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Sublethal effects in *Perinereis gualpensis* (Polychaeta: Nereididae) exposed to mercury-pyrene sediment mixture observed in a multipolluted estuary

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Abstract Sediment-living organisms can be subjected to a multi-pollution condition due to an increase in the diversity of contaminants. Sediment mixtures of Mercury (Hg) and some polycyclic aromatics hydrocarbons like Pyrene (Pyr) are common in heavily industrialized coastal zones. In the present study, greater than (>) and less than (<) probable effect concentration levels (PELs) of Hg and Pyr were assessed using spiked sediments in order to determine combined (Hg + Pyr) effects in uptake, metabolization and oxidative balance in the polychaete *Perinereis gualpensis* at short and medium-term exposure. Hg + Pyr significantly influenced the uptake/kinetics of Hg and Pyr metabolite 1-OH-pyrene in polychaete tissues during the exposure time compared with separate treatments of each analyte ($p < 0.05$). Both the Hg-only and Pyr-only exposures significantly influenced both enzymatic and non-enzymatic responses respect to control groups ($p < 0.05$). The Hg-only treatment showed the worst scenario related to the activation and subsequent inhibition of glutathione S- transferase (GST) and peroxidase (GPx) activities, high levels of Thiol-groups (SH-groups), low antioxidant capacity (ACAP) and enhanced lipid peroxidation (TBARS) in the last days of exposure ($p < 0.05$). In contrast, ragworms exposed to Hg + Pyr showed a significant increase in both enzymatic and non-enzymatic activity during the first days of exposure and the absence of lipid peroxidation during the whole experiment. Our results suggest different oxidative stress scenarios in *P. gualpensis* when exposed to >PEL Hg concentration with <PEL Pyr in sediments. Results also reveal the importance of the exposure time, endpoints involved as well as of the contaminant monitoring during the whole experiments in assessing the interactive effects of the contaminant mixture.

Keywords Mercury · Pyrene · Mixture · Oxidative stress · Spiked sediments

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Introduction

Estuarine sediments are important sinks of the contaminants originated by several anthropogenic activities (Amiard-Triquet and Rainbow 2009). These activities have led to an increase in aquatic contaminants not only in terms of quantity but also in terms of variety (Newman and Unger 2003; UNEP 2013). Interactions of contaminants in mixtures can lead to diverse biochemical pathways and consequently trigger different and unpredictable toxicological responses in aquatic organisms (Maria and Bebbiano 2011; Wang et al. 2011).

Polycyclic aromatic hydrocarbons (PAHs) and non-essential metals represent aquatic contaminants of high environmental concern due to their ubiquity and well-documented effects on the biota (Gauthier et al. 2014). Due to the occurrence of industrial clusters linked to chlor-alkali and oil-related industries, Mercury (Hg) and Pyrene (Pyr) are likely to appear individually and simultaneously in coastal/estuarine environments in concentrations above and below to some SQG respectively (MacDonald et al. 2000; CCME 2002; Mai et al. 2002; Cachot et al. 2006; Pozo et al. 2011; Díaz-Jaramillo et al. 2013; Yañez et al. 2013). Pyr, which is related to fossil combustion and oil spill events, is one of the dominant PAHs in coastal environments (Richardson et al. 2008; Pozo et al. 2011; Oliveira et al. 2013; Almeida et al. 2012). The toxicity and metabolism of Pyr in marine animals, following short- and long-term exposure, where 1-OH-Pyrene is one of the most important intermediate metabolites, have been studied by several authors (Giessing et al. 2003; Oliveira et al. 2013). Hg, which comes from natural and anthropogenic sources, is a highly toxic non-essential trace element of global concern, whose inputs have substantially increased during the last century (Colacevich et al. 2011). In coastal sediments, urban runoff, industrial discharge and atmospheric deposition are the main anthropogenic sources of the trace element (Stoichev et al. 2004). Although methylmercury (MeHg) is the form of Hg that represents the most environmental concern regarding Hg, inorganic forms as Hg^{2+} are also involved (Colacevich et al. 2011). Inorganic Hg forms are also toxic, being the most important source of biotic and abiotic Hg methylation and representing more than 95% respect its organic forms in estuarine sediments (Lund et al. 1993; Stoichev et al. 2004; Yañez et al. 2013). Real scenarios (including estuarine environments) are complex in terms of sediment biogeochemistry and Hg bioavailability (Ouddane et al. 2008). However, since naturally contaminated sediments generally contain mixtures of toxicants, it is difficult to establish cause-effect relationships (Hutchins 2005). Tracking pollutant uptake/biotransformation during exposure time can yield valuable data to explain the bioavailability and biochemical responses of pollutants.

In terms of sublethal toxicity, trace elements and PAHs cause oxidative stress in aquatic organisms by different toxicological pathways (Almeida et al. 2012; Gauthier et al. 2014). These include direct or indirect formation of Reactive Oxygen Species (ROS) generating oxidative stress burst (Kopecka-Pilarczyk and Correia 2009; Colacevich et al. 2011; Rodrigues et al. 2013). Oxidative stress responses include those of glutathione S-transferase (GST) as a biotransformation phase II enzyme, the antioxidant enzyme glutathione peroxidase (GPx), the intracellular amount of Thiol-groups (SH-groups), the total antioxidant capacity against peroxyl radicals (ACAP) as the sum of enzymatic and non-enzymatic defenses, and Thiobarbituric Reactive Substances (TBARS) as biomarkers of lipid oxidative damage (also referred as LPO). TBARS represent endpoints with high ecotoxicological relevance and thus allow us to elucidate potential oxidative stress scenarios in aquatic organisms (Oakes and Van Der Kraak 2003).

During the last years, the interaction between trace elements and PAHs in aquatic organisms has been increasingly assessed (Gauthier et al. 2014). However, studies have been focused on aqueous media and different metals and PAH compounds (Ahmad et al. 2005; Almeida et al. 2008; Banni et al. 2009; Bouraoui et al. 2009; Maria and Bebbiano 2011; Wang et al. 2011; Vega-López et al. 2013; Gauthier et al. 2014). Therefore, studying the effects of Hg-Pyr mixtures using sediments and soft-bottom benthic animals represents a valuable contribution to understanding the effects of Hg pollution on multi-polluted environments.

The aim of this study was to examine the single and combined effects of Pyr and Hg at environmental relevant concentrations on oxidative stress responses (GST, GPx, SH-groups, ACAP and TBARS), including measurements of Hg and Pyr in sediments, overlying and pore water plus total Hg bioaccumulation and 1-OH-pyrene concentrations in the key estuarine species *Perinereis gualpensis* (ragworm) during short- and medium-term exposure.

Perinereis gualpensis was selected as test organism because it has been used as an effective biomonitor species of Hg bioaccumulation in sediments (Díaz-Jaramillo et al. 2013). *P. gualpensis* also provides measurable sub-individual responses during short- and medium-term exposure to contaminants (Díaz-Jaramillo et al. 2011).

Material and methods

Test design

To determine single and combined effects of Hg and Pyr on oxidative stress responses, adult individuals of *P. gualpensis* in non-evident reproductive stage (0.26 ± 0.04 g w. w) from the aquaculture facility of the Coastal Laboratory of Aquatic Resources of Calfuco (Universidad Austral de Chile) were exposed separately to sediments spiked with Hg and Pyr and their mixture. Concentrations were based on environmental Hg and Pyr levels reported in previous studies from estuarine polluted areas characterized by Hg pollution legacy from chlor-alkali and Pyr levels related to oil industries. (Table 1; Pozo et al. 2011; Díaz-Jaramillo et al. 2013; Yañez et al. 2013).

Before the experiments, ragworms from aquaculture sediment hatchery were transferred to laboratory keeping with filtered seawater and similar sediment conditions (salinity: 20 PSU, see below test sediment) for 48 h. Four

Table 1 Ranges and means (in parenthesis) of Hg and Pyr sediment concentrations observed in different areas from a multipolluted estuary including values of international Sediment Quality Guidelines (SQG) for marine sediments

Estuarine area	Hg (mg/Kg d.w)	Pyr (ng/g d.w)
Mouth	0.4–1.22 (0.79)[a,b]	91–369 (189)[c]
Central	1.61–83.0 (14.7)[a,b]	36–1017 (442)[c]
Head	1.00–129 (43.5)[b]	86–953 (394)[c]
Total average	19.2	342
SQG		
PEL	0.70	1398

PEL probable effect level

[a] Díaz-Jaramillo et al. (2013)

[b] Yañez et al. (2013)

[c] Pozo et al. (2011)

treatments were assessed to obtain sediment exposures from single Hg and Pyr sediments and their mixture. A non-spiked control group was run in parallel with the same sediments. Then, 5–7 individuals were transferred to plastic containers with 500 g of spiked and non-spiked sediments ($n = 3$ containers per treatment/sampling time) with filtered and continuous aerated seawater (salinity: 20 PSU). At the end of each exposure time, surviving ragworms were gently removed from the sediments and frozen in liquid N_2 and subsequently stored at −80 °C for biochemical analysis. Ragworms were sampled at different times (2, 7, 14 and 21 days) in the control sediments and during the single and combined Hg + Pyr exposure. Additionally, dead ragworms were daily removed and summarized every 7 days, total mortality was recorded at the end of the exposure time from each treatment. For chemical analysis of Hg and Pyr metabolites, ragworms were also sampled at different times (2, 7, 14 and 21 days) and transferred to plastic containers with filtered seawater for 6 h to allow them to clean their guts, and then stored at −20 °C, previous to chemical analysis (see below).

Test sediment

Estuarine natural sediment (Valdivia river estuary; 39° 51' 44.9" S; 73° 20' 59.5" W) also utilized for cultured ragworms was used to prepare the control and spiked sediments. The sediment was dried at 80 °C for 96 h to homogenize it evenly and eliminate other organisms. The sediment was also sieved through a 400-µm mesh to exclude gross sediment particles. This sandy-mud sediment contained 0.5% of organic carbon, 93.4% of sand, 4.7% of clay and 1.9% of silt. Sediments were spiked with solutions made from Hg^{2+} as Mercury chloride ($HgCl_2$; Merck, Germany) and certified Pyrene standard (Dr Ehrenstorfer, Germany). Desired sediment concentrations for single/mixture exposures were 20 mg/kg and 1000 µg/kg d.w for Total Hg and Pyr respectively.

$HgCl_2$ and Pyr were dissolved in ultra-pure water and acetone, respectively and then transferred to the sediments. The sediments were then stored in the dark and placed in a well-ventilated fume hood to dry and evaporate the solvent. Then, dry control and spiked sediments were mixed in overhead shaker for 14 days and stored in the dark at room temperature for 30 days to age and equilibrate. Sediments were additionally equilibrated with filtered seawater (salinity: 20 PSU) for 24 h prior to the addition of ragworms. Test sediments were run in a temperature-controlled room (15 °C) and exposed to a photoperiod of 10:14 h of light: dark, respectively. Experimental conditions of pH and temperature of seawater were 8.1 (±0.1) and 13.2 (±0.6 °C) respectively.

Chemical analysis

Sample preparation

For chemical analysis of Hg and Pyr in sediments, overlying and pore water samples were obtained at 2 and 21 days of exposure. Before ragworms collection, overlying waters and sediments were gently removed from test containers. Test sediments were transported to Geosciences Institute, Universidad Austral de Chile for pore water extraction. Pore water from test sediments was obtained following the methodology described by Mason et al. (1998) with modifications. Briefly Nitrogen positive gas pressure was applied to the top of the core with sediments through the core tube lid. Porewater was squeezed out of the sediment, filtering pore fluid through 0.4 µm membrane filter and contained into precleaned bottles.

Hg in water and sediments

Total sediment and water Hg were determined from freeze-dried sediment, overlying and pore water by cold vapor atomic absorbance spectrometry (CVAAS; Perkin–Elmer FIMS-400, Perkin–Elmer Corp., USA). Sediment and water samples were determined by EPA method 245.5 (USEPA 1991) and standard 3111 method (APHA 2012) respectively. Calibration was done using certified standard (Merck 170226, Darmstadt, Germany) traceable to Standard Reference Materials (SRM). Procedural blanks and SRM PACS-2 from the National Research Council of Canada (NRC, Canada) was included (106% recovery average; $n = 3$). The detection limit for Hg in the sample solutions was 0.5 µg/L.

Pyr in water and sediments

The sample preparation for Pyr analysis was performed according to UNEP/IOC/IAEA (1992). Blanks were prepared to check any possible contamination during the analytical procedure. Samples were analyzed using a Shimadzu GCMS-QP2100ULTRA-AOC20i with a column Zebron ZB-5MS (30 m × 0.25 mm × 0.10 µm) in splitless mode and the injection volume was 2 µl. The interface and the ionization source were kept at 300 and 280 °C respectively. Electron impact ionization was used at 70 eV in the SIM mode. The oven temperature program was as follows: 80 °C, held for 1 min, increased at 5 °C/min to 320 °C, held for 4 min. Surrogate recoveries were estimated from the labeled compounds (napthalene d8; acenapthene d10, Phenanthene d10) spiked prior to extraction. Average surrogate recoveries estimated from napthalene d8, acenapthene d10 and Phenanthene spiked prior to extraction were 92, 91 and 72% respectively. All data were recovery corrected. The target compounds were not detected in procedural blanks. The detection limit for Pyr in the sample solutions was 0.5 ng/ml.

Hg in P. gualpensis tissues

Total Hg concentration in *P. gualpensis* tissues was analyzed in freeze-dried ragworms from each sampling time in Hg and Hg + Pyr treatments ($n = 3$ per treatment/sampling time) with their respective controls. Total Hg was determined by CVAAS (see above) according to Díaz-Jaramillo et al. (2013). Procedural blanks and SRM DORM-2 (NRC, Canada) were included (112% recovery average; $n = 3$). The method detection limit was 0.016 mg/kg.

1-OH-Pyr concentration in P. gualpensis tissues

Quantification of 1-hydroxypyrene (1-OH-Pyr) in *P. gualpensis*, the most important intermediate Pyr metabolite observed in polychaetes (Giessing et al. 2003) was performed in order to quantify indirectly *P. gualpensis* Pyr exposure from test sediments. Quantification of 1-OH-Pyr was performed according to Giessing et al. (2003) with modifications and was analyzed in ragworms from each sampling time in Pyr and Hg + Pyr treatments ($n = 3$–5 per treatment/sampling time) with their respective controls. Briefly, tissues from ragworms were homogenized in 250 µL of methanol (1:3 weight:solvent). Samples were then centrifuged (500 g) to precipitate any debris and supernatant was filtered through a 0.22-mm syringe filter and transferred directly to amber HPLC-MS vials. Calibration curves were made by standard of 1-OH-Pyr (Sigma-Aldrich, Germany) in methanol (regression coefficient: $r^2 > 0.99$). 1-OH-Pyr was determined by the LC system coupled with a MSD VL quadrupole with an electrospray ionization interface (1100, Agilent Technologies Inc., USA). The chromatographic separations were performed on a C-18 column on isocratic mode (Acetonitrile: Formic Acid 0.1%). The electrospray ionization was operated at 330 °C on positive mode. Analytical procedures were validated using recoveries made with spiked a known standard concentration of 1-0H-Pyr on polychaete tissue. Average recovery of 1-0H-Pyr on spiked polychaete tissue was 70%.

Biochemical analysis

Protein content, GST and GPx activities and SH-groups concentrations and ACAP were biochemically measured using anterior/medium body regions of ragworms (5–9 organisms/tissues per treatment/sampling time) homogenized in Tris-Sucrose buffer, centrifuged (9000 g) and stored in ultrafreezer (−80 °C) for further analysis (Díaz-Jaramillo et al. 2011). The activity of GST was evaluated according to Habig and Jakoby (1981), the activity of GPx according to Sies et al. (1979), and sulfhydryl content (SH-groups) according to Sedlak and Lindsay (1968). Total Antioxidant capacity against peroxyl radicals (ACAP) was measured according to Amado et al. (2009) high relative difference areas reflecting lower antioxidant capacity. For TBARS measurements, posterior body end of the same ragworm tissues were evaluated according to Oakes and Van Der Kraak using KCl buffer (2003).

Data analysis

Changes in total Hg and 1-OH-Pyr tissue concentrations and biochemical responses were evaluated by analysis of variance (ANOVA) using Newman–Keuls test for post-hoc comparisons (α: 0.05; p below to 0.01 expressed as <0.01). Data were checked to meet the assumptions of normality and homogeneity of variances prior to analysis. Data without normal distribution were analyzed using Kruskal–Wallis non-parametric test. Additionally, to visualize the overall biochemical responses and their differences between single treatments and the Hg + Pyr mixture, a principal component analysis (PCA) was performed.

Results

In the bioassay test, total ragworm mortality at the end of the exposure period (21 days) was as follows: 13% in the control, 31% in the treatment with Hg, 29% in the treatment with Pyr and 23% in the treatment with both Hg and Pyr. Statistical analysis showed no significant differences between groups ($p > 0.05$). Total Hg and Pyr concentrations in the spiked dried sediments prior to used in bioassays

were 21.5 ± 1.5 mg Hg/kg d.w. and 1328.7 ± 262.8 µg Pyr/kg d.w respectively.

Hg and Pyr in sediments and water

Regarding Hg and Pyr concentrations, test sediments (controls) had 0.01 to 0.09 mg of total Hg/kg d.w. and 0.28 to 7.93 µg Pyr/kg d.w (Table 2). In terms of overlying and pore water, Hg and Pyr concentrations from controls showed values below detection limits (bdl) in most samples and exposure days (Table 2). Sediments single treatments at 2 and 21 days of exposure showed values ranged from 15.2 to 17.4 mg/kg and from 432 to 1048 µg/kg d.w for Hg and Pyr respectively (Table 2). Overlying and pore water from single treatments ranged from bdl to 0.27 µg/ml and bdl to 5.20 ng/ml for Hg and Pyr respectively and showed a decrease in their values after 21 days of exposure (Table 2). In Hg + Pyr treatments, sediments showed similar Hg values to those from single treatments ranged from 16.3 to 17.4 mg/kg d.w at 2 and 21 days of ragworm exposure respectively (Table 2). Pyr concentrations from Hg + Pyr treatments showed lower values compared to single Pyr treatments ranged from 168 to 213 µg/kg d.w at 2 and 21 days of ragworm exposure respectively (Table 2). Overlying waters from mixture treatments only showed measurable concentrations of Hg ranged from 0.15 to 0.39 µg/ml at 2 day of exposure (Table 2). Pore water from Hg + Pyr treatments showed values from bdl to 0.59 µg/ml and from 0.12 to 0.54 ng/ml for Hg and Pyr respectively, decreasing their values after 21 days of exposure (Table 2).

Table 2 Total Hg (bold) and Pyr (cursive) ranges ($n = 2$) in sediments, overlying and pore water from control, single Hg/Pyr and combined Hg + Pyr mixture at 2 and 21 days of *P. gualpensis* exposure

Compartment	Treatment	Day 2	Day 21
Sediments*	Ct	0.01–0.09	0.03–0.05
		7.69–7.93	*0.28–0.37*
	Single Hg	15.2–16.2	16.7–17.4
	Single Pyr	*432–640*	*335–1048*
	Hg + Pyr	16.3–18.0	17.3–17.4
		168–182	*172–213*
Overlying water**	Ct	bdl	bdl
		bdl	*bdl*
	Single Hg	0.18–0.27	0.001-bdl
	Single Pyr	*1.87–5.20*	*bdl*
	Hg + Pyr	0.15–0.39	bdl
		bdl	*bdl*
Pore water**	Ct	bdl-0.003	bdl
		bdl	*bdl*
	Single Hg	bdl-0.06	bdl
	Single Pyr	*0.29–0.38*	*bdl-0.22*
	Hg + Pyr	0.27–0.59	bdl
		0.50–0.54	*bdl*

bdl below detection limits

*mg/kg d.w for Hg and µg/kg d.w for Pyr, **µg/ml for Hg and ng/ml for Pyr

Hg in *P. gualpensis* tissues

Total Hg concentration in ragworms from the control treatments ranged from bdl to 0.04 mg/kg w.w. (Fig. 1a). In ragworms exposed to Hg-only, total Hg concentration increased significantly during the exposure period at 2 days compared to Hg concentrations at 21 days (p:0.04; Fig. 1a). In those exposed to Hg + Pyr, total Hg concentration increased from 0.17 mg/kg w.w. at 2 days to 0.22 mg/kg w.w. at 21 days, and the difference between these two sampling times was not significant ($p > 0.05$, Fig. 1a). The differences in total Hg concentration in *P. gualpensis* tissues between the treatments with Hg-only and those with Hg + Pyr were not significant ($p > 0.05$, Fig. 1a).

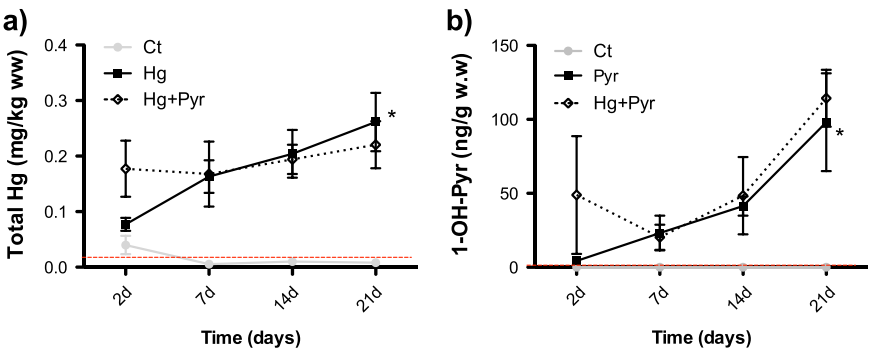


Fig. 1 **a** Total Hg concentrations after Hg-only and Hg + Pyr exposure ($n = 3$) plus controls ($n = 2$) in *Perinereis gualpensis* tissues during 21 days. Measurements represent the mean ± SE. *Same color asterisks* indicate significant differences ($p < 0.05$) in the same treatment vs. the initial condition (day 2). *Red arrow lines* indicate method detection limits reported. **b** 1-OH-Pyrene concentrations after Pyr-only and Hg + Pyr exposure ($n = 3$–5) plus controls ($n = 2$) in *Perinereis gualpensis* tissues during 21 days. Measurements represent the mean ± SE. *Same color asterisks* indicate significant differences ($p < 0.05$) in the same treatment vs. the initial condition (day 2). *Red arrow lines* indicate non-detected values

1-OH-Pyr in *P. gualpensis* tissues

No 1-OH-Pyr was detected in ragworms from control treatments at each sampling time, whereas significant amounts of this metabolite were quantified in organisms exposed to Pyr-only and Hg + Pyr at all exposure times (Fig. 1b). In ragworms exposed to Pyr-only, 1-OH-Pyr concentrations increased significantly at 21 days compared to 2 day exposure (p:0.04; Fig. 1b). In those exposed to Hg + Pyr, the difference between these two sampling times was not significant ($p > 0.05$, Fig. 1b). The differences in 1-OH-Pyr concentrations in *P. gualpensis* tissues between the treatments with Pyr-only and those with Hg + Pyr were not significant ($p > 0.05$, Fig. 1b).

Biochemical analysis in *P. gualpensis*

In general, biochemical responses showed different patterns between single and combined treatments. GST activity increased significantly relative to the control treatment in ragworms exposed to Hg-only or Pyr-only at 7 and 14 days of exposure ($p < 0.01$; Fig. 2a), but decrease significantly relative to control treatment in ragworms exposed to Hg-only for 21 days ($p < 0.01$; Fig. 2a). GST activity in ragworms exposed to Hg + Pyr showed significant differences at 2 and 14 days of exposure respect to the control treatment and a significant increase at 2 days regarding Hg-only and Pyr-only exposure ($p < 0.01$; Fig. 2a). GST activity after 7, 14 and 21 days of exposure to Hg, Pyr or Hg + Pyr showed significant differences respect to the initial activity (2 days) ($p < 0.01$; Fig. 2a). Regarding GPx activity, significant differences with the control group were detected in Hg + Pyr and Hg-only treatments at 2 days (p: 0.04; Fig. 2b). GPx activity increased significantly after Hg exposure as compared to control at 14 days ($p < 0.01$; Fig. 2b) and decreased significantly at 21days ($p < 0.01$; Fig. 2b). GPx activity after 7, 14 and 21 days of exposure to Hg + Pyr showed significant decrease respect to the initial activity (2 days) (p: 0.03; Fig. 2b) and after 21 days to Hg ($p < 0.01$; Fig. 2b). Total SH-groups increased significantly as compared to the control after Hg + Pyr and Hg-only exposure at 2 and 14 days respectively ($p < 0.01$; Fig. 2c). No significant differences in total SH-groups were observed between Hg + Pyr and the single treatments at all exposure times ($p > 0.05$; Fig. 2c). Total SH-groups from ragworms exposed to Hg-only and Hg + Pyr at 14 and 7 days respectively were different from those at the initial conditions (p: 0.03 for Hg; $p < 0.01$ for Hg + Pyr; Fig. 2c). Total ACAP activity showed a significant decrease in total antioxidant capacity in Hg-only and Pyr-only treatments at 14 and 21 days respectively compared to the control and Hg + Pyr treatments ($p < 0.01$ for Hg; p: 0.01 for Pyr; Fig. 2d). The Hg + Pyr mixture showed a major increase in terms of ACAP in the 1st days of exposure respect to the control and single treatments (day 2; $p < 0.01$; Fig. 2d). ACAP from ragworms exposed to Hg-only and Pyr-only at 14–21 days and Hg + Pyr at 21 days were different from those at the initial conditions ($p < 0.01$ for Hg and Pyr; p: 0.02 for Hg + Pyr; Fig. 2d). Lipid peroxidation in terms of TBARS levels showed differences between single and combined exposure ($p < 0.05$; Fig. 3). TBARS levels in ragworms exposed to Hg showed significantly higher at 7 day of exposure than Hg + Pyr treatment (p:0.01; Fig. 3). TBARS levels in ragworms exposed to Hg-only and Pyr-only showed significantly higher levels of lipid peroxidation at 14 and 21 days than each control and Hg + Pyr treatment ($p < 0.01$; Fig. 3). TBARS levels in ragworms exposed to Hg + Pyr showed no significant

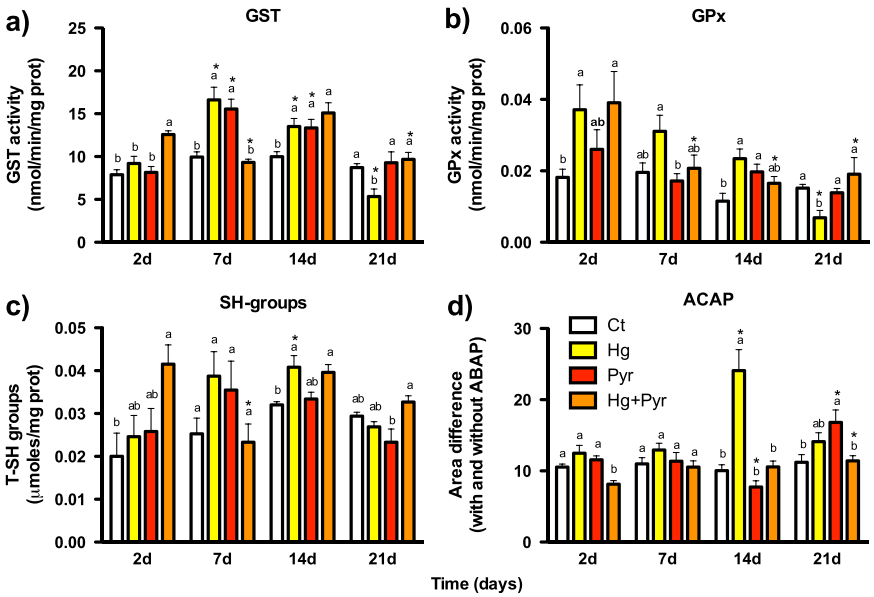


Fig. 2 Single and joint effects of Hg and Pyr on: **a** GST activity, **b** GPx activity, **c** Total SH-groups and **d** ACAP in *P. gualpensis* after 21 days of exposure. Measurements represent the mean ± SE. Different letters indicate significant differences ($p < 0.05$) between treatments at the same exposure time. Asterisks indicate significant differences ($p < 0.05$) in the same treatment vs. the initial condition (day 2)

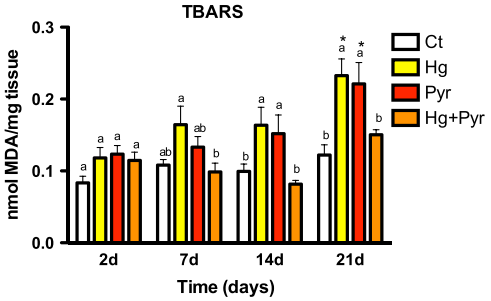
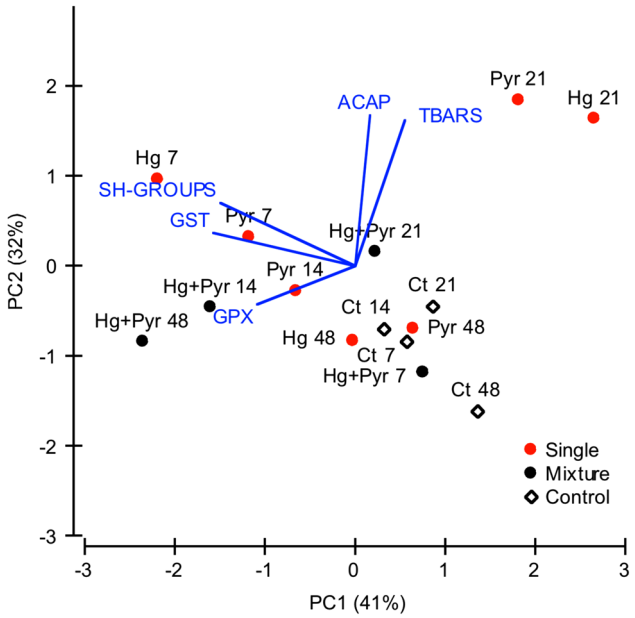


Fig. 3 Single and joint effects of Hg and Pyr on TBARS levels in *P. gualpensis* after 21 days of exposure. Measurements represent the mean ± SE. Different letters indicate significant differences ($p < 0.05$) between treatments at the same exposure time. Asterisks indicate significant differences ($p < 0.05$) in the same treatment vs. the initial condition (day 2)

differences from the control groups ($p > 0.05$; Fig. 4). TBARS levels were significant different from the initial ones only in ragworms exposed to Hg-only and Pyr-only at 21 days of exposure (p:0.01; Fig. 3).

To compare overall oxidative stress responses after single and Hg + Pyr exposure, the PCA was made with the biochemical responses found after the different exposure times (Fig. 4). PCA analysis indicated that the first two components explained 73% of the variation, being GST activity, SH-groups, ACAP and TBARS levels the most significant variables that explained the first two principal components (Fig. 4). It also indicated that ragworms exposed to Hg + Pyr in general showed enzymatic and non-enzymatic activities similar to those observed in controls, Hg-only and Pyr-only treatments on the first weeks of exposure (Fig. 4). In contrast, ragworms exposed to Hg-only or Pyr-only were characterized by lower antioxidant capacity and higher lipid peroxidation (mainly in Hg-only exposure) than those exposed to Hg + Pyr and controls at 14 and 21 days, (Fig. 4).



Variable	PC1	PC2
%Total variance	41	32
GST	-0.631	0.147
GPx	-0.436	-0.170
SH-groups	-0.599	0.280
ACAP	0.065	0.671
TBARS	0,220	0.649

Fig. 4 Principal component analysis (PCA) for the overall oxidative stress responses observed in ragworms from control, single and Hg + Pyr treatments at different exposure times. The variability percentage explained by each principal axis is provided. The direction of the blue lines indicates the steepest increase in the variable, and the length indicates the strength relative to other variables. A table of variable correlations and principal component percentages (PC1, PC2) of oxidative responses is provided

Discussion

Despite Hg concentrations in the sediments remaining relatively constant and similar in both single and mixture treatments during all exposure periods, Pyr concentrations in the sediments differed between single and mixture treatments. These results showed possible interactions between those contaminants to be taken in consideration when generating the spiked metals-PAHs sediment mixture. PAHs are known to modify and enhance solubility of metals in sediments (Almeida et al. 2008), this fact would explain the higher values of Hg in porewater mixture compared to single Hg treatment during the 1st days of exposure. Moreover, both Hg and Pyr concentrations in porewater at 21 days were low and/or non-detected in both single and Hg + Pyr treatments as compared to the initial conditions.

Considering only the 7-21-day exposure period, during which ragworms from Hg, Pyr and Hg + Pyr treatments showed similar accumulation trends, the influence of Hg + Pyr in the uptake/biotransformation processes was confined only to the 1st h/days of exposure. On the other hand, some authors have reported different 1-OH-Pyr concentrations when animals are exposed to Pyr together with other pollutants (Broerse et al. 2012; Oliveira et al. 2013). Moreover, in some invertebrate species, high and early Pyr exposure leads to high metabolization rates (Richardson et al. 2008). As observed in other annelids, the occurrence of 1-OH-Pyr metabolites in *P. gualpensis* tissues emerges as an effective biomarker of exposure to PAHs (Giessing et al. 2003) and suggests that the cytochrome P450 (Phase I) system involved in the metabolism of xenobiotics exhibits relatively high activity in this species.

Single Pyr exposure triggers oxidative stress responses of some polychaeta, as reported by some authors in aquatic organisms (Almeida et al. 2012; Luís and Guilhermino

2012). GST activation by this PAH could be related to the biotransformation of Pyr by catalyzing the conjugation of metabolite(s) generated in phase I with glutathione and/or the increase in GST activity in response to Pyr-induced oxidative stress (Luís and Guilhermino 2012). Nevertheless, the decreases observed in the present study in total antioxidant capacity and enhanced lipid peroxidation in ragworms at the end of Pyr exposure indicate harmful single effects at the environmental concentrations studied. However, the Hg-only exposure caused either induction or inhibition processes in most of the responses evaluated. Regarding GST and GPx activity, the activation and subsequent inhibition by the Hg-only exposure revealed the high disrupting capacity of Hg in glutathione-dependent enzymes (Colacevich et al. 2011). It is known that Hg causes oxidative stress via H_2O_2 production, where GPxs are crucial for peroxide removal and many GST isoforms possess peroxidase-like activity (Rodrigues et al. 2013). As the main thiol pool, glutathione contains SH-groups that play a role in intracellular Hg sequestration (Kovářová and Svobodová 2009). So, Hg^{2+} ions have a high affinity for SH-groups, and it has been reported that non-organic Hg compounds are able to induce the formation and subsequent depletion of SH compounds in aquatic organisms (Colacevich et al. 2011; Wu and Wang 2012). Decreased total antioxidant capacity and high levels of lipid peroxidation in polychaetes after the Hg-only exposure relates to the decreased levels of antioxidant/detoxification enzymes. The inhibition or down-regulated responses of crucial enzymes could be responsible for the oxidative damage caused by Hg forms, indicating that enzyme inhibition represents at least an important mechanism by which Hg causes deleterious effects (Rodrigues et al. 2013; Gauthier et al. 2014).

In contrast to that observed in the single treatments, polychaetes from the Hg + Pyr treatment reflected different patterns of the above-mentioned responses and suggest that >PEL Hg concentrations in sediments could exerts different toxic scenarios in the presence of certain < PEL Pyr concentrations. Since some of the biochemical responses observed during the 21 days of the experiment are idiosyncratic (e.g. total SH) and TBARS levels from posterior body end may be different from the rest of the body (Díaz-Jaramillo et al. 2011), the predictive value of these responses at this exposure time are questionable. The different toxic interactions suggested in this work doesn´t represent non-adverse scenarios, since long-term exposures to high levels of Hg and organic pollutants in sediments could trigger more ecological relevant adverse effects on this specie (Díaz-Jaramillo et al. 2011). On this basis, low densities, low adult survival and poor reproductive fitness comparing to non-impacted *P. gualpensis* populations were the main observed effects from this multi-polluted scenario (Díaz-Jaramillo et al. 2015).

However, equilibration time and medium-term exposure used following spiking sediments, would lead to Hg methylation in sediments. Unfortunately, MeHg concentrations in the sediments and worms were not determined, since inorganic forms as $HgCl_2$ are used for maximal Hg bioavailability to methylation process (Davis et al. 1997; Bloom and Preus 2003). Instead of spiked inorganic mercury, the lower toxicity of Hg + Pyr compared to Hg-only, could probably be due to the fact that Pyr impacts on MeHg production in sediments.

Finally, different types of mixtures between PAHs and metals and their concentrations, the exposure medium, test organism, test duration and endpoints studied result in a wide range of interactive effects and predominantly more than additive type responses (Shen et al. 2006, Gauthier et al. 2014). Unfortunately neither Hg nor Pyr studies were reported in similar test media or exposure time in order to obtain comparative results. Our preliminary results suggest the need for further studies to elucidated Hg interactions with other chemicals highlights the complexity that take place in multi-polluted sediments.

Conclusions

Results showed differences between single and combined treatments in terms of *P. gualpensis* enzymatic and non-enzymatic oxidative stress responses. An early enzymatic and non-enzymatic activation with the absence of oxidative damage in the mid-term suggests different oxidative stress scenarios for each contaminant. Single exposure to Hg elicited more relevant toxicological effects, often resulting in inhibition of some antioxidant/detoxification enzymes and enhanced lipid peroxidation. Our results suggest the importance of time-scale and tracking contaminant concentrations in elucidating potential effects on benthic species produced by this type of chemical mixture.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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