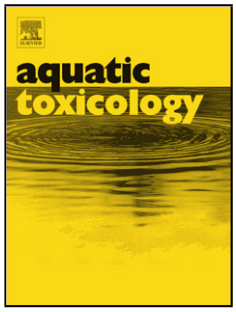

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Combined exposure to pyrene and fluoranthene and their molecular effects on the Sydney rock oyster, *Saccostrea glomerata*

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Highlights

- PAHs cause suppression of transcripts coding for pathogen recognition in *S. glomerata*
- Transcripts involved in phagocytosis and apoptosis are elevated in response to PAHs
- A small set of transcripts implicated in PAH metabolism is induced by PAHs

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitously detected in the water column, associated with particulate matter or in the tissue of marine organisms such as molluscs. PAH exposure and their resultant bioaccumulation in molluscs can cause a range of serious physiological effects in the affected animals. To examine the molecular response of these xenobiotics in bivalves, Sydney rock oysters (*Saccostrea glomerata*) were exposed to pyrene and fluoranthene for seven days. Chemical analysis of the soft-tissue of PAH stressed *S. glomerata* confirmed that pyrene and fluoranthene could be bioaccumulated by these oysters. RNA-Seq analysis of PAH-exposed *S. glomerata* showed a total of 765 transcripts differentially expressed between control and PAH-stressed oysters. Closer examination of the transcripts revealed a range genes encoding enzymes involved in PAH detoxification (e.g. *cytochrome P450*), innate immune responses (e.g. pathogen recognition, phagocytosis) and protein synthesis. Overall, pyrene and fluoranthene exposure appears to have resulted in a suppression of pathogen recognition and some protein synthesis processes, whereas transcripts of genes encoding proteins involved in clearance of cell debris and some transcripts of genes involved in PAH detoxification were induced in response to the stressors. Pyrene and fluoranthene exposure thus invoked a complex molecular response in *S. glomerata*, with results suggesting that oysters focus on removing the stressors from their system and dealing with the downstream effects of PAH exposure, potentially at the exclusion of other, less immediate concerns (e.g. protection from infection).

Keywords

Sydney rock oyster; *Saccostrea glomerata*; mollusc; RNA-seq; stress; oil; pyrene; fluoranthene; detoxification; immunity

1. Background

Polycyclic aromatic hydrocarbons (PAHs) are organic contaminants, ubiquitously observed in aquatic environments, where they are found in the water column as well as associated with particulate matter (Croxton et al., 2012; Mirza et al., 2012; Zhou et al., 1998). For example, a wide range of PAH compounds have been measured in the seawater and sediment of different inter-tidal areas off the coast of Bushehr province (Persian Gulf) (Mirza et al., 2012), from surface sediments in inter-tidal areas of Dar es Salaam (Tanzania) and the coastline of Guadeloupe (Gaspare et al., 2009; Ramdine et al., 2012), and in surface sediments and suspended particulate matter in the Humber Estuary (United Kingdom) (Zhou et al., 1998). The lipophilic PAHs can be produced naturally or are released during fossil fuel combustion and enter aquatic habitats through oil spills, surface runoff, atmospheric deposition and industrial and domestic effluent, where they can persist for a long period of time (Bustamante et al., 2012; Croxton et al., 2012; Mirza et al., 2012; Neff et al., 1976; Teal and Howarth, 1984). Other sources releasing PAHs into the aquatic environment include two-stroke engines (e.g. Jet Ski) and creosote-treated timber (Kelly et al., 2005; Smith, 2008; Thompson et al., 1993).

Bivalves, which have been assessed as potential biomonitors for aquatic contamination due to their often sessile nature and ability to bioaccumulate many contaminants, take up PAHs from the water column through passive diffusion or by ingestion of contaminated sediment that has been resuspended by bioturbation or water currents (Ciarelli et al., 1999; Croxton et al., 2012). Bivalve uptake and bioaccumulation of these PAHs depends on the molecular and steric properties of the respective PAHs (Croxton et al., 2012; Kanaly and Harayama, 2000), with PAH bioaccumulation observed in *Crassostrea gigas* (Bustamante et al., 2012), *Crassostrea virginica* (Neff et al., 1976), *Saccostrea cucullata* (Gaspare et al., 2009; Mirza et al., 2012), *Mytilus trossulus* (Turja et al., 2013), *Crassostrea*

rhizophorae (Castillo et al., 1992), and in wild oysters from the Hunter River estuary (NSW, Australia) (Lincoln-Smith and Cooper, 2004), exposed to a range of PAHs in the laboratory or along pollution gradients in the field.

Exposure of molluscs to the toxic and carcinogenic PAHs and their metabolites has been shown to decrease digestive epithelial thickness, cause gill morphological anomalies (e.g. detachment and degeneration of epithelial cells, loss of cilia) and DNA damage, delay gametogenesis, affect respiration and filtration rates, lysosomal stability and lysosome number and levels of RNA polymerase. Furthermore, long-term exposure to PAHs results in high mortalities and decreased growth rates of affected molluscs (Banni et al., 2010; Bayne et al., 1982; Cappello et al., 2013; Frouin et al., 2007; Kim et al., 2007; Liu et al., 2012; Sindermann, 1982; Weinstein, 1997). Considering the widespread distribution of aquatic PAH contamination, and the serious physiological effects PAH exposure can have on molluscs and other marine animals, further knowledge regarding the molecular effects of the contaminant on marine organisms would be beneficial.

For this study, adult Sydney rock oysters (*Saccostrea glomerata*) were chosen as this is the most important oyster species cultivated on the east coast of Australia, where it currently forms the basis of a AU$36 million/annum industry (Livingstone, 2016). In order to determine the molecular response of these oysters to PAHs, *S. glomerata* were exposed for one week to two PAH compounds: pyrene, which is highly toxic and persistent in the environment (Ceyhan, 2012), and fluoranthene, which is toxic, mutagenic and carcinogenic (Liu et al., 2006). Both of these compounds are on the United States Environmental Protection Agency (US EPA) priority pollutant list (Yan et al., 2004) and have previously been demonstrated to be accumulated by bivalves (Castillo et al., 1992; Lincoln-Smith and Cooper, 2004; Neff et al., 1976). In addition, bivalves in nature are generally exposed to a mixture of PAHs, which could potentially interact with each other. Therefore, to closer mimic the natural state, more than one PAH contaminant was chosen. At the conclusion of the exposure trial, RNA-Seq analysis was carried out on control and PAH exposed oysters to determine their molecular response to the two compounds.

2. Methods

2.1 PAH stress exposure and sample collection

Wild, adult *Saccostrea glomerata*, collected from Cromarty Bay, Port Stephens (NSW, Australia), were experimentally exposed to PAH for one week, with full details of animal husbandry, experimental set-up and exposure described in Supplementary File 1. In this experiment, rice flour (particle sizes up to 50 μm) was used as a carrier of pyrene and fluoranthene (Sigma-Aldrich, Australia), two common components of oil contamination. 200 g of rice flour was spiked with either a) 40 mL of dichloromethane (Honeywell, USA) (control) or b) 17.96 mg/kg pyrene and 17.64 mg/kg fluoranthene dissolved in 40 mL of dichloromethane (total PAH). The level of total PAH used in this study was based on results of a sediment monitoring study conducted in 2005 in the Port Stephens estuary to investigate residual PAH levels in oyster farming areas where the timber culture infrastructure had been treated with coal tar to protect against damage by marine boring organisms (DPI, unpublished data). *S. glomerata* were acclimated to seawater of ambient salinity and temperature for four days, after which oysters were fed 20 mg/L/oyster of either control or total PAH flour five times a day for one week, with each treatment replicated ten times. In order to depurate the digestive tract of the experimental *S. glomerata*, all oysters were fed twice with control flour over a time period of at least 8 h at the conclusion of the exposure experiment. Six random oysters per treatment were then collected for RNA-Seq analysis. For the chemical analysis, tissue from the same oysters used for molecular analysis was pooled for each individual oyster sample, homogenised and stored short-term at -80°C in acid-washed glass vials wrapped with aluminium foil until chemical analysis was carried out.

2.2 Sample preparation and sequencing

For RNA-Seq analysis, total RNA was extracted from 25 mg of pooled tissue (5 mg each of gill, mantle, adductor muscle, gonad and digestive tissue) per individual *S. glomerata*, using the Direct-zol

RNA MiniPrep kit (Zymo Research Corporation, USA) including the DNase I digestion step according to the manufacturer's protocol. Quality and quantity of the extracted RNA of the 12 samples were tested with gel electrophoresis, the 2100 Bioanalyzer (Agilent Technologies, USA) using the RNA 6000 Nano Chip kit (Agilent Technologies) and with the Quantus^{TM} fluorometer (Promega, Australia). ERCC RNA spike-in control mixes (Ambion, Australia) were diluted 1:100, then 2 µL of the diluted mix1 and mix2 added to 1 µg of total RNA of control and total PAH samples, respectively. RNA-Seq libraries were then prepared from each of these samples, using the TruSeq RNA sample prep kit-v2 (Illumina, Australia) according to the kit's preparation guidelines. Quality and quantity of the 12 cDNA libraries were tested with the 2100 Bioanalyzer, using the High Sensitivity DNA chip kit (Agilent Technologies) and with the Quantus^{TM} fluorometer (Promega). Paired-end (100 bp) sequencing of the libraries was carried out by AGRF (Australia) as described in Supplementary File 1. Raw reads were submitted to the sequence read archive and can be accessed under the SRA study accession number SRP058070.

2.3 *De novo* reference transcriptome and differential transcript expression analysis

Sequence quality was assessed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) pre- and post-trimming, with details of read trimming, *de novo* assembly of a *S. glomerata* reference transcriptome, redundancy removal and transcriptome assessment described in Supplementary File 1. For differential transcript expression analysis, post-processed reads of the 12 experimental *S. glomerata* samples were mapped to the *S. glomerata* reference transcriptome with Bowtie (Langmead et al., 2009), using a RSEM (Li and Dewey, 2011) internal script. Read counts for each sample were estimated with RSEM, sample data of all 12 oysters combined into one matrix file and read count data of transcripts with zero estimated counts for all 12 samples removed from the matrix file. The new matrix file, along with a ngvector file produced with the rsem-generate-ngvector RSEM script were then imported into EBSeq (Leng et al., 2013) in R (version 3.1.1) for further analysis. As gene-isoform relationships are unknown in *de*

novo assembled transcriptomes, the mapping ambiguity clusters detailed in the ngvector file are necessary for the differential expression analysis. Median normalisation was carried out on the imported matrix file, and then EBTest run for 14 iterations until convergence was reached. Isoforms/transcripts found to be significantly differentially expressed between control and total PAH samples, using a false discovery rate (FDR) threshold of 0.05, were closer examined. Fold change values used throughout the text were based on the posterior fold change values. EBSeq uses an empirical Bayesian approach and was chosen in this study as it takes the estimation uncertainty inherent in isoform expression analysis into consideration (Leng et al., 2013). Graphical results of the standard diagnostics on the differential transcript expression analysis with EBSeq are presented in Supplementary Figures 1 to 4. Differential transcript expression analysis method used in this study was previously validated with qPCR, with details of the analysis described in Supplementary File 1.

2.4 Functional annotation of differentially expressed transcripts

Similarity searches of significantly differentially expressed transcripts were carried out against the NCBI non-redundant (nr) database (downloaded 08.09.14) using blastx. E-value cut-off for the similarity searches was set at $1e^{-5}$ with a hit number threshold of 25. Blast2GO (Conesa et al., 2005) with standard parameters (hit adjusted to 25) was used for mapping and functional annotation of the transcripts. Furthermore, the InterProScan functionality of Blast2GO was run on transcripts and the results merged with the already existing annotations. Where domain/family information was available for transcripts with a sequence description of "---NA---" or "hypothetical protein/uncharacterised protein", with the information offering an indication as to the potential identity of the transcript, it was added to the respective transcript.

2.5 Chemical analysis of experimental samples

2.5.1 Preparation of standards

100 ppm stock solutions were prepared in analytical grade acetonitrile (Merck, Kilsyth, Australia) for pyrene and fluoranthene. Spike and standard solutions were then prepared in hexane (Merck) from the stock solutions. Spike solutions used in the analysis were 4 ppm and 1 ppm composite solutions, while standard solutions for the calibration curves had the following concentrations: 1000, 120, 100, 80, 60, 40 and 20 ppb.

2.5.2 Processing and extraction of samples

The PAH extraction method used in this study was based on two published methods (AOAC International, 2007; Johnson, 2012). Here, 2 mL of deionised water and 4 mL of analytical grade acetonitrile were added to either 2.0 g (± 0.2 g) of each homogenate or 2.0 g (± 0.2 g) untreated control samples spiked with a) 100 µL of the 4 ppm composite solution (200 ppb recovery) or b) 100 µL of the 1 ppm composite solution (50 ppb recovery). Next, 1.6 g of anhydrous $MgSO_4$ (Sigma-Aldrich) and 0.4 g of anhydrous NaCl (Thermo Fisher Scientific, Australia) were added to each sample, the samples vigorously shaken for 1 min, after which they were centrifuged for 10 min at 3500 rpm. After centrifugation, 3 mL of the acetonitrile layer containing the analyte were transferred into a new PAH free glass centrifuge tube along with 150 mg primary secondary amine sorbent (Agilent Technologies, Australia), 900 mg $MgSO_4$ and 150 mg graphitised carbon black (Banksia Scientific, Australia). The tubes were then capped, vortexed for 1 min and centrifuged for 10 min at 3500 rpm. For the final extraction step, 1 mL of the acetonitrile extraction solvent containing the analyte was transferred to a fresh centrifugation tube, 3 mL of deionised water and 1 mL of hexane (acetonitrile saturated) added, the mixture shaken for 5 min and then centrifuged for 3 min at 3500 rpm. The top hexane layer was then transferred into 2 mL gas chromatograph vials for direct gas chromatograph and mass spectrometer (GC-MS) analysis.

2.5.3 GC-MS analysis

The GC-MS used in this study was a Clarus 580 gas chromatograph (Perkin Elmer, Australia) with a Clarus SQ 8S single-quadrupole mass spectrometer (Perkin Elmer) controlled by the TurboMass software. The column used was a Zebron ZB-5MS (30 m x 0.25 mm with a 0.25 μm film thickness), with ultra-pure helium as a carrier gas at a constant flow rate of 1.00 mL/min. Injection volume was 1.0 μL splitless for -0.5 to +1.0 min then split at 20:1, inlet temperature set to 280°C with the temperature program set to 100°C hold for 0.5 min, then 25.0°C/min ramp to 200°C, followed by 15.0°C/min ramp to 310°C for a total run time of 11.83 min. Quantitation of PAHs utilised selective ion monitoring, with quantitation at 202 m/z and qualifier 101 m/z between 4.0 and 11.8 min.

3. Results and Discussion

In this study, *S. glomerata* were exposed to pyrene and fluoranthene, two common components of oil contamination for one week, and their molecular response to the contaminants analysed with Illumina RNA-Seq. The presence of the PAHs was chemically measured in the soft tissue of control oysters (below the limit of quantitation for both PAHs) and oysters exposed to total PAH (fluoranthene: 155.1 ± 31.9 μg/kg; pyrene: 94.8 ± 19.9 μg/kg). The molecular responses of the same oysters were analysed to allow observations to be directly linked to the PAH stressor. We found that *S. glomerata* readily bioaccumulated both pyrene and fluoranthene (Table 1). However, even though the feed was spiked with a slightly higher level of pyrene than fluoranthene, higher concentrations of fluoranthene were measured in the oyster tissues, suggesting that a) fluoranthene uptake and/or retention was greater than that of pyrene, or b) that *S. glomerata* could metabolise pyrene faster than fluoranthene. Considering that a range of bacteria (e.g. *Mycobacterium* sp., *Proteus vulgaris*) have been shown to be able to degrade PAHs such as pyrene and fluoranthene by using them as their sole source for carbon and energy (Boldrin et al., 1993; Ceyhan, 2012), it is also possible that the natural microbiota present in the digestive system of *S. glomerata* could have eliminated some of the pyrene parent compound. Differential PAH bioaccumulation has also been observed in the clam *Mya arenaria*

exposed to a range of PAH sources for 30 days (Frouin et al., 2007). Similar to our study, the pattern of fluoranthene and pyrene accumulation in the digestive gland of *Mya arenaria* did not follow the ratio of these PAHs in the different treatments (Frouin et al., 2007), showing that PAHs are not necessarily equally accumulated in the soft tissue of molluscs. The ratio and concentration of total PAH used for *S. glomerata* exposure were based on the results of a monitoring study in the Port Stephens estuary (DPI, unpublished data), and expected to be potentially stressful but not lethal to the exposed oysters. As anticipated, no mortality was observed throughout the exposure trial, showing that the exposure was sublethal. Similar to our study, *Crassostrea virginica* exposed to clay particles spiked with different concentrations of PAHs (64.6, 159 and 242 µg PAHs/g wet clay particles) for 40 days resulted in no mortality (Cruz-Rodríguez and Chu, 2002).

Of the 206,585,654 paired-end raw reads sequenced, 98.7% survived processing (Supplementary Table 1) and were mapped to a previously assembled *S. glomerata* reference transcriptome (Supplementary File 1) for differentially transcript expression analysis.

3.1 Differential transcript expression analysis of PAH samples

A total of 765 *S. glomerata* transcripts were found to be differentially expressed (DE) between control and total PAH exposed *S. glomerata*, using a false discovery rate (FDR) threshold of 0.05. Functional annotation of the DE transcripts with Blast2GO against NCBI's non-redundant database with an e-value cut-off of $1e^{-5}$ resulted in the annotation of 68.5% of the DE transcripts, whereas InterProScan matches were found for 71.4% of DE transcripts when transcripts were searched against the InterProScan database through Blast2GO.

The most common GO-terms of the functionally annotated DE transcripts were associated with cellular and metabolic processes, cell, organelle and membrane, and catalytic activity and binding (Figure 1a, 1b and 1c). While the DE transcripts present only a small section of the *S. glomerata* reference transcriptome, this pattern of GO-terms is comparable to the most common GO-terms found in the transcriptomes of other molluscs (Huan et al., 2012; Zhang et al., 2014). Aside from the main

GO-terms, DE transcripts were also associated with transporter activity, localization, biological regulation, response to stimulus and immune system process, suggesting that the DE transcripts had roles in a variety of processes and functions.

In marine invertebrates, PAH metabolism is divided into phase I (biotransformation) and phase II (conjugation) reactions, with enzymes such as cytochrome P450 (phase I) and glutathione S-transferases (GST) (phase II) involved in the metabolism, where parent compounds are converted into more water soluble metabolites that can be excreted (Bustamante et al., 2012; Livingstone, 1985). Exposure of *S. glomerata* to pyrene and fluoranthene resulted in the 4-fold and higher up-regulation of *cytochrome P450* and *carbonyl reductase* transcripts, while *glutathione S-transferase* and transcripts of the *aldo-keto reductase family* were 4-fold and higher down-regulated (Figure 2). *Cytochrome P450s*, which catalyse the first step of PAH metabolism (Shimada, 2006), were also observed to be induced in *Chlamys farreri* after a 10 day exposure to a mixture of PAHs (Jin et al., 2015), and in *C. gigas* exposed to a hydrocarbon mixture for 7 and 21 days (Boutet et al., 2004). *Carbonyl reductase* (CBR), one of the other two phase I enzymes found to be differentially expressed in *S. glomerata*, are NADPH-dependent short chain dehydrogenases/reductases that are localised to the cytosol and found in a wide range of organisms (e.g. insects and fish) (Forrest and Gonzalez, 2000). These enzymes reduce carbonyl compounds (e.g. aldehyde and keto groups of steroids and biogenic amines) and PAH derived quinones, with the best PAH substrates being *K*-region ortho-quinones from PAHs such as pyrene, benzo(a)pyrene or benzo(a)anthracene (Forrest and Gonzalez, 2000; Zhang et al., 2012), which could contribute to the lower level of pyrene seen in our study. Quinone reduction by CBRs yields hydroquinones that can be conjugated (phase II reaction) to more hydrophilic compounds; however, quinone reduction can also result in redox cycling and the production of reactive oxygen species (ROS) in the absence of the antioxidant superoxide dismutase (SOD) (Forrest and Gonzalez, 2000; Oppermann, 2007; Penning et al., 1999). While no *SOD*, *catalase* or *glutathione peroxidase* were found to be differentially expressed in the PAH-exposed *S. glomerata* of this study, a basic level of antioxidants such as SOD would need to exist in a healthy organism to maintain the balance between ROS produced as a by-product of aerobic respiration and

the level of ROS removed to avoid oxidative stress and its deleterious downstream effects on the organism (Birben et al., 2012; Matés et al., 1999). It is possible that basal antioxidant levels in *S. glomerata* are sufficient to limit the production of ROS during the reduction of quinones by CBR. In addition, two transcripts that encode proteins which are part of complex II (*succinate dehydrogenase cytochrome b560 subunit*) and complex III (*cytochrome b-c1 complex subunit 7*) of the mitochondrial respiratory chain were 4-fold and higher down-regulated in PAH-exposed *S. glomerata* (Supplementary Table 2). Although complex I and complex III are the key sites of ROS production in the mitochondrial respiratory chain, complex II has also been shown to produce ROS in mammals (Chen et al., 2003; Lenaz, 2001). Down-regulation of components from complex II and complex III in PAH challenged *S. glomerata* might be a protective measure to restrict the amount of ROS produced during PAH exposure, as limiting ROS production could preserve energy that the oyster would have to invest otherwise into antioxidant defence.

Similar to *CBR*, *aldo-keto reductases* (AKRs), the genes encoding the other NADP(H)-dependent phase I enzymes (Oppermann, 2007) that were found to be down-regulated in response to PAH in our study, can also lead to futile redox cycling. These enzymes reduce aldehydes and ketones, as well as catalyse the oxidation of PAH-derived *trans*-dihydrodiols to ketols that then rearrange to catechols, which in turn can autooxidise first to *o*-semiquinone anion radicals in an one-electron oxidation and then to *o*-quinones in a second one-electron oxidation (Jin and Penning, 2007; Oppermann, 2007; Penning et al., 1999; Shimada, 2006; Zhang et al., 2012). In the presence of NADPH, PAH *o*-quinones can be reduced to catechols, establishing a futile redox cycle that amplifies the ROS produced during each of the one-electron oxidation steps (Jin and Penning, 2007; Zhang et al., 2012). While conjugation of PAH hydroquinones, *o*-quinones and catechols is an important step in PAH metabolism, thio-conjugated *o*-quinones and hydroquinones conjugated with glutathione are still able to redox-cycle and produce ROS (Forrest and Gonzalez, 2000; Zhang et al., 2012). These ROS can cause DNA damage and production of reactive lipid aldehydes through lipid peroxidation and glycoxidation (Ellis, 2007; Jin and Penning, 2007; Oppermann, 2007; Penning et al., 1999), whereas *o*-quinones can react with proteins, DNA and RNA (Jin and Penning, 2007; Zhang et al., 2012).

Although lipid peroxidation and DNA damage were not measured in the *S. glomerata* of this study, increased lipid peroxidation was found in the gill and digestive tissue of *Ruditapes decussatus* 14 days after translocation to a PAH contaminated area (Bebianno and Barreira, 2009), and significant DNA damage was observed in *Mytilus galloprovincialis* exposed to benzo(a)pyrene for 72 h (Banni et al., 2010). Increased lipid peroxidation, along with decreased cell membrane stability was seen in *Pecten maximus* exposed to 200 μg/L phenanthrene for 7 days (Hannam et al., 2010), and high protein carbonyl levels were found in *M. galloprovincialis* collected after an oil spill (Sureda et al., 2011). High levels of protein carbonyl, increased lipid peroxidation and a high rate of DNA protein crosslinks were observed in *C. farreri* grown at a metal and PAH contaminated site (Liu et al., 2012). Furthermore, while lipid peroxidation was not significantly changed in *S. glomerata* exposed to metal and PAH contaminated estuaries for four weeks, an increase in lysosomal membrane destabilisation was observed (Edge et al., 2012). Reactive carbonyls (e.g. reactive aldehydes) produced by lipid peroxidation and glycoxidation may affect the function of enzymes and other proteins, impact signalling pathways, damage DNA, trigger apoptosis and lower intracellular glutathione levels (Ellis, 2007). Enzymes such as CBR and AKR, which are able to reduce aldehydes to alcohols (Ellis, 2007), protect the oysters from harmful downstream effects of ROS production. Based on the expression pattern of *CBR* and *AKR* transcripts (*CBR* up-regulated, *AKRs* down-regulated) in PAH stressed *S. glomerata*, the oysters appear to preferentially activate *CBR* over *AKRs* in response to the stressor. As both enzymes are able to contribute to the metabolism of PAH and deal with the effects of oxidative stress, it is possible that a) *CBR* induction is a specific response to the combination of pyrene and fluoranthene and b) increasing the expression of only one type of enzyme allows *S. glomerata* to preserve NADPH and limit the amount of ROS produced during the seven day exposure to PAH.

Furthermore, members of the cytochrome P450 family (CYP3A and CYP4A) are also able to oxidise aldehydes produced by lipid peroxidation (Ellis, 2007), and the up-regulation of both *cytochrome P450* and *CBR* in PAH stressed *S. glomerata* might have been sufficient to cope with the effects of oxidative stress. Similar to our study, the expression of *CBR1* was up-regulated in human lung tissue

and lung cancer cells that had been exposed to the PAH benzo(a)pyrene (Kalabus et al., 2012), and in juvenile rainbow trout exposed to silver nanoparticles for 96 h (Gagné et al., 2012). In addition, up-regulation of *CBR1* in the rainbow trout was significantly correlated with an increase in lipid peroxidation (Gagné et al., 2012). Up-regulation of the *S. glomerata CBR* transcript of our study in response to total PAH might serve a similar dual purpose of PAH metabolism and reduction/detoxification of carbonyl compounds produced by lipid peroxidation. Aside from lipid peroxidation, DNA damage has also previously been observed in molluscs and although DNA damage was not measured in the PAH exposed *S. glomerata* of this study, one transcript putatively coding for deltex-3-like protein (DTX3L or BBAP) and two out of three *uracil-DNA glycosylase* transcripts were 4-fold and higher up-regulated in PAH stressed oysters (Supplementary Table 2), suggesting that DNA damage might have occurred to some degree during the PAH stress exposure. In mammals, the expression of BBAP, which protects cells from DNA damaging agents, was induced in response to DNA damage and has been shown to localize to the area of the damage (Yan et al., 2009; Yan et al., 2013). Uracil-DNA glycosylase, on the other hand, is involved in base excision repair where it identifies and excises single base DNA lesions caused by oxidative damage (Bridge et al., 2014; Svilar et al., 2011). These protective mechanisms might allow the PAH stressed *S. glomerata* to counteract the deleterious effect of ROS on its DNA.

Aside from phase I enzymes, phase II enzymes such as GSTs are also involved in PAH metabolism. In this study, one transcript putatively coding for a *pi-class GST* was found to be down-regulated in PAH exposed *S. glomerata*. A similar down-regulation of *GSTs* has been observed in *C. farreri* exposed to benzo(a)pyrene for ten days (Cai et al., 2014) and in *C. gigas* exposed to a mixture of hydrocarbons for 3 weeks, where two *GSTs* were down-regulated and one *GST* up-regulated after seven days of exposure (Boutet et al., 2004). Contradictory to our results, *GST* was up-regulated in *C. farreri* exposed to a mixture of PAHs for ten days (Jin et al., 2015), and in the gill of *Crassostrea brasiliana* exposed to 1000 μg/L of phenanthrene for 24 h, where two out of four *GSTs* were up-regulated, with no significant difference in the expression of the *GSTs* from the digestive gland of these oysters (Lüchmann et al., 2014). The range of *GST* expression patterns seen in the different

molluscs might be due to differences in the concentration, tissue analysed, length of exposure and type of PAH or PAH mixture the animals were exposed to. Down-regulation of *GST* in the *S. glomerata* of this study, suggests that a) glutathione, the GST conjugate, might have been depleted after seven days of exposure to PAHs, affecting *GST* expression, or b) *S. glomerata* temporarily down-regulated *GST* expression to limit the possibility of redox-cycling of glutathione conjugated PAH intermediates.

Interestingly, *laccase* that appears to play a role in antibacterial defence in marine organisms (Li et al., 2015) and was also found to be differentially expressed in the *S. glomerata* of this study (Figure 2), has also been implicated in PAH metabolism in fungi. For instance, a laccase of *Trametes versicolour* was shown to oxidise a range of PAHs (e.g. acenaphthylene, anthracene, fluoranthene and pyrene) *in vitro* (Majcherczyk et al., 1998), and a commercial laccase was able to metabolise 1-hydroxypyrene (a metabolite of pyrene) *in vitro* (Roper and Pfaender, 2005). It is unclear whether *S. glomerata laccases* found in our study might have a similar ability to metabolise/detoxify pyrene and fluoranthene *in vivo*.

Closer examination of the DE transcripts also showed a range of transcripts with putative immune functions differentially expressed between control oysters and *S. glomerata* exposed to total PAH (Supplementary Table 2). Some of these transcripts are coding for pattern recognition receptors (PRRs) such as peptidoglycan recognition proteins (PGRPs), C1q domain containing proteins, toll-like receptors (TLRs), c-type lectins, c-type mannose receptors/macrophage mannose receptors, galectin, gram-negative bacteria binding protein (GNBP), scavenger receptor (SR), 2'-5'-oligoadenylate synthase 1 and fibrinogen-related proteins (tenascin and fibrinogen c domain-containing proteins), with overall slightly more *PRRs* (53.1%) down-regulated than up-regulated. These PRRs recognise pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide and peptidoglycan and trigger innate immune responses (Hornung et al., 2014; Lee and Kim, 2007; Wang and Wang, 2013; Yang et al., 2014) to protect the oyster from invading pathogens. An overall down-regulation of pathogen recognition indicates that the innate immune system of PAH exposed *S. glomerata* might be suppressed by the stressor, making the oysters more susceptible to infections. A similar pattern of *PRR* differential expression was seen in *C. gigas*

exposed to a hydrocarbon mixture for up to 21 days (Boutet et al., 2004). After seven days of exposure, a putative *PRR* (similar to *C1q-related factor precursor*) was down-regulated in exposed oysters, whereas 21 days of exposure resulted in the up-regulation of a *lipopolysaccharide and β-1,3-glucan* clone and the down-regulation of a *c-type lectin* (*hepatic lectin*) (Boutet et al., 2004). Aside from *PRRs*, transcripts coding for the antimicrobial peptides big defensin (4-fold and higher) and bactericidal permeability increasing protein (2- to 4-fold) were up-regulated in *S. glomerata* exposed to pyrene and fluoranthene for seven days (Supplementary Table 2). In addition, *cathepsin L* was 4-fold and higher up-regulated and *cathepsin K* less than 2-fold down-regulated in PAH stressed *S. glomerata* (Supplementary Table 2). Both antimicrobial peptides and cathepsins are associated with phagocytosis, which recognises and removes foreign bodies (e.g. bacteria) and dead cells (Flannagan et al., 2012; Underhill and Goodridge, 2012). General up-regulation of these transcripts, in concert with the 4-fold and higher up-regulation of a *SR* transcript, which is also linked to phagocytosis (Flannagan et al., 2012), suggests an up-regulation of phagocytosis in *S. glomerata* in response to PAH exposure, possibly to deal with an increase in cell debris removal or as a pre-emptive measure to maintain the health of the oyster. Comparable to the results of our study, *defensin*, *cathepsin L2* and *cathepsin D* were up-regulated in *Venerupis philippinarum* exposed to benzo(a)pyrene (Liu et al., 2014), and *cathepsin 1*, *procathepsin L* and *cathpesin L like protease precursor* were up-regulated in *C. gigas* exposed to a hydrocarbon mixture, whereas *cathepsin 8* was down-regulated (Boutet et al., 2004). In addition, while *P. maximus* exposed to 200 µg/L phenanthrene for seven days showed decreased phagocytic activity (Hannam et al., 2010), cytotoxicity and phagocytic activity were increased in *M. edulis* challenged with oil-well produced water (Hannam et al., 2009). Another transcript associated with phagocytosis was *deoxyribonuclease-2-alpha* (*DNase II*), which was 4-fold and higher down-regulated in *S. glomerata* exposed to total PAH (Supplementary Table 2). Comparable to our results, *DNase II* expression was also decreased in rat cells exposed to low-level laser irradiation (Wu et al., 2012). DNase II is found in lysosomes and functions in the degradation of engulfed DNA (Seong et al., 2006). Studies in *Drosophila* showed that deficiency of DNase II led to a higher susceptibility to bacterial infection, as well as to an increase in the expression of antimicrobial peptides in flies with reduced DNase II activity (Seong et al., 2006). These results are consistent with

the expression pattern seen in *S. glomerata*, where *DNase II* was strongly down-regulated while antimicrobial peptides were up-regulated in PAH stressed oysters.

Transcripts associated with apoptosis, a mechanism that removes damaged cells without inducing inflammation (Ashe and Berry, 2003; Kiss, 2010), were also found to be differentially expressed in PAH stressed *S. glomerata* (Supplementary Table 2). Of these transcripts, *caspase-7* and *TRPM2* (*transient receptor potential cation channel subfamily M member 2*) were 4-fold and higher up-regulated, while *Bcl-2-like protein*, *inhibitor of apoptosis protein (IAP) family proteins* and *death domain-containing protein cradd* (*CRADD*) were found to be down-regulated in *S. glomerata* exposed to pyrene and fluoranthene. Caspases are involved in apoptotic signalling cascades, with IAP family members playing a role in the inhibition of apoptosis (Ashe and Berry, 2003; Dubrez-Daloz et al., 2008; Kiss, 2010). TRPM2, which has been shown to be activated by hydrogen peroxide, is a Ca^{2+}-permeable cation channel that has been shown to be involved in the induction of cell death in rat cells (Takahashi et al., 2011). CRADD, an adaptor protein, plays a role in the tumor necrosis factor receptor (TNFR) mediated apoptosis pathway where it interacts with RIP (receptor-interacting protein) to promote inflammation and apoptosis (Ashe and Berry, 2003; Thakar et al., 2006). Bcl-2-like protein 13 appears to be an apoptosis inducer and has been indicated to interact with the adenine nucleotide translocator (ANT) leading to the loss of mitochondrial membrane potential and caspase activation (Kim et al., 2012; Zheng et al., 2004). Overall, these results suggest that apoptosis was induced in the PAH stressed *S. glomerata* of this study, which would allow the oysters to clear cells damaged by pyrene and fluoranthene and downstream effects of the PAH exposure; however, long-term PAH exposure may compromise the oyster's survival.

Besides detoxification and immune related transcripts, *heat shock proteins* were found to be differentially expressed in *S. glomerata* exposed to total PAH (Supplementary Table 2). Heat shock proteins (Hsps) are molecular chaperones with roles in protein folding, assembly, translocation and the degradation of misfolded proteins (Craig et al., 1993; Parcellier et al., 2003; Srivastava, 2002) that can be modulated by different stressors such as extreme temperature, heavy metal, PCBs and PAHs (Feder and Hofmann, 1999; Taylor et al., 2013). In our study, two *Hsp70* transcripts, along with three

sacsin transcripts were 4-fold and higher up-regulated in PAH stressed *S. glomerata*, while one *Hsp70*, one *Hsp20* and one *Hsp90* transcript were 4-fold and higher down-regulated in the PAH exposed oysters. Another member of the Hsp family, *78-kDa glucose-regulated protein* (*HSPA5*) that functions in protein folding in the endoplasmic reticulum (Galligan et al., 2014; Suyama et al., 2014), was also found to be less than 2-fold down-regulated in PAH stressed *S. glomerata*. *Sacsins* assist in protein folding (Pespeni et al., 2013) and similar to our study have also been found to be up-regulated in juvenile corals exposed to elevated pCO_2 (Moya et al., 2015). Analysis of *Hsp* expression in *C. gigas* in response to a hydrocarbon mixture showed an up-regulation of *Hsp70* after seven days of exposure and an up-regulation of *Hsp90α* after 21 days of exposure (Boutet et al., 2004), whereas expression of *Hsp70* was down-regulated in *C. farreri* exposed to a PAH mixture for 10 days (Jin et al., 2015) and in *C. farreri* challenged with benzo(a)pyrene for 10 days (Cai et al., 2014). Also, *S. glomerata* exposed to different metals for four days showed a decreased *Hsp70* expression in response to copper, zinc and lead, with no difference in response to cadmium, while *Hsp90* expression was up-regulated under cadmium and zinc exposure but down-regulated when exposed to copper (Taylor et al., 2013). Results of our study suggest that after seven days of exposure to pyrene and fluoranthene, *S. glomerata* Hsp70 proteins were the main proteins involved in the stress recovery process, with Hsp20 and Hsp90 either temporarily suppressed after longer-term PAH exposure to conserve energy or depleted.

Among the DE transcripts were also transcripts with products putatively involved in protein synthesis, with the majority of the transcripts down-regulated (Supplementary Table 2). One of these transcripts codes for nuclear respiratory factor-1 (NRF-1), and was 4-fold and higher down-regulated in *S. glomerata* exposed to total PAH. NRF-1 is a nuclear transcription factor with a role in the transcription of a range of mitochondrial genes (e.g. components of the mitochondrial respiratory chain and mitochondrial transcription factor) (Huo and Scarpulla, 2001; Zhang et al., 2011). The down-regulation of *NRF-1* in PAH stressed oysters might be linked to the down-regulation of respiratory chain complex II and complex III that was also observed, suggesting that the maintenance of the respiratory chain could have been compromised in the stressed *S. glomerata*, potentially leading

to a reduced energy supply. Aside from *NRF-1*, *5-methylcytosine rRNA methyltransferase nsun4* (4-fold and higher down-regulated) and *28S ribosomal protein S29* (4-fold and higher up-regulated) are also associated with the mitochondria. 5-methylcytosine rRNA methyltransferase nsun4 has been shown in mammals to function in the modification of mitochondrial 12S rRNA (small ribosomal subunit) and in combination with mitochondrial transcription termination factor 4 (MTERF4) is involved in the assembly of functional ribosomes (Metodiev et al., 2014). Down-regulation of *Nsun4* as well as *NRF-1* in PAH-exposed *S. glomerata* indicates that expression of mitochondrial genes might be further compromised in these oysters. In comparison, 28S ribosomal protein S29 (DAP3) plays a role in mitochondrial protein synthesis and has also been shown to be involved in apoptosis (TNF-α and Fas-induced apoptosis) in mammals (Kolanczyk et al., 2011; Suzuki et al., 2001). Based on the function of DAP3 in mammals, it is possible that the up-regulation of *DAP3* in the PAH stressed *S. glomerata* might be linked to the overall induction of apoptosis that was also observed in these oysters.

Several transcripts linked to transcription initiation were also found to be differentially expressed in PAH-exposed *S. glomerata*. Transcription initiation involves a pre-initiation complex (PIC) that consists of a DNA template, RNA polymerase II, general transcription factors (e.g. TFIID) and Mediator (Kandiah et al., 2014). TFIID, one of the general transcription factors, is comprised of the TATA-binding protein (TBP) and multiple TBP-associated factors (TAFs) such as TAF6, with human TAFs recognising and interacting with promoter elements (Kandiah et al., 2014; Sadhale et al., 2007). One transcript putatively coding for transcription initiation factor TFIID subunit 6 (TAF6) was found to be 4-fold and higher down-regulated in *S. glomerata* in response to total PAH. Based on TAF function in humans, down-regulation of *TAF6* in *S. glomerata* might affect the recognition and/or interaction with specific promoter elements, potentially impacting on transcription and downstream gene expression in the stressed oysters. PAH exposure of *S. glomerata* also resulted in the down-regulation of one transcript coding for mediator of RNA polymerase II transcription subunit 12 (MED12) and one coding for MED15, while two *MED15* transcripts were 4-fold and higher up-regulated. Subunit MED12, as well as MED15 are part of the Mediator complex that functions as a

master coordinator with a multitude of functions (e.g. role in transcription initiation, elongation, termination and mRNA processing) (Allen and Taatjes, 2015; Yin and Wang, 2014). Biological function of the Mediator complex can be affected by changes in the subunit composition (subunit loss or addition), with some subunits seen to be over- or under-represented in human and yeast cells (Allen and Taatjes, 2015). Aside from transcription factors, the spliceosome can also interact with the Mediator complex (Yin and Wang, 2014). *Gem-associated protein 5* (*Gemin5*) is a transcript associated with the spliceosome and was 4-fold and higher down-regulated in PAH stressed *S. glomerata*. The spliceosome functions in pre-mRNA splicing and its major components are small nuclear ribonnucleoprotein particles (snRNPs) that are composed of small RNAs, Sm proteins and proteins unique to each snRNP. Gemin5 is a component of the SMN (survival motor neuron) complex that assembles the Sm protein cores and brings both, Sm proteins and snRNAs together. Studies in mammals have shown that Gemin5 has a key role in snRNA recognition as it can bind directly and specifically to snRNAs (Battle et al., 2006; Yong et al., 2010). It is possible that down-regulation of *Gemin5* in PAH exposed *S. glomerata* could affect the specificity of the SMN complex and its ability to assemble SM protein cores, with downstream effects on pre-mRNA splicing. Changes seen in the transcription factor TFIID and the SMN complex of PAH exposed *S. glomerata* might also be responsible for the differential transcript expression of the *MED12* and *MED15* subunits of the Mediator complex, causing an adjustment of the subunit composition of the Mediator complex and potentially affecting the biological function of the complex.

Other *S. glomerata* transcripts putatively linked to protein synthesis were *ribosomal RNA processing protein 36 homology* (*RRP36*), *26S proteasome non-ATPase regulatory subunit 5, symplekin, elongation factor 2 kinase, eukaryotic translation initiation factor 2 subunit 2* and *peptidyl-prolyl cis-trans isomerase FKBP14*. *RRP36* (2- to 4-fold down-regulated) has been shown to function in early cleavages of pre-rRNA in yeast and mammalian cells, with RRP36 depletion resulting in decreased levels of mature 18S rRNA (Gérus et al., 2010). While levels of mature 18S rRNA were not measured in our study, it is conceivable that the decrease in *RRP36* transcript expression might have a similar effect on 18S rRNA in exposed *S. glomerata* as was observed in yeast and mammalian cells, with

potential down-stream effects on protein synthesis. Protein degradation, in turn, might be affected by the 4-fold and higher down-regulation of *S. glomerata 26S proteasome non-ATPase regulatory subunit 5* which is a regulatory component of the proteasome that catalyses protein degradation (Pittà et al., 2013).

Another transcript with a regulatory role was *elongation factor 2 kinase* (*eEF2 kinase*), which was 4-fold and higher down-regulated in *S. glomerata* exposed to pyrene and fluoranthene. This kinase catalyses the phosphorylation of elongation factor 2 (eEF2), inhibiting eEF2's ability to bind ribosomes and to mediate ribosome movement along the mRNA during peptide elongation (Browne et al., 2004; Ryazanov et al., 1997). Decreased *eEF2 kinase* transcript expression in *S. glomerata* in response to total PAH might be a compensatory measure of the oyster to maintain a necessary level of protein synthesis of important genes in an environment that appears to promote a general suppression of protein synthesis. *Symplekin*, a component of the polyadenylation complex with an essential role in canonical and histone pre-mRNA processing (Kennedy et al., 2009), was also 4-fold and higher down-regulated in PAH exposed *S. glomerata*. *Peptidyl-prolyl cis-trans isomerase FKBP14* aids and accelerates the folding of nascent proteins (Pittà et al., 2013), and its 4-fold and higher up-regulation in PAH exposed *S. glomerata* indicates that even though many of the protein synthesis and modification processes were impaired, energy was extended to ascertain that the expressed proteins were correctly folded and could carry out their respective functions. Overall, it appears that a range of protein synthesis mechanisms of *S. glomerata* have been negatively affected by the seven day exposure of the oysters to pyrene and fluoranthene.

4. Conclusions

This study analysed the molecular effects of the combined exposure of pyrene and fluoranthene on *S. glomerata*, using RNA-Seq. Overall, slightly more transcripts were down-regulated than up-regulated, with the expression pattern of immune related transcripts suggesting that the PAH treatment resulted in a suppression of pathogen recognition, and an induction of phagocytosis and

apoptosis. These results indicate that PAH stressed *S. glomerata* concentrated on responding to the internal effects of PAH exposure rather than extending additional energy into protecting itself from external pathogens, therefore potentially increasing its susceptibility to infections. Aside from immune related transcripts potentially responding to the downstream effects (e.g. lipid peroxidation, DNA damage) of PAH exposure/detoxification, several transcripts involved in phase I and phase II PAH detoxification were differentially expressed in PAH stressed *S. glomerata*. Responses indicate that *S. glomerata* employ a small set of enzymes to carry out pyrene and fluoranthene detoxification and to limit downstream effects of ROS potentially produced through redox cycling during the detoxification process. PAH exposure also appears to have affected stress protective mechanisms (heat shock proteins) and protein synthesis, with PAH stressed *S. glomerata* potentially adjusting their protein synthesis machinery to preserve energy during the prolonged exposure to pyrene and fluoranthene while maintaining the necessary expression levels of essential genes. In summary, *S. glomerata* fed with pyrene and fluoranthene for seven days showed a complex molecular response to the stressors, with the oyster appearing to divert energy from general protective and homeostasis mechanisms into processes that allow the oyster to deal with the stressor and its downstream effects. Furthermore, insights gained from this RNA-Seq study could aid future proteomic and metabolomic studies by highlighting areas of interest for a targeted approach to obtain a more comprehensive picture of the stress response of oysters to PAHs.

Authors' contributions

NGE: designed the study, prepared feed, carried out the exposure experiment, collected samples, carried out all relevant laboratory and bioinformatics work, as well as the differential gene expression analysis, drafted manuscript. WAO: designed study, advised on the experimental design, provided oysters and necessary equipment for the experiment, reviewed manuscript. PB: advised on experiment and chemical analysis of the soft-tissues, prepared feed. MK: determined chemical analysis method and carried out the chemical analysis of the oyster soft-tissues. AE: designed study, reviewed manuscript. All authors have read and accepted the manuscript.

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Figures

Figure 1 GO analysis of *S. glomerata* DE transcripts.

GO-terms were determined with Blast2GO, using default parameters. Level 2 GO-terms are associated with **a)** biological process, **b)** cellular component and **c)** molecular function are depicted.

Figure 2 Heatmap of *S. glomerata* DE transcripts putatively involved in PAH metabolism.

Heatmap shows the posterior fold change values of the DE transcripts putatively involved in PAH metabolism, with transcripts depicted in red up-regulated and transcripts in green down-regulated in oysters exposed to pyrene and fluoranthene when compared to control oysters.

Fig.1

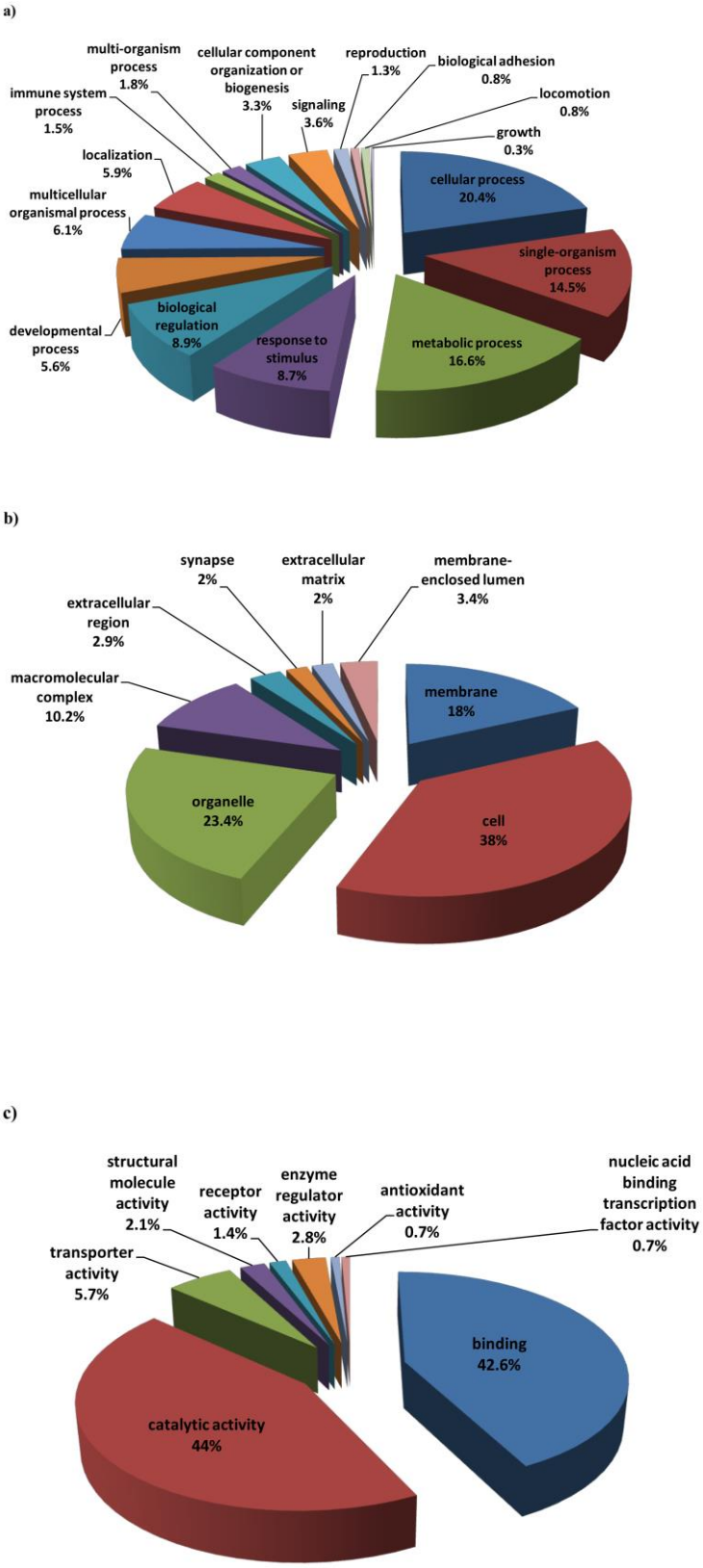
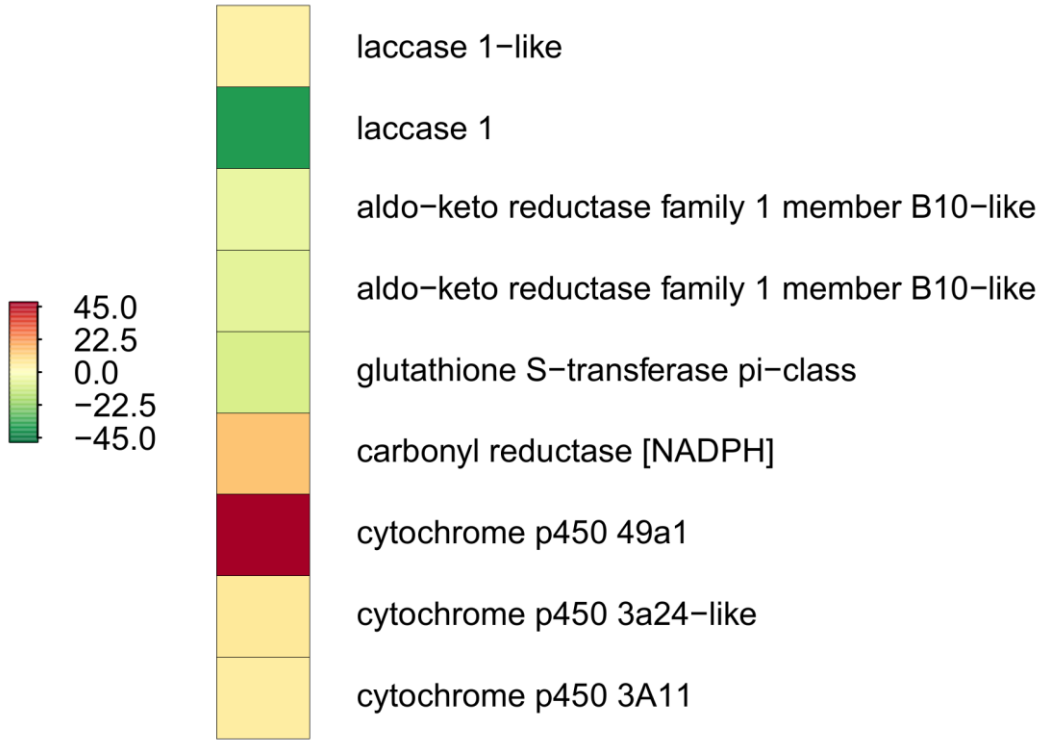


Fig.2



Tables

Table 1 Levels of pyrene and fluoranthene in *S. glomerata* soft-tissue.

Sample ID	Treatment	Concentration (µg/kg)	
		Fluoranthene	Pyrene
475	control	below LOQ	below LOQ
476	control	below LOQ	below LOQ
484	control	below LOQ	below LOQ
485	control	below LOQ	below LOQ
486	control	below LOQ	below LOQ
487	control	below LOQ	below LOQ
481	total PAH	115.5	76.3
482	total PAH	132.5	56.9
490	total PAH	128.5	85.3
492	total PAH	168.6	96.9
499	total PAH	195.3	106.9
500	total PAH	124.3	120.4

*Limit of quantitation (LOQ) = 50 µg/kg

Supporting information

Supplementary Figure 1 Variance versus mean plot for each Ng group (C1).

This plot shows the mean-variance relationship (using polynomial regression) for each isoform (Ng) group of condition 1 (control samples). Mapping ambiguity clusters were produced with RSEM (rsem-generate-ngvector), while the plot was visualised in R using EBSeq's PolyFitPlot function.

Supplementary Figure 2 Variance versus mean plot for each Ng group (C2).

This plot shows the mean-variance relationship (using polynomial regression) for each isoform (Ng) group of condition 2 (total PAH samples). Mapping ambiguity clusters were produced with RSEM (rsem-generate-ngvector), while the plot was visualised in R using EBSeq's PolyFitPlot function.

Supplementary Figure 3 Quantile-quantile plot.

QQ-plots show the fitted Beta prior distributions within each condition and each Ig group (uncertainty group) and were visualised in R using EBSeq's QQP function.

Supplementary Figure 4 Density plot.

Plot shows the prior distribution fit within each condition and each Ig group, visualised in R using EBSeq's DenNHist function.

Supplementary Table 1 Bowtie alignment statistics.

Table shows the number of raw reads and reads surviving the processing, with the total alignment percentage based on the post-processed reads aligning to the *S. glomerata* reference transcriptome, using Bowtie.

Supplementary Table 2 *S. glomerata* DE transcripts.

List of 765 DE transcripts determined with Bowtie-RSEM-EBSeq, using a FDR threshold of 0.05. Sequence descriptions are based on blast homology searches against the NCBI nr database (e-value cut-off: 10^{-5}, hit number threshold: 25), and on InterProScan domain/family information. Posterior fold change (FC) was based on the normalised data, whereas real FC was based on the raw data. C1 stands for control, C2 for treatment condition.