Pollutants bioavailability and toxicological risk from microplastics to marine mussels

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ABSTRACT

Microplastics represent a growing environmental concern for the oceans due to their potential of adsorbing chemical pollutants, thus representing a still unexplored source of exposure for aquatic organisms. In this study polyethylene (PE) and polystyrene (PS) microplastics were shown to adsorb pyrene with a time and dose-dependent relationship. Results also indicated a marked capability of contaminated microplastics to transfer this model PAH to exposed mussels *Mytilus galloprovincialis*; tissue localization of microplastics occurred in haemolymph, gills and especially digestive tissues where a marked accumulation of pyrene was also observed. Cellular effects revealed immunological responses, lysosomal compartment, peroxisomal proliferation, antioxidant system, neurotoxic effects, onset of genotoxicity; changes in gene expression profile was also demonstrated through a new DNA microarray platform. The study provided the evidence that microplastics adsorb PAHs, emphasizing an elevated bioavailability of these chemicals after the ingestion, and the toxicological implications due to responsiveness of several molecular and cellular pathways to microplastics.

Capsule.

Pyrene adsorbed on microplastics is accumulated in tissues of marine mussels. Transcriptional and cellular responses highlight the potential risk of virgin and contaminated polymers.

Keywords: microplastic, PAHs, bioavailability, biomarkers, mussels, transcriptomics

1. INTRODUCTION

The global production of plastic dramatically increased in the last decades, from 0.5 million tons/yr^{-1} in 1960 to 280 million tons in 2012 (Plastic Europe, 2012). Almost 10% of the annual production ends up into the oceans, and plastic debris accumulation has been reported as a global scale phenomenon for the marine environments, including polar areas and abyssal regions (Barnes *et al*., 2009).

Adverse effects of plastics have been documented in terms of entanglement and physical damages to locomotory, respiratory or digestive appendages in marine mammals, turtles, seabirds and crustaceans (Andrady, 2011). In addition, since plastics degrade very slowly, they also act as floating substrates for several organisms, and thus contribute to long-range transport of alien species, representing an additional risk to local biodiversity (Andrady, 2011).

In the recent years, a great scientific interest is being directed toward microplastics, i.e. fragments with a grain size lower than 5 mm, which are manufactured *ex novo* for their use in cosmetics, industrial or medical applications, or derive from macroscopic debris after chemical, physical and biological fragmentation (Barnes et *al*., 2009).

Ingestion of microplastics has been demonstrated in various marine organisms with different feeding strategies; this phenomenon may negatively influence both the feeding activity and nutritional value of a plankton-based diet, particularly in those species which can not discriminate the food source (Moore *et al*., 2001; Browne *et al*., 2008).

Recent evidences also suggest the potential role of microplastics as vectors of chemical pollutants, either used as additives during the polymer synthesis, or adsorbed directly from seawater (Rios *et al*., 2007; Teuten *et al.,* 2009; Engler, 2012). The hydrophobicity of organic xenobiotics and the large surfaces of floating polymers facilitate the adsorption of these chemicals on microplastics at concentrations orders of magnitude higher than those detected in seawater (Ogata *et al*., 2009). The possibility for plastic particles to adsorb chemical pollutants from the surrounding environment has been also characterized in laboratory conditions. Different particles polymers, like polyvinyl chloride, polyethylene, polypropylene, polystyrene, were shown to have a high sorption capacity for DDTs, polycyclic aromatic hydrocarbons (PAHs), hexachlorocyclohexanes and chlorinated benzenes (Bakir *et al*., 2012; Lee *et al*., 2014). Consistent with these studies, several persistent organic pollutants (POPs), polychlorinated biphenyls (PCBs), organo-halogenated pesticides, nonylphenol, PAHs and dioxins have been detected in plastic pellets stranded on different beaches of the world (Endo *et al*., 2005; Ogata *et al*., 2009; Hirai *et al* 2011; Heshett *et al.*, 2012).

Despite the importance of microplastics in adsorption and transport of hydrophobic pollutants, it is still unclear whether they also represent a potential source of chemical exposure within marine

food webs. Various evidences, including the use of a thermodynamic approach and of models simulating physiological conditions in the gut, suggested that both adsorbed pollutants and chemical additives of plastics might be released to organisms (Gouin *et al.*, 2011; Tanaka *et al*., 2013; Bakir *et al*., 2014a).

In laboratory conditions, microplastics have been shown to be ingested by amphipods, barnacles, and lugworms (Thompson *et al*., 2004); in mussels, *Mytilus edulis*, plastic particles (3-9.6 µm) were accumulated in digestive tissues and translocated to haemolymph (Browne *et al.*, 2008). In the same organisms, the uptake of microplastics caused notable histological changes in digestive cells with strong inflammatory responses, formation of granulocytomas and lysosomal destabilization which increased with exposure time (Von Moos *et al*., 2012).

To further assess the possible risk of microplastics as environmental contaminants, the present investigation aimed at a multidisciplinary approach to characterize the chemical adsorption of hydrophobic pollutants, as well as bioaccumulation, chemical release and onset of potential health effects in the filter feeding mussels *Mytilus galloprovincialis*. Two different polymers, polyethylene (PE) and polystyrene (PS) were exposed to various doses of pyrene, selected as one of the more commonly represented PAHs adsorbed on plastic marine debris (Rios *et al.*, 2007); virgin and contaminated PE and PS were then used in a trophic transfer experiment with mussels. Tissue localization of microplastics was integrated with measurement of pyrene bioaccumulation and a wide battery of cellular biomarkers to detect the early onset of adverse effects. Such analyzed responses included immunological parameters, lysosomal membrane stability, peroxisomal proliferation, antioxidant defences and oxidative stress biomarkers, neurotoxic effects and onset of genotoxicity; for the first time, effects of microplastics were also investigated at the transcriptomic level through a new *M. galloprovincialis* DNA microarray platform, to better elucidate pathways and molecular mechanisms of action (MOA).

Obtained results have been elaborated with a classical Weight Of Evidence (WOE) approach that combine and differently weight various typologies of data, or lines of evidence (LOEs), providing multidisciplinary characterization of hazard indices and risk evaluation (Chapman *et al.*, 2002; Chapman, 2007). WOE methods are considered as key components of Ecological Risk Assessment (ERA) procedures, according to recent European Directives which require member states to evaluate and classify the ecological status of water bodies integrating different quality elements. Among the available WOE procedures, the Sediqualsoft model elaborates data from sediment chemistry, bioavailability of pollutants and onset of adverse effects at different levels of biological organization (Piva *et al.*, 2011; Benedetti et al., 2012); the computational rules have been successfully validated in filed conditions for the characterization and classification of risk from industrial and harbour

sediments, natural hydrocarbon seepage in coastal areas or the recent Costa Concordia wreck at Giglio Island (Piva *et al*., 2011; Benedetti *et al*., 2012, 2014; Regoli *et al*., 2014). In this study we have applied the flow-charts and mathematical algorithms developed for elaborating data and summarizing the hazard index for bioavailability and biomarker responses, thus providing a synthetic judgment on the biological relevance of these observed effects.

The overall results of this study were expected to increase our knowledge on the potential toxicological risk of microplastics in the marine environment.

2. MATERIALS AND METHODS

2.1 *Experimental design*

Polyethylene (PE) and polystyrene (PS) powders were obtained from a private plastic company. Particles were size-sorted in a 1000-100 µm group used for characterization of the pyrene adsorbing capacity, and in a <100 µm group for the exposure of mussels to virgin and contaminated polymers.

The adsorption of pyrene to PE and PS was assessed by mixing solutions of microplastics (20 g/L in seawater) with pyrene dosed at final concentrations of 0.5 µg/L (low, L), 5 µg/L (medium, M) and 50 µg/L (high, H). While the L and M treatments are environmentally realistic for pyrene, the H dose is uncommon but still possible, i.e. after heavy oil spill or in highly contaminated sewage (Neff, 2002). The mixing solutions were maintained in continuously rotating 50 mL glass tubes for 6 days; water was changed and pyrene re-dosed after 3 days. Levels of pyrene adsorbed on polymers were measured after three and six days of treatment.

For the exposure of mussels to microplastics, specimens of *M. galloprovincialis* (5 ± 1 cm shell length) were obtained from a local farm (Numana, Ancona, Central Adriatic Sea) and acclimatized for 10 days to laboratory conditions with aerated seawater, at 18 ± 1 °C and 35 ‰ salinity. Contaminated plastics were prepared according to the above description, by maintaining a solution of <100µm microplastics with pyrene (50 µg/L) in rotating conditions for 6 days. A total of 150 organisms were distributed into fifteen 6 L glass-beakers and exposed to virgin or pyrene-contaminated plastics for 7 days with three replicates for each of the 5 following treatments: Control (CNTR), Polyethylene (PE), Polystyrene (PS), Pyrene-treated Polyethylene (PE-PYR), Pyrene-treated Polystyrene (PS-PYR). Water was changed daily and both virgin and pyrene-treated particles re-dosed at a nominal concentration of 1.5 g/L.

No mortality of mussels was observed during the experiments. After the exposure period, haemolymph, digestive glands and gills were rapidly removed from 30 specimens for each treatment,

pooled in 10 samples (each with tissues of 3 specimens), frozen in liquid nitrogen and maintained at -80°C for chemical, biochemical and histochemical analyses; for haemolymph samples, an aliquot was also immediately processed for lysosomal neutral red retention time assay (NRRT), phagocytosis activity, and DNA damage, and another aliquot fixed in Carnoy's solution (3:1 methanol, acetic acid) for the microscopic evaluation of granulocytes and chromosomal alteration. Four additional pools, each with digestive glands of three specimens, were prepared from CNTR, PS and PS-PYR groups for DNA microarray analysis.

2.2 Chemical analyses of pyrene on plastics and exposed mussels

Pyrene adsorbed on microplastics (PE and PS) or accumulated in mussels tissue (gills and digestive glands) was determined after extraction of samples in 0.5 M potassium hydroxide and methanol (1:10 w:v) with microwave at 55°C for 15 min (Benedetti *et al*., 2014). After centrifugation for 5 min at 1000 × g, the methanolic solutions were concentrated in speedvac and purified with solid phase extraction (Octadecyl C18, 500 mg × 6 mL, Bakerbond). A final volume of 1 mL was recovered with pure, analytical HPLC gradient-grade acetonitrile, and HPLC analyses were carried out with water–acetonitrile gradient and fluorimetric detection. Pyrene was identified by the retention time of appropriate pure standard solutions (EPA 610 Polynuclear Aromatic Hydrocarbons Mix). Quality assurance and quality control were tested by processing blank and reference samples (mussel tissues SRM 2977, NIST); concentrations obtained for the SRM were always within the 95% confidence interval of certified value. The water content in tissues was determined and concentrations of pyrene expressed as ng/g dry weight (d.w.).

2.3 Biological analyses in mussel tissues

To evaluate the possible presence of plastic particles in different tissues, cryostatic sections (8 µm thick) of gills and digestive glands, and haemolymph smears were histologically examined. After staining with haematoxilyn and eosin, the occurrence and localization of microplastics was assessed through polarized light microscopy.

Standardized protocols were used for measurement of biomarkers in tissues of control and exposed organisms (Gorbi *et al*., 2013, Benedetti *et al*., 2014). Detailed methods are given in Supplementary Material 1 for the following measurements: immunological alterations in terms of granolocytes/hyalinocytes ratio, phagocytosis activity and lysosomal membrane stability (NRRT) in haemocytes; neurotoxic responses as acetylcholinesterase (AChE) in haemocytes and gills; cellular and oxidative stress biomarkers in digestive tissues, i.e. acyl-CoA oxidase (AOX), antioxidant defenses (catalase glutathione S-transferases, glutathione peroxidases, glutathione reductase, total glutathione), total oxyradical scavenging capacity (TOSC), lysosomal latency period (LP),

malondialdehyde (MDA), lipofuscin, neutral lipids; genotoxic effects in haemolymph in terms of DNA strand breaks, micronuclei frequency (MN) and nuclear alterations (NA).

2.4 Mytilus galloprovincialis oligonucleotide microarray

Gene transcription analyses were performed using an 8X60K Agilent oligo-DNA microarray platform designed within the European project REPROSEED (REsearch project to improve PROduction of SEED of established and emerging bivalve species in European hatcheries). Information about sequencing, assembly, annotation and microarray design are summarized in Supplementary Material 2 and 3. Probe sequences and other details on the microarray platform can be found in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GPL18667. Microarrays were synthesized in situ using the Agilent non-contact ink-jet technology including default positive and negative controls.

2.5 Labelling, microarray hybridization and data acquisition

Sample labeling and hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol with the Low Input Quick Amp Labeling kit. Full details about labeling, hybridization and data acquisition are reported in Supplementary Material 3.

Raw gene expression data were deposited in the GEO database under accession number GSE57460. Due to technical problems during the hybridization step, one of the four pools of the PS exposed mussels was excluded by the gene transcription analyses. Normalization procedures included quantile normalization which always outperformed cyclic loess, and further adjustment by the parametric Combat in R to account for the between-experiments batch effects of the oligonucleotide microarray (Johnson *et al.*, 2007). Normalized data were deposited in GEO archive under accession number GSE57460.

2.6 Microarray data processing and analysis

Statistical analyses were performed on 52.988 out of 59.971 probes with signal higher than the background in 8 out of 11 analysed samples. The TIGR Multi Experiment Viewer 4.5.1 statistical software (TMeV; Saeed *et al*., 2003) was used to perform T-test statistics (p-value<0.01; 200 permutations) comparing CNTR to both PS and PS-PYR groups. The resulting T-test genes lists were then filtered and only probes with fold change (FC) > 1.5 have been considered as differentially expressed genes (DEGs). A more systematic functional interpretation of differentially transcribed genes was obtained through an enrichment analysis using Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Huang *et al.*, 2009). Since these databases contain

functional annotation data for a limited number of species, transcripts of *M. galloprovincialis* were matched to *Danio rerio* Gene IDs using dedicated Blast searches performed with blastx (E-value < 10-5). The choice of *D. rerio* allowed the assignment of a putative homologue to a larger number of *M. galloprovincialis* transcripts (see Supplementary Material 2), and was previously demonstrated a useful option for *Ruditapes philippinarum* functional analyses (Milan *et al*., 2011). A functional annotation was obtained for genes differentially expressed in each T-test pairwise comparison, setting DAVID for gene count=2 and ease=0.1.

2.7 Statistical analyses and toxicological risk assessment

Statistical analyses were performed with the statistical R-software (R. development Core Team, 2010). Adsorption of pyrene to microplastics was tested by analysis of variance (ANOVA) according to typology of polymer (PE-PS), time of exposure (3-6 d), dose of pyrene (L, M, H); bioaccumulation of pyrene and biomarker responses in exposed mussels were also compared by one-way ANOVA and post-hoc comparison (Bonferroni) was used to discriminate between means of values. Level of significance was set at $p < 0.05$, homogeneity of variance was checked by Cochram C and mathematical transformation applied if necessary. For biomarkers data, multivariate principal component analysis (PCA) was combined to hierarchical clustering of the PCA patterns which visualize the relationships among the different treatments and organize the samples into groups of homogeneous observations (Husson et al., 2010). The proposed methodology is available in the HCPC (Hierarchical Clustering on Principal Components) function of the FactoMineR package (Lê et al., 2008).

Results on bioaccumulation of pyrene and biomarkers responses in mussels exposed to virgin and contaminated microplastics were further elaborated within a classical Weight Of Evidence WOE approach, using a previously developed quantitative and software-assisted model (Sediqualsoft). According to WOE principles, different typologies of data are initially evaluated with appropriate criteria to provide synthetic indices of hazard for each of considered line of evidence, before their final integration in a quantitative WOE evaluation (Piva *et al*., 2011). Whole calculations, detailed flow-charts, rationale for weights, thresholds and expert judgments have been fully given elsewhere (Piva *et al*., 2011; Benedetti *et al*., 2012, 2014; Regoli *et al*., 2014).

Briefly, the bioavailability hazard was calculated from the initial calculation of a weighted Ratio to Reference (RTRw), reflecting the magnitude of pyrene accumulation in tissues of exposed organisms, corrected for both the statistical significance of the difference compared to controls and the typology of chemical. Depending on the magnitude of such variations, the model assigns the hazard to 1 of 5 classes: from Absent to Slight if the calculated increase of pyrene tissue concentration

is lower than 2.6 folds compared to control organisms, Moderate between 2.6 and 6.5 folds , Major between 6.5 and 13 folds, Severe if greater than 13 folds (Piva *et al*., 2011; Benedetti *et al*., 2012).

For the evaluation of biomarkers results, the model contains a large selection of responses among those more widely used by scientific community in different bioindicator organisms (Piva *et al*., 2011); according to species and tissue, each biomarker, has a "weight" based on the relevance of biological endpoint, and a "threshold" for changes of biological significance which consider both inductions and/or inhibitions of various responses. For every analysed biomarker, the measured variation is compared to the threshold, then corrected for the weight of the response and the statistical significance of the difference compared to controls. Depending on the magnitude of the calculated effect, each biomarker response is assigned by the model to 1 of 5 classes of effect (from Absent to Severe); the calculation of the Hazard Quotient for biomarkers (HQ_{BM}) does not consider the contribution of responses with an effect lower or equal to threshold (Absent or Slight), calculates the average for those with an effect up to two-fold compared to the threshold (Moderate) and adds the summation (Σ) for the responses more than 2 fold greater than the respective threshold, i.e. Major or Severe (Piva *et al*., 2011):

$$HQ_{BM} = \left(\frac{\sum_{j=1}^{N} Effect_W(j)_{1<Effect(j)\leq 2}}{num\ biomark_{1<Effect(j)\leq 2}} + \sum_{k=1}^{M} Effect_W(k)_{Effect(j)>2} \right)$$

According to variations measured for various biomarkers, the model summarizes the level of cumulative HQ_{BM} in one of five classes of hazard for biomarkers, from Absent to Severe (Piva *et al*., 2011).

The elaborations of results on bioavailability of pyrene and biomarker variations were integrated after normalization of hazard indices to a common scale; the resulting level of toxicological risk was finally assigned to 1 of 5 classes from Absent to Severe (Piva *et al*., 2011).

3. RESULTS

Microplastics showed an elevated capability to adsorb pyrene with a dose- and time-dependent trend (Figure 1). After 6 days of M treatment, concentrations of adsorbed pyrene were 145 ± 35 and 126 ± 35 ng/g on PE and PS microplastics respectively, with an accumulation factor of 29 and 25.2 calculated as the ratio to nominal levels dosed in seawater. Concentrations of adsorbed pyrene were

even greater after H dose experiment (305 ± 89 and 244 ± 52 ng/g for PE and PS) but the accumulation factors were 6.1 and 4.8 for the two polymers.

Concentrations of pyrene on contaminated microplastics used for the laboratory experiments with mussels (< 100 μm) were in the range of 200-260 ng/g for both PE and PS. After 7 days of exposure, a significant increase of pyrene was observed in gills, and a more marked bioaccumulation occurred in digestive glands, with concentrations much greater than those measured directly on contaminated particles (Figure 2). Tissue levels of pyrene in exposed mussels (up to 470 ng/g) increased by more than 13 folds compared to control specimens, reflecting an hazard index for bioavailability summarized as Severe by the proposed WOE model (Table 2, LOE1).

Histological analyses of treated mussels revealed the presence of microparticles in haemolymph, gills and, especially, in digestive glands where numerous aggregates could be observed in the intestinal lumen, epithelium, and tubules (Figure 3). No qualitative differences in tissue localization were evident between organisms treated with the two polymers (PE, PS), both as virgin or contaminated particles.

Among immunological responses of haemocytes, phagocytosis activity did not exhibit particular variations, while a decrement of granulocytes versus hyalinocytes type cells was observed in mussels exposed to virgin and to pyrene-contaminated PE; lysosomal membrane stability decreased in almost all the treatment groups (Figure 4).

The comet assay indicated a significant enhancement of DNA strand breaks in haemocytes of mussels treated with virgin microplastics, while nuclear anomalies were higher in all the treatments with either virgin or contaminated polymers; the frequency of micronuclei significantly increased only in specimens exposed to pyrene-treated PS (Figure 4). Acetylcholinesterase did not vary in haemolymph and decreased in gills of mussels exposed to both virgin and contaminated microplastics, while activity of AOX was not influenced in any of experimental treatments (Figure 4).

Antioxidant defenses did not reveal variations in the levels of glutathione and activities of glutathione reductase, glutathione S-transferases, and sum of Se-dependent and Se-independent glutathione peroxidases (Figure 5). A significant inhibition was observed in all the treatments for Se-dependent glutathione peroxidases and a similar trend appeared also for catalase; the overall significance of those effects was reflected in slight variations of the Total Oxyradical Scavenging Capacity (TOSC) toward both peroxyl and hydroxyl radicals (Figure 5). The moderate pro-oxidant challenge induced by microplastics on mussels was supported by the lack of relevant variations for malondialdehyde, lipofuscin and neutral lipids in digestive tissues; lysosomal integrity appeared more sensitive, and decreased after exposure to both virgin and contaminated microplastics (Figure 5).

Considering the magnitude of variations observed for various biomarkers, their statistical significance and the toxicological relevance of each biological endpoint, the WOE model summarized the hazard for cellular responses as ranging from Slight to Moderate, typically higher for PS compared to PE, and for contaminated compared to virgin microplastics (Table 2, LOE3). The combination of hazard indices elaborated for bioavailability and biomarker data resulted in an overall WOE risk classified as Slight or Moderate for virgin PE and PS (reflecting only the cellular effects), Major or Severe for contaminated polymers (integrating both bioaccumulation and cellular perturbations, Table 2, WOE).

The principal component analysis (PCA) carried out on the whole set of biomarkers produced a two dimensional pattern explaining 72 % of total variance (Figure 6). The hierarchical clustering on PCA pattern indicated a clear separation between control and exposed mussels, dividing as homogeneous groups those treated with virgin or pyrene-treated microplastics respectively; the parameters determining the separation along to the PC1 axis were lysosomal membrane stability in haemocytes and digestive glands, AChE in gills and some antioxidant responses (catalase, glutathione reductase, Se-dependent glutathione peroxidase, TOSC-HO·). On the other side, genotoxic effects (DNA strand breaks, nuclear anomalies, micronuclei), phagocytosis, AChE in haemolymph and levels of glutathione determined the separation along the PC2 axis between mussels exposed to virgin compared to pyrene-contaminated microplastics (Figure 6); the typology of polymer (PE vs PS) did not appear to influence the observed responses.

The analysis of transcriptional responses revealed a total of 2.143 and 1.320 differentially expressed genes (DEGs, $p<0.01$; FC>1.5) in response to PS and PS-PYR exposures, respectively (Supplementary Material 4). Among these, 280 transcripts were significantly affected after both exposures (Figure 7), but the majority of transcripts appeared specifically modulated within each treatment (1.863 in PS and 1.040 in PS-PYR). Functional annotation and enrichment analysis was applied to DEGs to highlight the most significantly affected Biological Processes (BP), Molecular Functions (MF), Cellular Component (CC) and KEGG pathways (KP), which are detailed in Supplementary Material 5.

Some of the most interesting enriched KEGG pathways/GO terms are reported in Table 1: Lysosome (with 16 and 15 DEGs in PS and PS-PYR exposed mussels respectively), Coated membrane (9 and 6 DEGs), Endosome (6 and 3 DEGs), NOD-like receptor signalling pathway (4 and 7 DEGs), Response to bacterium (5 and 3 DEGs), Apoptosis (7 and 8 DEGs), Regulation of programmed cell death (5 and 8 DEGs), Citrate cycle (8 and 3 DEGs) and Arachidonic acid metabolism (5 and 3 DEGs).

Beside the above mentioned GO terms and KEGG pathways, mussels exposed to PS and PS-PYR showed the modulation of several genes involved in DNA repair (i.e. *growth arrest and DNA-damage-inducible protein, GADD45A* and *GADD45G*; *excision repair cross-complementing rodent*

repair deficiency, complementation group ERCC; *aprataxin, APTX*), detoxification (i.e. *Glutathione S-transferase pi, GSTP1* and *GSTP2*; *glutathione S-transferase M, GSTMU*; *sulfotransferase family 4A, member 1,* S*ULT4A1*) and oxidative processes (i.e. *glutathione peroxidase, GPX2* and *GPX3*; *superoxide dismutase mitochondrial,* SOD2; see Supplementary Material 4).

4. DISCUSSION

The present investigation aimed to provide new insights on the potential role of microplastics as a source of chemical exposure and ecotoxicological challenge to marine organisms. A growing concern is being raised for the possibility of these polymers to adsorb environmental pollutants, and our results clearly confirmed such hypothesis. Using environmentally realistic levels of dissolved pyrene, the concentrations on exposed microplastics markedly increased with a time- and dose-dependent trend. Worthy to note, the comparison of various experimental conditions did not reveal a linear relationship with levels of pyrene dosed in seawater, since the greatest adsorption efficiency was obtained for the Moderate treatment (5 µg/L). Adsorption of pyrene did not particularly differ between PS and PE, and chemical values measured on both polymers were comparable to those previously reported in plastic pellets from beaches and industrial sites in California, Hawaii and Greece (Rios *et al.*, 2007; Karapanagioti *et al.*, 2011). These data support the potential of microplastics in trapping and transporting marine pollutants, as already suggested by studies on equilibrium kinetics and partition coefficients of several hydrophobic chemicals on various typologies of plastic polymers (Zarfl and Matthies, 2010; Bakir *et al.*, 2012; Lee *et al.*, 2014); a transport model for persistent organic pollutants by microplastics has been recently proposed also for estuarine conditions, demonstrating a relatively little effect of salinity compared to chemical concentration in water, plastic density and particle residence time in estuaries (Bakir *et al.*, 2014b).

Several species have been shown to ingest and accumulate microplastics, and the ecological impact of this phenomenon would be greatly influenced by the desorption of toxic chemicals. The release of additives or adsorbed chemicals from plastics to organisms has been suggested (Engler, 2012; Tanaka *et al.*, 2013; Bakir *et al.*, 2014a), but a clear demonstration is still lacking because organisms in field conditions can accumulate the same classes of chemicals from other sources. In our experimental conditions, mussels were exposed to microplastics containing adsorbed pyrene at concentrations of 200-260 ng g^{-1}. Despite variable levels of PAHs have been measured worldwide, such values are within the range of pyrene concentrations recently measured in plastic pellets sampled in differently impacted sites of Portuguese coast (5-530 ng g^{-1}, Mizukawa *et al.*, 2013) and beaches (20-320 ng g^{-1}, Frias *et al.*, 2010). The results obtained with exposed mussels provided the first clear evidence that pyrene adsorbed on contaminated microplastics was transferred to organisms and concentrated in tissues. Despite the analyses might have been partly influenced by the presence of

400 still un-excreted, contaminated particles, this effect can be probably considered as negligible. Average
401 concentrations higher than 50 ng g^{-1} were measured in gill samples and, assuming that all the pyrene
402 was that adsorbed on microplastics, we should expect at least 0.2-0.25 g of particles for each gram of
403 gill tissue, a possibility certainly excluded by histological analyses. The bioaccumulation of pyrene
404 was particularly marked in digestive glands where concentrations of pyrene appeared up to 3 folds
405 higher than those present on contaminated polymers, thus necessarily reflecting a major contribution
406 of the chemical accumulated in tissues to the total content of analyzed pyrene: this results clearly
407 demonstrated an elevated desorption and bioconcentration process of this chemical from
408 microplastics to tissues under physiological gut conditions (Teuten *et al*., 2009; Bakir *et al*., 2014a).
409 The histological analyses qualitatively supported the bioaccumulation data, with observation
410 of particles in the digestive tissues of exposed mussels and, to a lower extent, in gills and
411 haemolymph. Uptake and tissue distribution of microplastics has already been described in the blue
412 mussels *M. edulis* after laboratory exposures to high density polyethylene and polystyrene (Browne
413 *et al*., 2008; Von Moos *et al*., 2012). In those studies, a first site of particles uptake was shown at the
414 gill surface, mediated by microvilli activity and endocytosis, while a second pathway occurred via
415 ciliae movement in the stomach, intestine and digestive tubules, followed by accumulation within the
416 lysosomal compartment (Von Moos *et al*., 2012): polystyrene particles smaller than 9.6 or 3.0 µm
417 could translocate from the gut cavity to the haemolymph and inside the haemocytes (Browne *et al*.,
418 2008). Despite our observations were not quantitatively assessed, they almost reflected the above
419 mechanisms of uptake, with conspicuous aggregates within intestinal lumen and digestive tissues,
420 and more limited occurrence of particles in branchial epithelial cells and in haemolymph; the lack of
421 microplastics within the haemocytes may be the consequence of a dimensional difference of plastic
422 particles used in our experimental conditions.
423 A large battery of biochemical and cellular biomarkers were analyzed in this study to
424 characterize the ecotoxicological potential of both virgin and contaminated microplastics. Significant
425 immunological effects were observed on haemocytes with a strong shift of the haemocytic cell
426 population, a limited variations of phagocytosis and a significant reduction of lysosomal membrane
427 stability. The lower granulocytes/hyalinocytes ratio did not probably reflect a decrease of
428 granulocytes, which are primarily involved in phagocytic activity, but rather a sharp increase in
429 hyalinocytes, less differentiated cells and potential precursors of granulocytes (Carball *et al*., 1997).
430 On the other hand, the lower lysosomal membrane stability of haemocytes could be reasonably related
431 to the over-production of prooxidant reactive oxygen species involved in the immune responses,
432 typical during microbial attack and recently observed also toward nanoparticles (Canesi *et al*., 2002;
433 Jovanovic and Palic, 2012). Similarly to our results, the ingestion and translocation of polystyrene
434 did not cause measurable changes in the viability and phagocytic activity of haemocytes in *M. edulis*

(Browne *et al.*, 2008), while inflammatory responses and lysosomal membrane destabilization occurred as a cellular host response to high density polyethylene microplastics (Van Moos *et al.*, 2012). Organisms exposed to virgin or contaminated microplastics exhibited similar effects, suggesting that immunological responses were mostly induced by the physical ingestion of the particles, more than the chemical toxicity of adsorbed pyrene; in this respect, the exposure to irregular particles with potentially sharp surfaces might have contributed to exacerbate these effects compared to the use of microspheres with smooth surfaces (Van Moos *et al.*, 2012).

Exposure to microplastics also determined the onset of various forms of genotoxicity in haemocytes. While strand breaks were higher in organisms exposed to virgin PE, nuclear alterations appeared more consistently distributed among all the treatments, resulting in an increased frequency of micronuclei after the exposure to pyrene-contaminated PS. This pattern of genotoxic effects allows to hypothesize that DNA strand breaks represent the first form of damage caused by the enhanced production of reactive oxygen species in response to microplastics: a more elevated prooxidant challenge caused by PS compared to PE or by pyrene-contaminated compared to virgin polymers would determine an irreversible loss of DNA integrity (i.e. nuclear alterations), leading to enhanced frequency of micronuclei in the worst condition. In this respect, oxyradical production was already shown to modulate immune responses, lysosomal dysfunction and pre-apoptotic processes in haemocytes of mussels exposed to TiO2 nanoparticles (Barmo *et al.*, 2013), while genotoxic proprieties of PAHs have been widely reported to produce chromosomal alterations (Benedetti *et al.*, 2012).

The activity of AChE was not affected in haemocytes of exposed mussels, but significantly reduced in gills after treatments with both typologies of either virgin or contaminated polymers. The ability of microplastics to depress AChE was recently described also in juveniles of the common goby *Pomatoschistus microps* exposed to polyethylene microspheres, dosed alone or in combination with pyrene (Oliveira *et al.*, 2013); despite mechanisms of action still remain to be elucidated, our results support the hypothesis that anticholinesterasic effects of microplastics should be taken in adequate consideration due to the abundance of these particles in the marine environment and the role of AChE in neurotransmission of fundamental physiological processes (Oliveira *et al.*, 2013).

The marked accumulation of microplastics in digestive tissues caused a significant destabilization of lysosomal compartment as also reported in *M. edulis* upon fusion with endocytotic vacuoles containing microplastic particles (Von Moos *et al.*, 2012). Lysosomal membranes are highly susceptible to oxidative effects of ROS which can be generated throughout a complex network of direct reactions and indirect mechanisms (Regoli and Giuliani, 2014). In this respect, lowered activities were measured for catalase and Se-dependent glutathione peroxidases which are known as particularly sensitive in revealing the early onset of a prooxidant challenge even at low levels of

environmental disturbance (Regoli and Giuliani, 2014). These enzymes are both involved in the removal of hydrogen peroxide, the main precursor of hydroxyl radical in aquatic organisms (Regoli and Giuliani, 2014): while glutathione peroxidases are mainly responsible for eliminating metabolically produced H_2O_2, catalase acts as defense mechanism also toward the exogenous source of this molecule. The contemporary variation of these enzymes might thus suggest different mechanisms and cellular pathways for H_2O_2 formation in tissues exposed to microplastics. However, the overall results on oxidative stress biomarkers indicated that short-term exposures to microplastics do not induce major perturbations, as revealed by the limited effects on the total antioxidant capacity and the lack of oxidative damages like lipofuscin, malondialdehyde and neutral lipids accumulation, in agreement with previous data on mussels and fish (Von Moos *et al.*, 2012; Oliveira *et al.*, 2013).

The overall evaluation of biomarker results by multivariate PCA and hierarchical clusterin analysis provided a clear separation between control and microplastics exposed mussels indicating that the majority of observed biological variations (immunological, lysosomal, cholinesterasic and antioxidant effects) were not influenced by the typology of polymer (PE vs PS) or contamination: only genotoxic responses further separated virgin from pyrene-contaminated polymers. The relatively limited impact of pyrene adsorbed on microplastics might suggest that energy resources were primarily directed to activate mechanisms of defense toward the physical rather than the chemical stressor; on the other hand, presence of microplastics was shown to delay the pyrene-induced lethal effects in *P. microps*, thus acting as a transitory mechanism of protection toward chemical toxicity (Oliveira *et al.*, 2013).

To better summarize the biological significance of pyrene accumulation and cellular responses in mussels exposed to virgin and contaminated microplastics, these data were elaborated according to the weighted criteria of the Sediqualsoft model. The level of pyrene bioavailability was classified as Severe while the toxicological hazard calculated from biomarkers ranged from Slight to Moderate in various treatments, depending on the number, magnitude and biological importance of measured variations: the combination of chemical and cellular effects summarized as Slight the hazard for mussels exposed to virgin polymers, Major or Severe for those exposed to pyrene-contaminated PE or PS respectively.

Transcriptional profiles provided additional insights on molecular mechanisms modulated by microplastics in digestive glands of mussels. In analogy with cellular biomarker, the enrichment of KEGG pathways involved in lysosomal metabolism and immunological functions, appeared as a primary response to either virgin or contaminated PS. The up-regulation of several genes coding for lysosomal enzymes, putative coating proteins and endosome indicates a coordinated increase of this cellular defense pathway following microplastics accumulation. The synthesis and maturation of lysosomal enzymes occur in the endoplasmic reticulum/trans-Golgi system and the following

trafficking of such proteins is regulated by specific recognition mechanisms and packaging into clathrin-coated vesicles for their transport to late endosomes (Bonifacino and Traub, 2003). The over-expression of several proteins involved in endosomes maturation, endocytic trafficking and lysosomal degradation, suggests increased uptake of microplastics via endocytosis and their endolysosomal degradation (Bucci *et al.*, 2000). The complete list of DEGs involved in "lysosome", "coated membrane", and "endosome", i.e. cathepsins, *clathrin heavy and light chain*, sorting nexins, is reported in Supplementary Material 6.

Mussels exposed to microplastics exhibited also the enrichment of the NOD-like receptor signaling pathway, involved in the innate immune defences, such as regulation of inflammatory and apoptotic responses. The NOD-like receptors (NLRs) act as intracellular sensors which recognize both pathogenic patterns entering the cell via phagocytosis, and damage-associated molecules produced during cellular stress and activating the non-infectious inflammatory response. The PS-PYR particles enhanced transcription of genes putatively involved in signal transduction and auto-modulation of the stress response NF-kB (i.e. TRAF6 and IκBα), while virgin PS up-regulated various components of the innate immune system, such as putative peptidoglycan recognition proteins (PGRPs).

Molecular analyses supported cellular biomarkers also regarding the transcriptional modulation of antioxidant defences, detoxification enzymes and responses to genotoxic effects. The up-regulation of putative *GPX2*, *GSTP1*, *GSTP2* and down-regulation of putative SOD2 were observed in PS-exposed mussels, while *GPX3*, *GSTMU* and *SULT4A1* were differentially expressed after exposure to both PS and PS-PYR (Supplementary Material 4). On the other hand, the onset of DNA damage in mussels exposed to PS and PS-PYR could be related to the up-regulation of *GADD45A* and *GADD45G* (Supplementary Material 4) which have a pivotal role in control of cell cycle checkpoint, DNA repair process and cellular responses to a variety of DNA-damaging agents (Fornace *et al.*, 1992); the increased transcription of GADD45 was also reported in the Manila clam *Ruditapes philippinarum* exposed to ibuprofen (Milan *et al.* 2013), corroborating its involvement in counteracting genotoxic stress in bivalves species. In mussels exposed to PS-PYR, the significant enhancement of micronuclei frequency was interestingly paralleled by the up-regulation of *ERCC1*, *ERCC2* and *APTX* which are required for the repair of DNA lesions, being mainly involved in nucleotide excision repair, single-strand break, double-strand break and base excision repair.

Substantial differences were observed in the transcriptional profile of genes related to apoptosis and the citrate cycle TCA. Despite such data may suggest molecular hypotheses on the down-regulation of apoptotic processes and energetic metabolism after exposures to virgin or contaminated microplastics, the functional implications at cellular level still remain to be elucidated.

In conclusion, this study confirmed that microplastics can efficiently adsorb organic contaminants like pyrene from the marine environment, providing the first experimental evidence for

the potential transfer and bioaccumulation of this chemical in mussel tissues. Both virgin and contaminated microplastics induced several effects at transcriptional and cellular levels highlighting the potential risk for organisms' health condition, especially under conditions of long-term, chronic exposure. Despite in the present study only bioaccumulation and cellular responses were considered, the clear evidence of a toxicological potential of microplastics supports the WOE approach and the integration of multiple indicators (physical, chemical, biological, ecological), toward a more comprehensive risk assessment analysis, in line with actual European Directives like Marine Strategy Framework Directive. Further studies are needed to better understand the effects of other typologies of chemicals or chemical mixtures adsorbed on microplastics, as well as the natural exposure conditions in terms of presence, concentration and magnitude of chemical load in microplastics in the marine environment.

Acknowledgements. The study was partly supported by Centro Assistenza Ecologica ECOCAE, Ancona, Italy and by the Italian Ministry for University and Research MIUR (project Tetris, PRIN 2010-2011).

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Table 1. Lists of the main enriched GO terms/KEGG pathways. Numbers and "gene name" of differentially expressed genes (DEGs) in each comparisons/terms are also reported. Down- and up-regulated transcripts in exposed groups are reported in green and red, respectively. Gene names reported in black indicate transcripts represented by multiple probes showing opposite responses (Supplementary Material 6). Full names of differentially expressed genes are reported in Supplementary Material 6 and the complete list of enriched KEGG/GO terms in Supplementary Materials 5.

CNTRvsPS			**CNTRvsPS-PYR**		
GO_TERM/KEGG	**N° DEGs**	**GENE NAME**	**GO_TERM/KEGG**	**N° DEGs**	**GENE NAME**
dre04142:Lysosome	16	*MAN2B1*, *AGA*, *CTSLA*, *CTNS*, *PSAP*, *NPC1*, *CTSC*, *ATP6V0B*, *GGA1*, *LGMN*, *CLTCA*, *CLTA*, *CTSBA*, *CTSD*, *AP1S2*, *CTSBB*	**dre04142:Lysosome***	15	*CD164*, *CTSLA*, *AGA*, *CTNS* *CD63*, *GLB1* *GGA1*, *LGMN* *AP3S2*, *CTSZ* *CTSBB*, *AP1S2* *PSAP*, *LDLR*, *CTSBA*
GO:0048475~coated membrane	9	*COPA*, *COPE*, *SEC23B*, *GGA1* *COPB2*, *CLTCA* *CLTA*, *COPB1*, *AP1S2*	**GO:0048475~coated membrane***	6	*LDLR* , *AP2S1*, *GGA1*, *AP3S2*, *COPB1*, *AP1S2*
GO:0005768~endosome	6	*CHMP2BB*, *CHMP4B*, *CHMP1A*, *SNX5* *RAB5C*,*TMEM55B*	**GO:0005768~endosome**	3	*CHMP1A*, *CHMP6B*, *VPS29*
dre04621:NOD-like receptor signaling pathway	4	*XIAP*, *SGUT1*, *BIRC2*, *BIRC7*	**dre04621:NOD-like receptor signaling pathway***	7	*HSP90B1*, *NFKBIAB*, *XIAP* *TRAF6*, *BIRC2*, *BIRC7*
GO:0009617~response to bacterium	5	*PGLYRP6*, *PGLYRP2*, *CTSD*, *RHOGB*, *PCNA*	**GO:0009617~response to bacterium**	3	*RHOGB*, *MYD88*, *TRAF6*
dre04210:Apoptosis	7	*DFFB*, *BAXA* *BIRC2*, *BIRC7*, *CASP3A*, *PRKAR2AA*, *XIAP*	**dre04210:Apoptosis***	8	*CASP9*, *NFKBIAB*, *BCL2L1*, *MYD88* *XIAP*,*PRKAR2AA*, *BIRC2*, *BIRC7*
GO:0043067~regulation of programmed cell death	5	*CASP3A*, *BAXA*, *TRAF3*, *BIRC2*, *CASP2*	**GO:0043067~regulation of programmed cell death***	8	*BIRC2*, *BCL2L1*, *CRADD*, *CASP2*, *CASP9*, *CCT3*, *MCL1B*, *TRAF6*

dre00020:Citrate cycle (TCA cycle)	8	*DLDH*, *PCK1*, *DLAT*, *MDH1AA*, *PCK2*, *IDH3G*, *ACO1*, *SDHB*	**dre00020:Citrate cycle (TCA cycle)**	3	DLST, PCK2, IDH3G
dre00590:Arachidonic acid metabolism	5	*PLA2G1B*, *CYP2P9*, *CPLA2*, *CYP2U1*, *ALOX5A*	**dre00590:Arachidonic acid metabolism**	3	*TBXAS1PLA2G1B GPX3*

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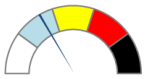
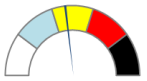
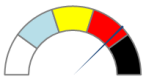
Table 2 Weight Of Evidence classification of bioaccumulation (LOE2) and biomarkers (LOE3) data, and integrated WOE risk in mussels exposed to virgin or pyrene-contaminated microplastics. The quantitative Hazard Quotients (HQ) for individual LOEs and the assigned classes of hazard or WOE risk are given. Treatments: PE= virgin polyethylene; PS= virgin polystyrene; PE-PYR= pyrene-contaminated polyethylene; PS-PYR= pyrene-contaminated polystyrene.

Treatments	**LOE2** (HQ and Class of hazard)	**LOE3** (HQ and Class of hazard)	**WOE**	
PE	-	1.87 Slight	SLIGHT	
PS	-	4.10 Moderate	MODERATE	
PE-PYR	13.01 Severe	6.44 Moderate	MAJOR	
PS-PYR	14.69 Severe	7.20 Moderate	SEVERE	

LEGENDS OF FIGURES

FIGURE 1. Time-course of pyrene adsorption to microplastics particles (polyethylene and polystyrene). Different lines indicate nominal doses of pyrene: solid line 50 µg/L, dashed line 5 µg/L, dotted line 0.5 µg/L. Data are expressed as ng/g dry weight (mean values ± standard deviation, n=5).

FIGURE 2. Concentrations of pyrene in gills and digestive glands of mussels exposed to various microplastics treatments: CNTR= control; PE= virgin polyethylene; PS= virgin polystyrene; PE-PYR= pyrene-contaminated polyethylene; PS-PYR= pyrene-contaminated polystyrene. Data are expressed as ng/g dry weight (mean values ± standard deviation, n=5); different letters indicate significant differences between groups of means (post-hoc comparison).

FIGURE 3. Polarized-light microscopy images showing the presence of plastic particles in haemolymph (A), gills (B), gut lumen and epithelium (C), digestive tubules (D).

FIGURE 4. Immunological, genotoxic, cholinesterasic and peroxisomal biomarkers in mussels exposed to various microplastics treatments: CNTR= control; PE= virgin polyethylene; PS= virgin polystyrene; PE-PYR= pyrene-contaminated polyethylene; PS-PYR= pyrene-contaminated polystyrene. Ach-E: acetylcholinesterase; AOX: Acyl CoA Oxidase. Data are expressed as mean values ± standard deviation, or standard error of mean for % of DNA in tail, n=5; different letters indicate significant differences between groups of means (post-hoc comparison).

FIGURE 5. Antioxidant defenses, total oxyradical scavenging capacity (TOSCA) toward peroxyl (·OOR) and hydroxyl (·OH) radicals, malondialdehyde (MDA), lipofuscin, neutral lipids and lysosomal membrane stability in digestive gland of mussels exposed to various microplastics treatments: CNTR= control; PE= virgin polyethylene; PS= virgin polystyrene; PE-PYR= pyrene-contaminated polyethylene; PS-PYR= pyrene-contaminated polystyrene. Data are expressed as mean values ± standard deviation, n=5; different letters indicate significant differences between groups of means (post-hoc comparison).

FIGURE 6. PCA analysis of biomarker data in mussels exposed to various microplastics treatments: CNTR= control; PE= virgin polyethylene; PS= virgin polystyrene; PE-PYR= pyrene-contaminated polyethylene; PS-PYR= pyrene-contaminated polystyrene. AchE: acetylcholinesterase; AOX: Acyl CoA Oxidase; CAT: catalase; DNA_COD: percentage of DNA in tail of comet assay; GST: glutathione S-transferases; GPX: glutathione peroxidases; GR: glutathione reductase; LIPO: lipofuscin accumulation; LP: lysosomal labilization period in digestive gland lysosomes; NRRT: neutral red retention time in haemocytic lysosomes; ORO: neutral lipids accumulation; TGSH: total glutathione; TOSCA: Total Oxyradical Scavenging Capacity.

FIGURE 7. Venn diagrams representing differentially expressed transcripts (total, down-regulated and up-regulated) in the comparisons CNTR vs PS and CNTR vs PS-PYR.

DATA ACCESSIBILITY

The following link has been created to allow review of record GSE57460 when still in private status: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=cxqdcokwnhwxzsf&acc=GSE57460

LIST OF SUPPLEMENTARY MATERIAL

Supplementary Material 1. Detailed analytical procedures for presented biomarkers.

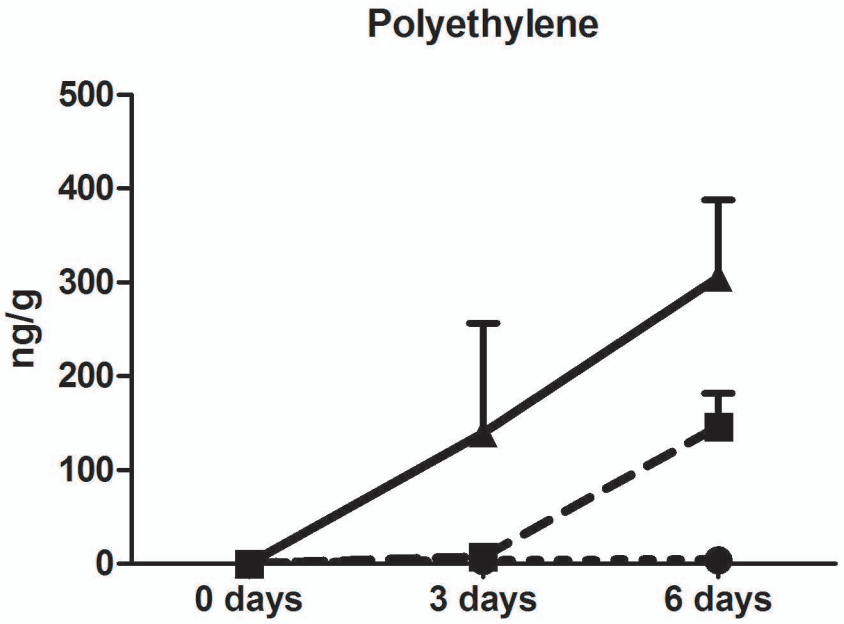
Supplementary Material 2. Summary of the origin of *Mytilus galloprovincialis* sequences. Information about assembly, annotation and microarray design have been also reported.

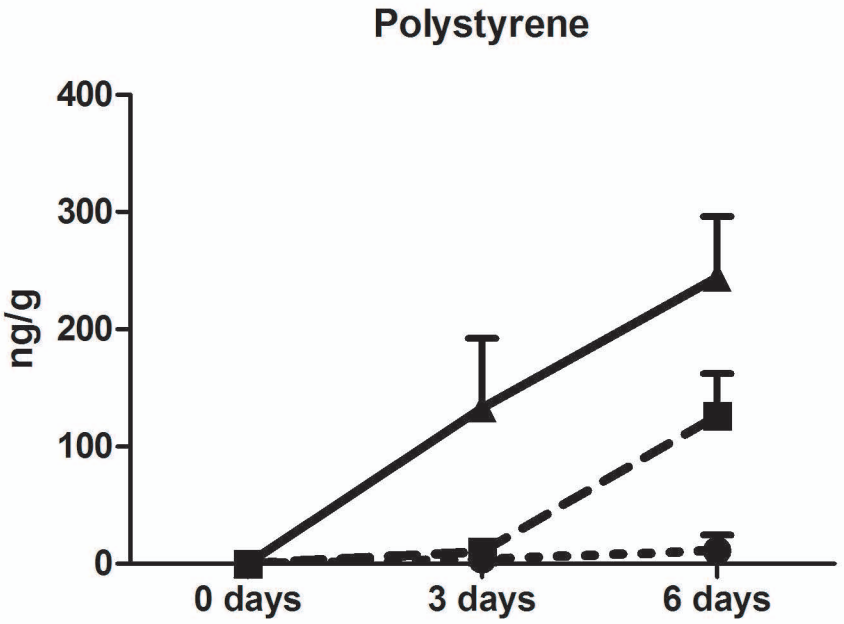
Supplementary Material 3. Additional information on methods and parameters used for DNA microarray design, labelling, microarray hybridization and data acquisition.

Supplementary Material 4. List of significant probes identified by T-Test analyses (p-value<0.01; 200 permutations) by comparing control vs PS-exposed mussels (CNTR vs PS), and control to PS-PYR exposed mussels (CNTR vs PS-PYR). Probes ID, mean fluorescence values, fold change and annotation (Swissprot, *Crassostrea gigas* and *Danio rerio* protein reference database) are reported for each comparison. Down- and up- regulated transcripts in exposed groups are reported in green and red, respectively. Lists of differentially expressed genes revealed in both comparisons has been also reported.

Supplementary Material 5. GO terms and KEGG pathways significantly enriched on differentially expressed genes revealed in each comparisons (CNTR vs PS and CNTR vs PS-PYR). Biological processes, cellular component and molecular function represented by at least two differentially expressed genes, and KEGG pathways significantly enriched are reported.

Supplementary Material 6. Lists of differentially expressed genes involved in GO terms/KEGG pathways which were enriched in at least one comparison (CNTR vs PS and CNTR vs PS-PYR). Probes ID, fold change and annotation (Swissprot, *Homo sapiens* and *Danio rerio* protein reference database) are reported for each GO term/KEGG pathway. Down- and up- regulated transcripts in exposed groups are shown in green and red, respectively. The asterisks (*) indicate GO and KEGG pathway significantly enriched in the considered comparison.


Polyethylene
500
400
300
200
100
0
ng/g
0 days
3 days
6 days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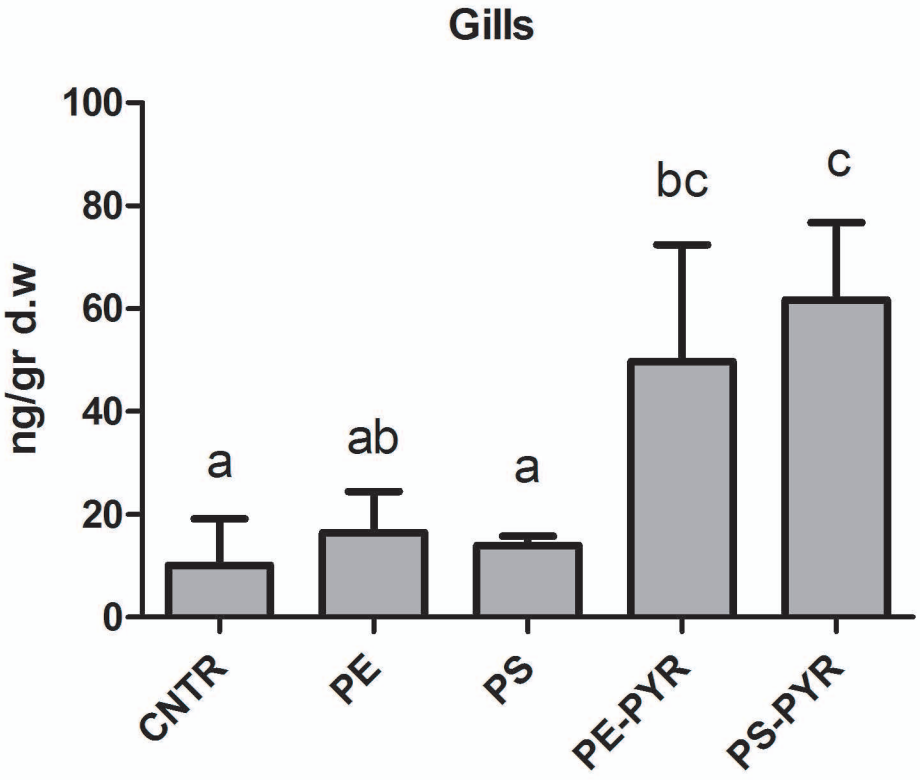
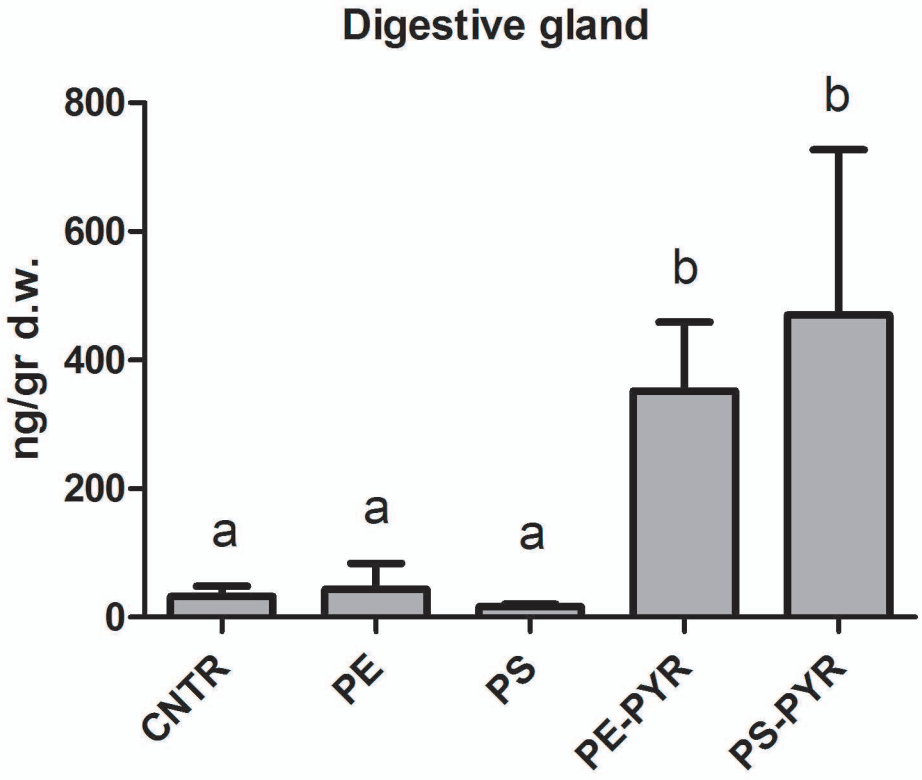
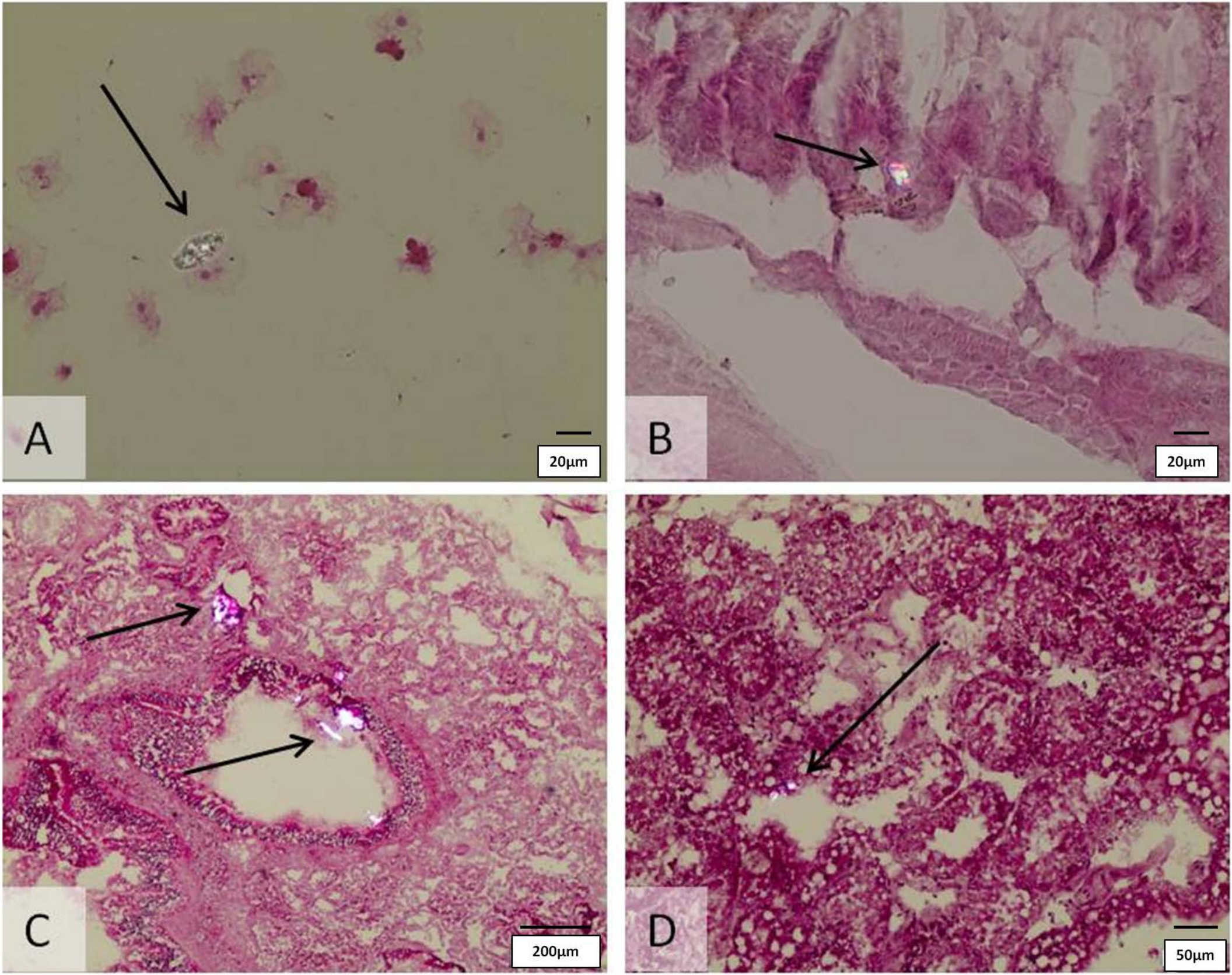
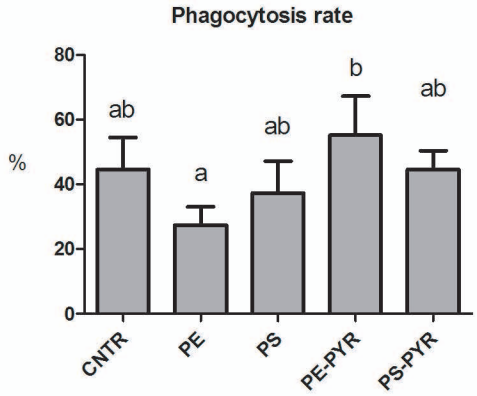
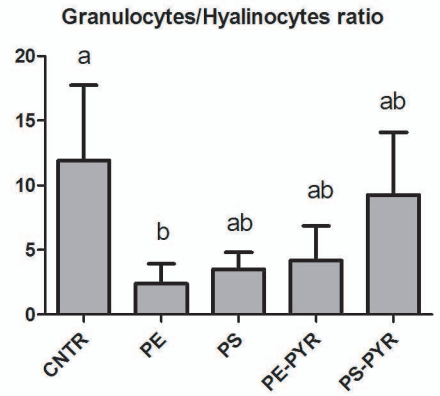
400
300
200
100
0
ng/g
0 days
3 days
6 days

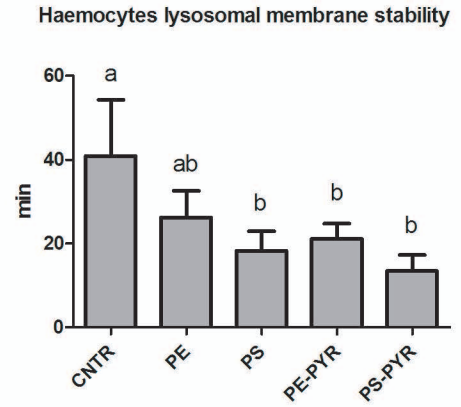

Gills
ng/gr d.w
100
80
60
40
20
0
a
ab
a
bc
c
CNTR
PE
PS
PE-PYR
PS-PYR

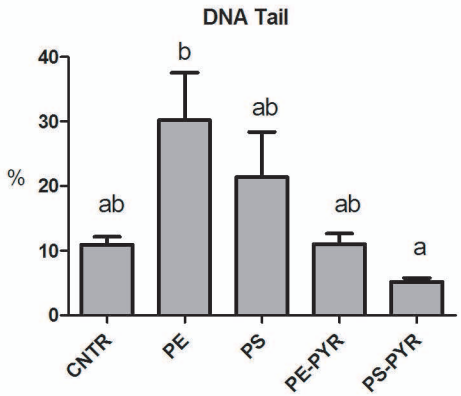

Digestive gland
ng/gr d.w.
800
600
400
200
0
a
a
a
b
b
CNTR
PE
PS
PE-PYR
PS-PYR

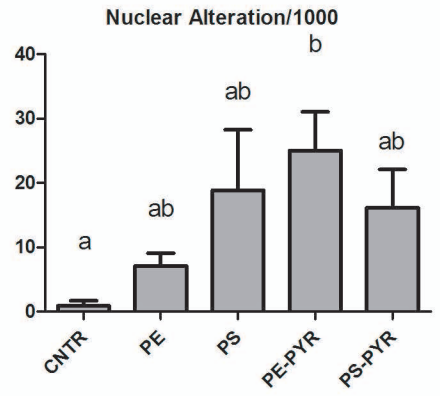

A
20µm
B
20µm
C
200µm
D
50µm

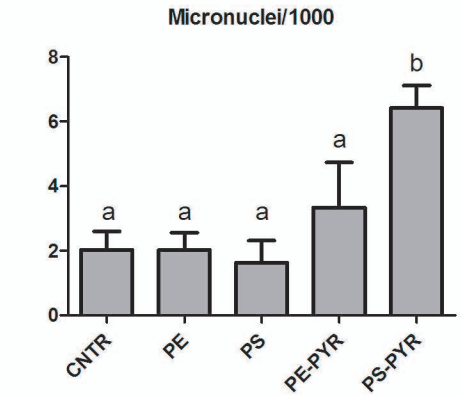

Phagocytosis rate
%
80
60
40
20
0
ab
a
ab
b
ab
CNTR
PE
PS
PE-PYR
PS-PYR

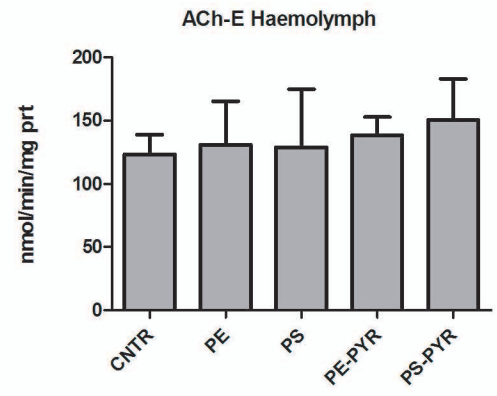

Granulocytes/Hyalinocytes ratio
20
15
10
5
0
a
b
ab
ab
ab
CNTR
PE
PS
PE-PYR
PS-PYR

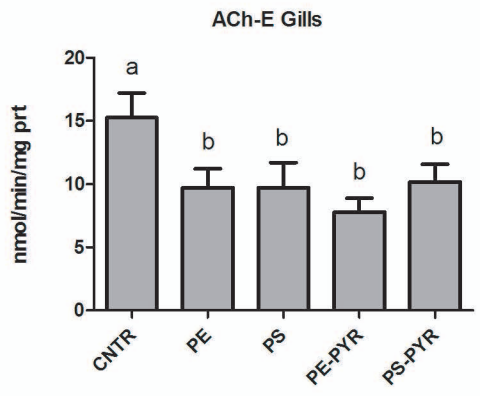

Haemocytes lysosomal membrane stability
min
60
40
20
0
a
ab
b
b
b
CNTR
PE
PS
PE-PYR
PS-PYR

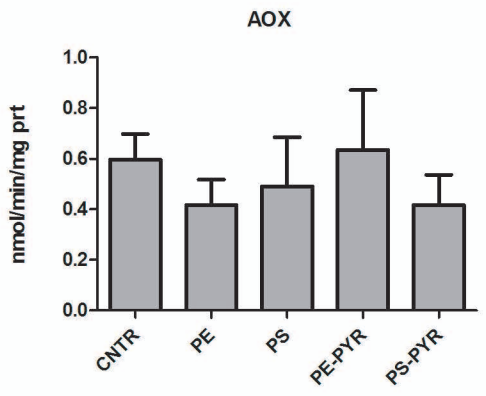

DNA Tail
%
40
30
20
10
0
ab
b
ab
ab
a
CNTR
PE
PS
PE-PYR
PS-PYR

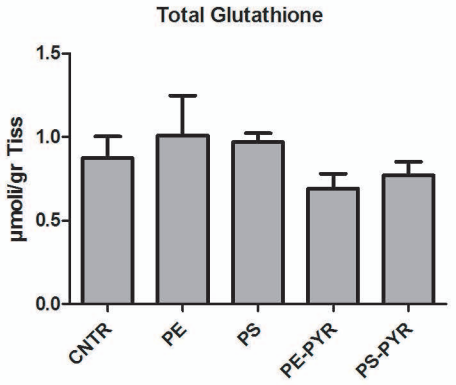

Nuclear Alteration/1000
40
30
20
10
0
a
ab
ab
b
ab
CNTR
PE
PS
PE-PYR
PS-PYR

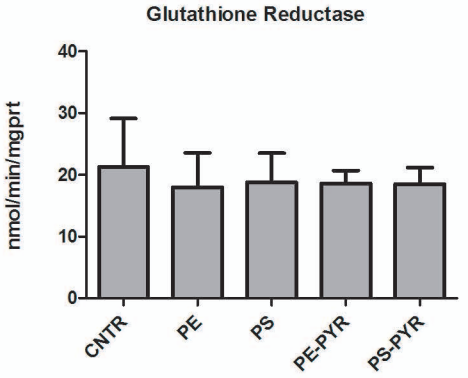

Micronuclei/1000
8
6
4
2
0
a
a
a
a
b
CNTR
PE
PS
PE-PYR
PS-PYR

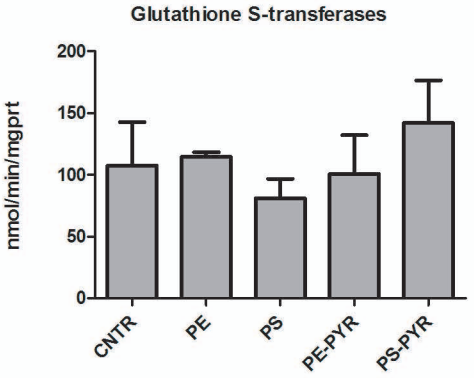

ACh-E Haemolymph
nmol/min/mg prt
200
150
100
50
0
CNTR
PE
PS
PE-PYR
PS-PYR

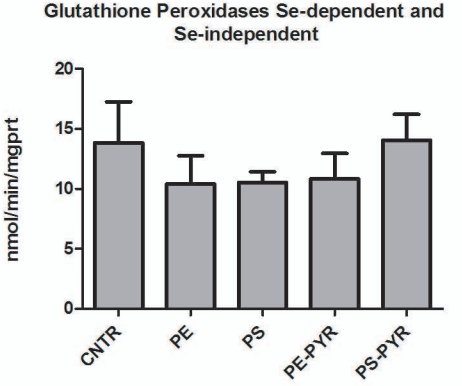

ACh-E Gills
nmol/min/mg prt
20
15
10
5
0
a
b
b
b
b
CNTR
PE
PS
PE-PYR
PS-PYR

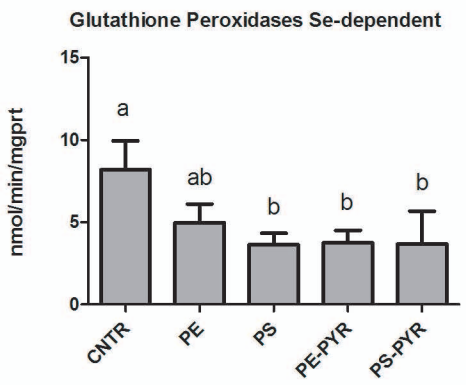

AOX
nmol/min/mg prt
1.0
0.8
0.6
0.4
0.2
0.0
CNTR
PE
PS
PE-PYR
PS-PYR

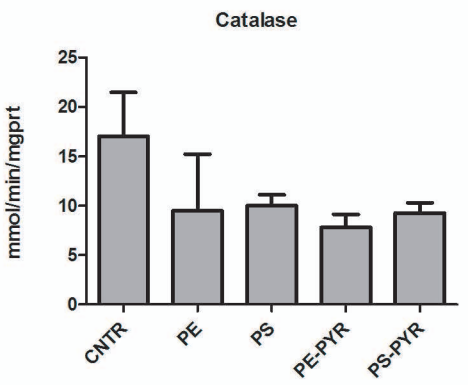

Total Glutathione
µmoli/gr Tiss
1.5
1.0
0.5
0.0
CNTR
PE
PS
PE-PYR
PS-PYR

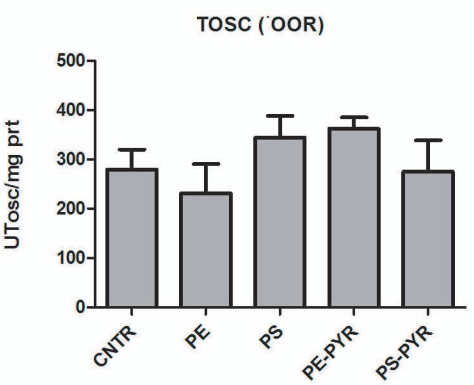

Glutathione Reductase
nmol/min/mgprt
40
30
20
10
0
CNTR
PE
PS
PE-PYR
PS-PYR

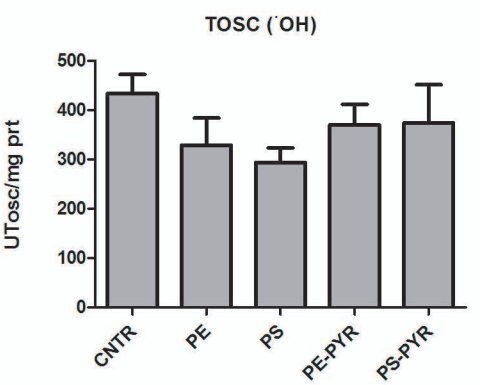

Glutathione S-transferases
nmol/min/mgprt
200
150
100
50
0
CNTR
PE
PS
PE-PYR
PS-PYR

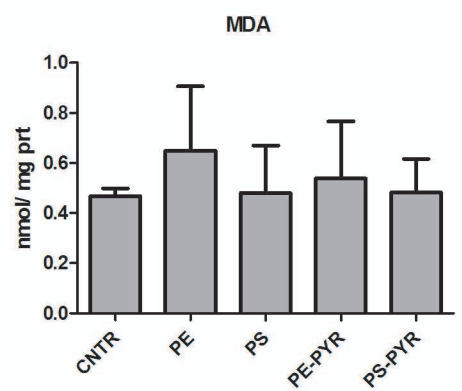

Glutathione Peroxidases Se-dependent and Se-independent
nmol/min/mgprt
20
15
10
5
0
CNTR
PE
PS
PE-PYR
PS-PYR

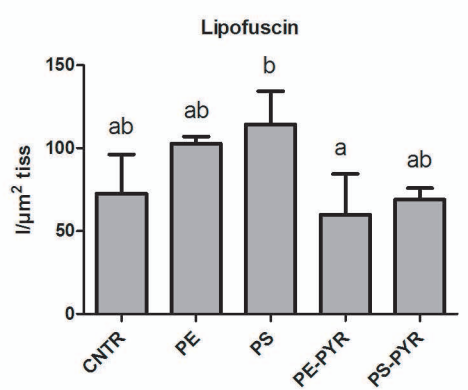

Glutathione Peroxidases Se-dependent
nmol/min/mgprt
15
10
5
0
a
ab
b
b
b
CNTR
PE
PS
PE-PYR
PS-PYR

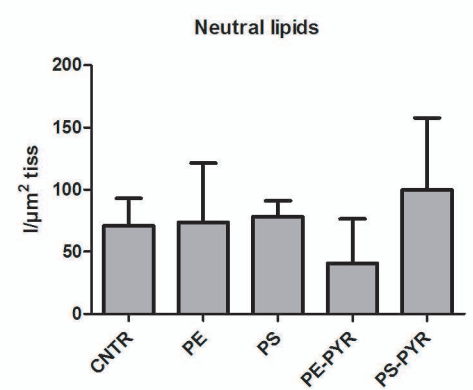

Catalase
mmol/min/mgprt
25
20
15
10
5
0
CNTR
PE
PS
PE-PYR
PS-PYR

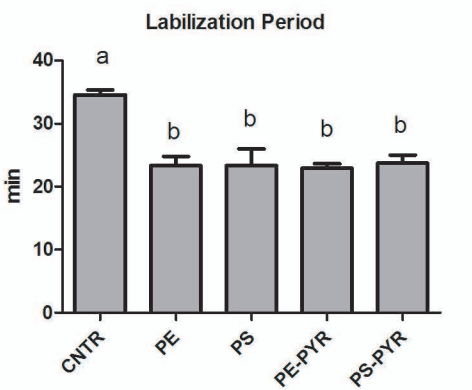

TOSC (˙OOR)
UTosc/mg prt
500
400
300
200
100
0
CNTR
PE
PS
PE-PYR
PS-PYR


TOSC (˙OH)
UTosc/mg prt
500
400
300
200
100
0
CNTR
PE
PS
PE-PYR
PS-PYR

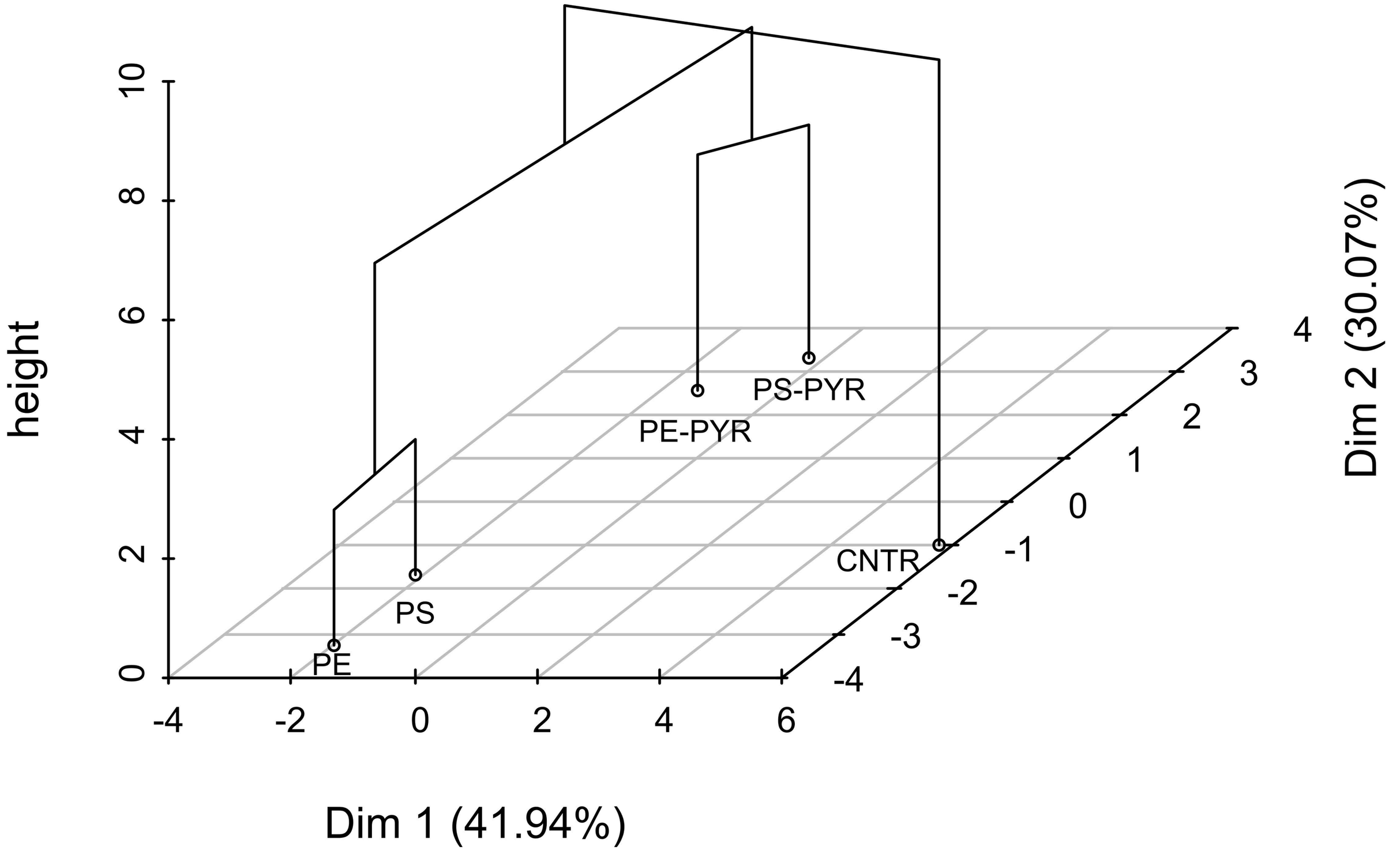

MDA
nmol/ mg prt
1.0
0.8
0.6
0.4
0.2
0.0
CNTR
PE
PS
PE-PYR
PS-PYR

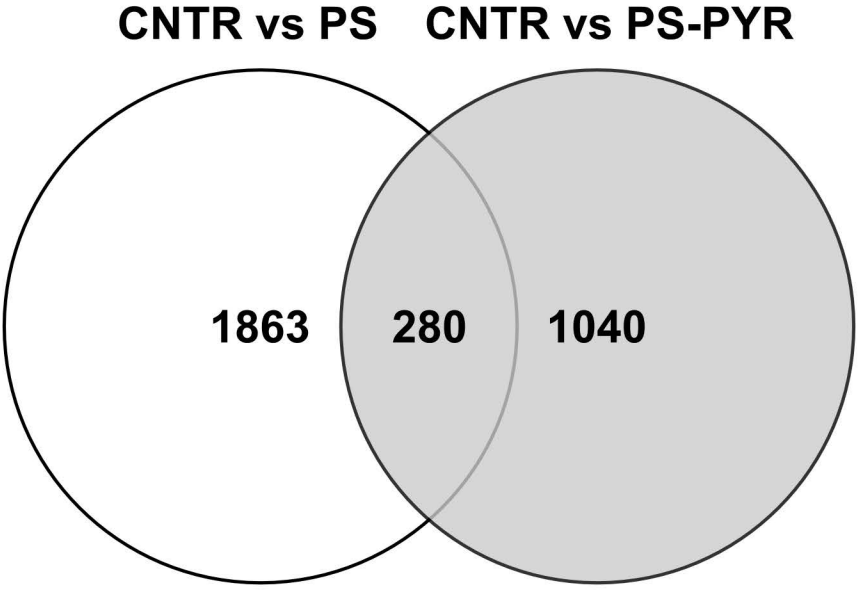

Lipofuscin
l/µm2 tiss
150
100
50
0
ab
ab
b
a
ab
CNTR
PE
PS
PE-PYR
PS-PYR


Neutral lipids
l/µm2 tiss
200
150
100
50
0
CNTR
PE
PS
PE-PYR
PS-PYR

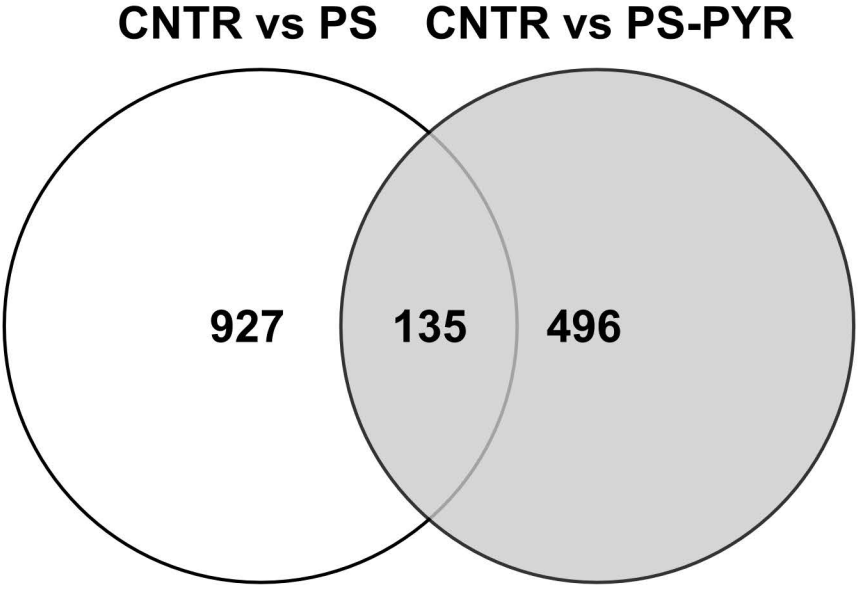

Labilization Period
min
40
30
20
10
0
a
b
b
b
b
CNTR
PE
PS
PE-PYR
PS-PYR

Hierarchical clustering on the factor map

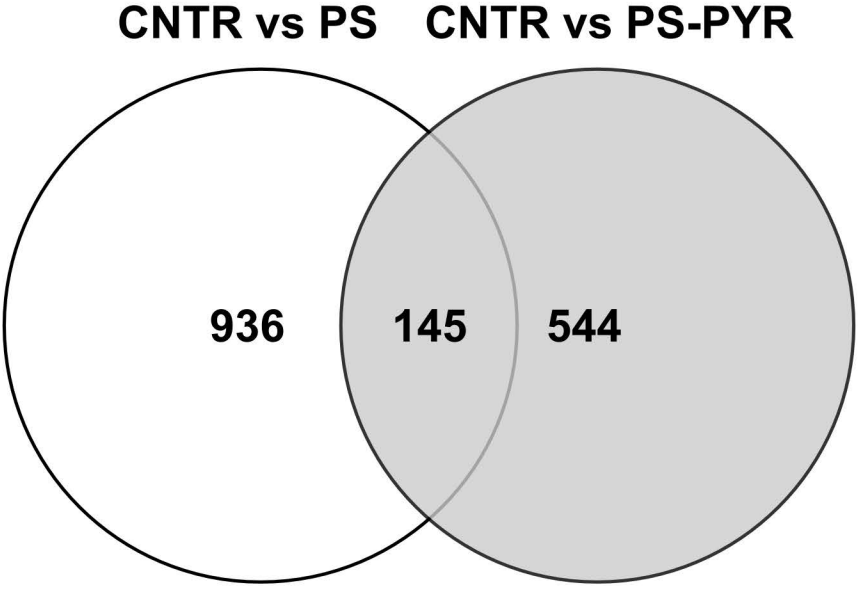




ALL DIFFERENTIALLY EXPRESSED GENES



DOWN-REGULATED GENES



UP-REGULATED GENES