated with the pHOi cluster (Figure S15).

*Invertebrate biochemical parameters*

Mean survival percentages of *P. ulvae and H. diversicolor* in Cont microcosms were 96 and 100, respectively. In general, the survival of organisms was more affected in individual microcosms with oil. In the four treatments with oil contamination (OnOi, UVOi, pHOi and UVTot) mean survival of gastropods and polychaetes ranged from 78.6 to 60.7% and 98.8 to 87.5%, while in the remaining treatments (Cont, OnUV, OnpH, UVpH) mean survival was greater than 81 and 98.8%, respectively.

The most consistent response detected in the studied biochemical markers was an independent effect of oil contamination in *H. diversicolor* (Figure 4). In this species, oil had a significant effect on the activity of GST (Adonis analysis: $F_{21,28}$ = 4.860, $R^2$ =0.147, P=0.049), LPO (Adonis analysis: $F_{21,28}$ = 27.599, $R^2$ =0.381, P=0.001) and AChE (Adonis analysis: $F_{21,28}$ = 8.012, $R^2$ =0.245, P=0.015) (Figure 4). However, while the activities of GST and LPO were approximately 1.6 and 2.4 times higher in oil contaminated than in uncontaminated treatments, the activity of AChE was 1.6 times higher in uncontaminated than in oil contaminated treatments. Here, we also detected a significant interaction between oil and pH on CAT activity (Adonis analysis: $F_{21,28}$ = 5.565, $R^2$ =0.154, P=0.044). In contrast, although LPO activity was on average 2.8 times higher in the pHOi treatment in comparison to all other treatments, no significant interaction was found between oil and pH. Interestingly, a significant interaction between oil and UV-B

(Adonis analysis: $F_{21,28}$ =6.597; R2=0.091; F=0.022), recorded for the latter biomarker, induced a reduction in this parameter when compared with other oil treatments; LPO activity was on average 1.6 times lower in UVOi when compared with other oil contaminated treatments.

In *P. ulvae* the response of the different biochemical markers showed high variability (Figure 4). In this species the most noteworthy shifts were the independent effects of oil (Adonis analysis: $F_{23,30}$ = 21.506, $R^2$ =0.282, P=0.001) and UV-B (Adonis analysis: $F_{23,30}$ = 11.152; $R^2$=0.146; P=0.002) on LPO and a significant interaction of oil, pH and UV-B on LPO (Adonis analysis: $F_{23,30}$ = 8.771, $R^2$ =0.115, P=0.005), with a higher expression of the effects, caused by the joint action of all the factors. LPO values in oil contaminated treatments were on average 2.15 times higher when compared to uncontaminated treatments; UV-B, however, seemed to maintain LPO values close to control values. Notably, LPO values under the combination of all treatments were 2.86 times higher when compared to other treatments. There was a significant interaction between oil and pH on AChE activity (Adonis analysis: $F_{23,30}$ = 9.280, $R^2$ =0.265, P=0.005), which was lower in the pHOi treatment than in all remaining treatments.

## Discussion

### *Experimental life support system simulation*

Water pH in estuarine and coastal environments is modulated not only by atmospheric $CO_2$ dissolution into water but also by other factors such as the influx of water from rivers that is generally more acidic. However, considering that the Ria de Aveiro is classified as a well-mixed estuary (Dias *et al.*, 2000), this effect is minimal. The pH control approach used in this study allowed us to simulate the environmental conditions near the mouth of the estuary in the Mira channel, one of the main channels of the Ria de Aveiro that is under the influence of marine coastal water (pH ~8) (Moreira *et al.*, 1993). Our model system simulates the conditions

predicted for the global sea surface for the year 2100 (Caldeira & Wickett, 2003). It should be noted, however, that other coastal systems could be subject to lower pH values than those modelled for the global sea surface. Coastal systems with higher levels of human impact and eutrophication than the relatively unperturbed Ria de Aveiro (Ferreira *et al.*, 2003, Lopes *et al.*, 2007)would be characterized by lower dissolved oxygen concentrations and more acidic waters (Wallace *et al.*, 2014). Reductions in seawater pH due to increasing eutrophication in coastal and estuarine ecosystems can greatly exceed the effect predicted for ocean acidification (Howarth *et al.*, 2011). Unlike the effect of increasing atmospheric $CO_2$ on ocean pH, there is a great deal of uncertainty about the projected changes of ultraviolet radiation that reaches the water surface (Correa *et al.*, 2013, Watanabe *et al.*, 2011). We therefore chose to simulate a moderate scenario by considering the typical DNA-damage UV dose observed at Lisbon (Portugal), in June. By supplementing a constant UV-B irradiation level during each day we did not consider small daily fluctuations of this parameter; for example, those caused by changes in cloud cover in natural environments. Although such fluctuations may be of importance in natural ecosystems, by applying a defined UV-B dose the experiment throughout we were able to discern the sole effect of this parameter on sediment community homeostasis and its additive impact when in synergism with water acidification and oil pollution.

In this study the ELSS operated during 36 days after oil contamination; until the oil from the water surface had almost completely disappeared. This period is consistent with previous studies, which have shown that microbes in temperate estuarine sediment can degrade most of the oil compounds after the first weeks of contamination (Coulon *et al.*, 2012, Pearson *et al.*, 2008). We focused our analysis on the last day of the experiment (57 days). After this period of time, the environment has had time to overcome the most acute effects of oil contamination. This study period also enabled us to learn more about the long-term interactions between ocean acidification and oil pollution.

The ability of bacterial communities to use oil hydrocarbons as a source of carbon and energy makes them key players in environmental detoxification and recovery (Head, 2006). There is increasing evidence that the predicted climate change scenarios, including ocean acidification and increased UV-B, have the potential to directly affect bacterial community structure and functioning (Coelho *et al.*, 2013b). However, there is a lack of information on how the net consequences of these multiple-stressors (synergism, antagonisms) will affect bacterial community structure and mediated processes, including detoxification and environmental recovery, in future marine coastal environments. One drastic effect detected in this study was a reduction in the relative abundance of the members of the *Desulfobacterales* order after exposure to oil contamination under reduced seawater pH. The order *Desulfobacterales* includes a diverse array of anaerobic bacteria known for their ability to use sulphate as a terminal electron acceptor in the oxidation of $H_2$ and organic compounds (sulphate-reducing bacteria) in a variety of habitats. This directly links this group of bacteria to carbon and sulphur cycling and to the degradation of organic contaminants, including oil hydrocarbons (Muyzer & Stams, 2008, Zhou *et al.*, 2011). In marine sediments, sulphate-reducing bacteria are frequently implicated in hydrocarbon degradation (McGenity, 2014). Therefore, this interaction has the potential to affect the anaerobic degradation of organic pollutants with possibly adverse consequences for the sediment oil composition and toxicity.

Our results showed that UV-B is a critical component in the interactive effect of oil contamination and reduced seawater pH on bacterial communities. For example, in the absence of UV-B the relative abundance of the order *Desulfobacterales* is reduced; in the presence of UV-B the relative abundance of this order, however, is similar to the control values. Hence, the presence of UV-B offsets the interactive effect between oil and pH on sulphate reducing bacteria, likely due to a direct effect of UV-B on oil chemistry. The influence of UV-B radiation on oil

chemistry is well-documented. Photooxidation processes, for example, can strongly increase mineralization rates changing the chemical structure of crude oil (Garrett *et al.*, 1998) with the rate of photooxidation directly related to the intensity of UVR in visible light (Wang *et al.*, 2010). The presence of UV-B also played a significant role in the interaction between oil and reduced seawater pH on the relative abundance of *Methylococcales*. Although the relative abundance of this order was lowest in the oil and reduced pH treatment, its maximum value was attained under the UVTot treatment. In this case, the increased abundance of *Methylococcales* might not only be related to UV-B induced photochemical modifications but also with a physiological effect of pH on this bacterial group. The *Methylococcales* order includes aerobic bacteria capable of oxidizing and utilizing methane (methanotrophic) and other $C_1$ compounds (methylotrophic) as sources of carbon and energy (Bowman, 2005). It is possible that the presence of UV-B induced the formation of C1 compounds through photochemical degradation of dissolved organic matter, whereas pH has been shown to exert a strong influence on other C1 degraders in ocean acidification experiments. Roy et al (2013) observed that the abundance of the genus *Methylotenera* was significantly correlated with $pCO_2$ with its highest abundance at pH 7.9 and 7.94.

*In silico predicted functional implications*

We used PICRUSt to predict the potential functional implications of the observed changes in bacterial community structure in response to all treatments evaluated in this study. PICRUSt allows for the inference of potentially meaningful differences in functional attributes using 16S rRNA surveys (Langille *et al.*, 2013). Given the observed changes in *Desulfobacterales* in microcosms exposed to oil and reduced seawater pH, we focused our analysis on the KEGG category for "xenobiotics biodegradation and metabolism". The predicted metagenome for this category was consistent with the taxon responses, with a reduced relative abundance of KOs assigned to "xenobiotics biodegradation and metabolism" pathways under the combined effect

of oil contamination and reduced pH. On the other hand, the relative abundance of KOs was similar to the control under the different treatment combinations in the presence of UV-B. This analysis further supports our finding that the combination of oil contamination and water acidification in the absence of UV-B has the potential to affect the anaerobic degradation of organic pollutants. It should be noted that, although the functional metagenome profiling obtained using PICRUSt represents an accurate inference of existing microbial genomes, it may be biased due to the lack of further reference genomes. Nevertheless, despite the limitations, predicted metagenomes have been shown to correlate well with environmental metagenomes (Langille et al., 2013). In this study, some of the changes in the predicted potential for xenobiotics degradation could be directly linked to the taxonomic results.For example, the reduction in the relative abundance of KOs linked to the ethylbenzene degradation pathway is most likely due to the lower relative abundance of *Desulfobacterales*, given the ability of sulphate reducing bacteria to degrade ethylbenzene (Harms *et al.*, 1999, Kniemeyer *et al.*, 2003). However, the metagenome prediction also revealed potential functional implications that were not clear from the community analysis. For example, the increase in the relative abundance of KOs assigned to cytochrome P450. It is possible that the increase in the metabolism of xenobiotic degradation by cytochrome P450 could be the result of changes in oil chemistry. For example, it has been shown that alkanes act as inducers of bacterial P450 cytochrome gene expression (Schneiker *et al.*, 2006).

*Sediment chemical status*

Although our analysis of the bacterial community structure and the predicted degradation pathways strongly suggests that oil removal will be affected by the interaction between oil and pH, we did not detect a significant effect of this interaction on the concentration of the 16 priority PAHs and the 20 most abundant aliphatic oil compounds. However, the interactive

effects of reduced water pH, UV-B and oil contamination could have affected other oil compounds. Indeed, the exploratory volatile compound analysis detected marked differences between treatments, thus supporting this hypothesis. Furthermore, there was a strong interdependence between the volatile compounds and bacterial composition datasets. However, at this point we cannot infer the nature of this correlation without the identification of the compounds detected. Although the GCxGC-TOFMS analysis offers the possibility of a non-target approach, the identification of compounds is still time consuming and in the optimization phase, particularly when analysing large datasets with several samples and replicates such as the one generated in this study (Hoffmann Nils & Jens, 2012). Nonetheless, when focusing our analysis on OnOi and pHOi treatments, the data showed that along with changes in bacterial communities and degradation pathways, the interaction between oil and reduced pH can also impact the sediment volatile profile. An increase in the number of compounds structurally related to the higher molecular weight fraction was observed in pHOi. In general, high molecular weight oil compounds are less soluble and more persistent in the environment than lower molecular weight compounds. For example, higher molecular weights PAHs often display long-term chronic toxicity including carcinogenesis and mutagenesis (Ray & McCormick-Ray, 2004).

*Invertebrate biochemical parameters*

During the previous calibration of the ELSS we showed that *H. diversicolor* was a suitable model for this type of microcosm study (Coelho *et al.*, 2013a). The survival percentages recorded in the present study support a similar conclusion for the gastropod *P. ulvae*. However, to the best of our knowledge this was the first time that a multi-biomarker approach was applied to this gastropod species making it difficult to ascertain whether the enzymatic levels recorded in the control organisms corresponded to basal levels for this species, i.e., whether the organisms were exposed

to stress factors inherent to the ELSS. Although other authors measuring lethal and sublethal endpoints (other than oxidative stress and neurological biochemical markers) argued that *P. ulvae* is as a suitable test species for routine sediment toxicity testing (Campana *et al.*, 2013, Krell *et al.*, 2011), further studies are required to confirm this supposition. Here, this species was selected due to its representativeness in estuarine ecosystems.

In the present study, the higher mortality recorded for both invertebrate species in the conditioned microcosms relative to the control ones, and particularly in oil contaminated microcosms, was probably due to volatile compounds that were quickly lost (Wernersser, 2002) and also by the physical effects of oil responsible for low dissolved oxygen levels and asphyxia (Saco-Álvarez *et al.*, 2008). In effect, the surviving polychaetes and gastropods were clearly under oxidative stress, particularly in oil treated microcosms.

With respect to *H. diversicolor*, the observed increased activity of the GST enzyme and the high levels of LPO in oil conditioned treatments demonstrated that the organisms were under oxidative stress. A significant increase in the activity of GST in polychaetes of the same species, promoted by B[a]P under laboratory conditions was shown by Bouraoui et al. (2009). Solé et al (2009) observed an increased trend in the activity of the same enzymes in *Nereis diversicolor* (presently *Hediste diversicolor*) and in *Scrobicularia plana* exposed to sediments contaminated with PAHs, polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDTs). Moreira et al. (2006) observed an increase in the activity of GST in ragworms collected in the Sado estuary (Portugal) at sites contaminated with PAHs and other contaminants. With respect to AChE, lower values of this biomarker were observed in *H. diversicolor* for all treatments containing oil contamination. This trend corroborates with previous studies from Bouraoui et al (2009) and Rodrigues et al. (2013). Both showed a strong inhibition of this enzyme caused by exposure to benzo[*a*]pyrene and to fluoranthene in *H. diversicolor* and *Carcinus maena.*

The interaction of UV-B and oil in LPO activity in both *H. diversicolor* and *P. ulvae* suggests that UV-B may attenuate the oxidative stress promoted by oil contamination. Interestingly, a

contrasting effect is often reported. It is know that UV light can induce PAH photoactivation, increasing their toxicity more than three orders of magnitude (Barron, 2007, Erickson *et al.*, 1999, Kirby *et al.*, 2007, Pelletier *et al.*, 2006). A possible explanation is that UV-B induced degradation of oil hydrocarbons overcomes the potential increase in their toxicity. Our data from the active bacterial community and the predicted functional analysis appears to corroborate this hypothesis. However, it is also possible that both species were able to protect themselves from the UV exposure levels applied. Such protection can be achieved by burying in the sediment and/or retreating inside their shell (Barron, 2007, Sinha & Häder, 2002), mitigating the effects of radiation since, at least in part, UV has to penetrate in the organisms for the photosensitization of bioaccumulated oil components (Barron, 2007, Wernersson, 2003). Both species would be then capable of protecting themselves from oxidative stress caused by UV-B exposure, but not by oil. Hydrocarbons dissolve progressively from the oil phase into the water phase with aliphatic hydrocarbons less soluble than aromatic hydrocarbons of the same molecular weight (Batelle, 2007). This dissolution in parallel with water movements caused by tidal simulation could contribute to the progressive penetration of hydrocarbons in the sediment column.

In general, the multi-biochemical marker analysis is in line with the results obtained for the active bacterial communities and predicted functional analysis. Here we could also detect a significant interaction between oil and pH on CAT activity in *H. diversicolor*. There was also an increase in LPO activity in pHOi treatments. However, in this case no significant interaction was detected. Most likely, the strong effect of the presence of oil masked the interaction between oil and pH. Furthermore, the significant interaction between these factors (pHOi treatment) also causes a reduction in the activity of Ache. As mentioned before, it has been shown that exposure to toxic oil hydrocarbon compounds inhibits the activity of this neurological biomarker. Importantly, the overall analyses of the invertebrate biochemical parameters revealed that the effect of oil alone overcomes the effect of pHOi. Nevertheless, LPO levels, a biomarker of true cellular oxidative stress effect, were higher for both species in pHOi. The non-target volatile compound profile

revealed an increase in HMW PAHs in pHOi when compared to OnOI. HMW PAHs are often associated with long-term, rather than acute, toxicological effects by overwhelming defenses against oxidative stress.

*Study implications*

The multidisciplinary approach used in this study shows that modest reductions in seawater pH in combination with oil contamination results in changes in the sediment chemical status, changes in the structure and putative function of active bacterial communities and increased oxidative stress in model benthic organisms. These effects could be a response to an increase in environmental toxicity due to i) a direct action of reduced pH on oil chemistry, increasing the overall toxicity ii) an indirect drop of sediment quality promoted by the reduction of oil degrading *Desulfobacterales* or iii) production of toxic compounds by the microbial community more adapted to this condition.

Our data also show that the magnitude of the interactions between reduced pH and oil will depend on the level of UV-B. Despite the potentially harmful effects of UV-B radiation, it appears to minimize the synergistic effects of reduced seawater pH and oil contamination on the structure and functioning of estuarine benthic communities. In the water column, shorter wavelengths (UV-B) are reduced more effectively than longer wavelengths (UV-A) (Tedetti & Sempéré, 2006). In areas exposed to UV-A with low or no UV-B, the interactive effects of ocean acidification and oil pollution may be more problematic. Ocean acidification will not be limited to surface waters and will occur in other regions of the ocean (Orr *et al.*, 2005). Given that oxidation of organic compounds coupled to the reduction of sulphate to sulfite may account for more than 50% of organic matter mineralization in the sea bed (Jorgensen, 1982), the degradation of oil in these environments may be adversely affected. Here we provide evidence that the interactive effects of ocean acidification and oil contamination have the potential to

aggravate the environmental condition of polluted coastal ecosystems, with possible direct and indirect consequences to the structure and functioning of sediment benthic communities.

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**Figures**

**Figure 1:** Principal coordinates analysis (PCO) of (bacterial) operational taxonomic unit (OTU) composition. Sequence reads (from 454 Genome Sequencer FLX Titanium) were assigned to OTUs with QIIME software (http://qiime.org). The PCO was generated using the cmdscale() function in the R base package and wascores() function in vegan. Prior to the PCO, the raw data was log10 (x+1) transformed and used to produce a distance matrix based on the Bray-Curtis distance with the vegdist() function in vegan (Oksanen *et al.*, 2012), and the first two explanatory axes are shown. **Cont** - control with no treatment; **OnpH** - reduced pH; **OnUV** - exposed to UV-B; **UVpH** - reduced pH and UV-B exposed; **OnOi** - contaminated with oil; **UVOi** - contaminated with oil and exposed to UV-B; **pHOi** - contaminated with oil and reduced pH; **UVTot** - contaminated with oil and exposed to UV-B and reduced pH.

**Figure 2:** Relative abundance of OTUs classified at order level under the independent and combined effects of reduced pH, UV-B exposure and oil contamination. Sequence reads (from 454 Genome Sequencer FLX Titanium) were assigned to OTUs and classified at order level with QIIME software (http://qiime.org). The four most abundant orders are shown.

**Figure 3:** Relative abundance of the predicted genes assigned to the KEGG metabolism category "xenobiotics biodegradation and metabolism" under the independent and combined effects of reduced pH, UV-B exposure and oil contamination. Metagenome functional content was predicted from the 16S rRNA sequence dataset against a set of reference metagenomes with

PICRUSt software (http://picrust.github.com/picrust/). The six most abundant degradation pathways are shown.

**Figure 4:** Values recorded for each biochemical parameter measured in *H. diversicolor* and *P. ulvae* under the independent and combined effects of reduced pH, UV-B exposure and oil contamination. CAT-catalase; SOD-superoxide dismutase; GST-glutatione S-transferase; AChE-acetylcholinesterase.