

Hepatic gene expression in flounder chronically exposed to multiply-polluted estuarine sediment: Absence of classical exposure ‘biomarker’ signals and induction of inflammatory, innate immune and apoptotic pathways.

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Abstract

The effects of chronic long term exposure to multiply-polluted environments on fish are not well understood, but environmental surveys suggest that such exposure may cause a variety of pathologies, including cancers. Transcriptomic profiling has recently been used to assess gene expression in European flounder (*Platichthys flesus*) living in several polluted and clean estuaries. However, the gene expression changes detected were not unequivocally elicited by pollution, most likely due to the confounding effects of natural estuarine ecosystem variables. In this study flounder from an uncontaminated estuary were held on clean or polluted sediments in mesocosms, allowing control of variables such as salinity, temperature, and diet. After 7 months flounder were removed from each mesocosm and hepatocytes prepared from fish exposed to clean or polluted sediments. The hepatocytes were treated with benzo(a)pyrene (BAP), estradiol (E2), copper, a mixture of these three, or with the vehicle DMSO. A flounder cDNA microarray was then used to measure hepatocyte transcript abundance after each treatment. The results show that long term chronic exposure to a multiply-polluted sediment causes increases in the expression of mRNAs coding for proteins of the endogenous apoptotic program, of innate immunity and inflammation. Contrary to expectation, the expression of mRNAs which are commonly used as biomarkers of environmental exposure to particular contaminants were not changed, or were changed contrary to expectation. However, acute treatment of hepatocytes from flounder from both clean and polluted sediments with BAP or E2 caused the expected changes in the expression of these biomarkers. Thus transcriptomic analysis of flounder exposed long-term to chronic pollution causes a different pattern of gene expression than in fish acutely treated with single chemicals, and reveals novel potential biomarkers of environmental contaminant exposure. These novel biomarkers include Diablo, a gene involved in apoptotic pathways and highly differentially regulated by both chronic and acute exposure to multiple pollutants.

1. Introduction

Estuarine sediments are sinks for chemical pollutants present in industrial and domestic effluents, and the health of fish which live in polluted estuaries is of concern. The European flounder lives in close association with estuarine sediments and is a key sentinel species for biological effects monitoring in initiatives such as the OSPAR Joint Assessment

and Monitoring Programme (OSPAR, 2003). Such biological effects monitoring programmes have concentrated on the use of biochemical, molecular, histopathological and morphological biomarkers to indicate exposure to harmful pollutants (van der Oost et al., 2003). The development of these biomarkers has most often been based on identifying endpoints which vary after single acute experimental exposure to model pollutants, such as polycyclic aromatic hydrocarbons (PAH), halogenated aromatic hydrocarbons, estrogenic chemicals, and metals. However, changes in these markers are not always observed in field surveys, even in fish exposed to high levels of contaminants (Eggens et al., 1996) (Reynolds et al., 2003).

The development of transcriptomic techniques for massively parallel, simultaneous measurement of expression of several thousands of genes may provide a means to assess, not only exposure but also mechanisms of action and effects of environmental contaminants. Transcriptomic profiling with targeted microarrays has been used to assess the effects of exposure to polluted sediments and in some of these studies it has been possible to obtain a toxicologically relevant signal from amongst the transcripts which were changed. Thus, when comparing a highly PAH-contaminated environment with a 'clean' reference site, flounder transcripts characteristic of acute PAH-response were changed (Williams et al., 2003). Similarly, when comparing hornyhead turbot (*Pleuronichthys verticalis*) from a site receiving an estrogenic effluent with a clean site, a signal characteristic of acute estrogen exposure was observed (Baker et al., 2009). However, in a larger study, involving several European estuaries of differing pollutant status, and using a more comprehensive microarray, a transcriptomic signal of pollutant effect was less clear (Falciani et al., 2008). These results demonstrated that gene expression profiles depended on site, and that the relative expression levels of a subset of 17 genes could predict the source of flounder with high accuracy. However, this subset only contained one currently described biomarker, CYP1A, and furthermore transcriptomic profiling of flounder experimentally exposed to acute doses of model pollutants (Williams et al., 2008) showed large differences to gene expression profiles in fish from polluted environments. These results have general similarities to studies on populations of *Fundulus heteroclitus*, a fish which inhabits severely contaminated estuarine sites in North America. Comparing gene expression profiles of *F. heteroclitus* from several polluted and unpolluted sites indicates that in each of the polluted populations 5 to 17% of genes are differentially expressed, yet only two genes had a consistent difference in expression among all polluted sites (Fisher and Oleksiak, 2007). Also notable was an apparent under-representation of the type of biomarker genes which might have been expected to be differentially expressed between polluted and unpolluted sites, although in the case of *F. heteroclitus* there appears to be a heritable resistance to the effects of some contaminants, which is believed to prevent CYP1A induction for example (Oleksiak, 2008).

It is perhaps not surprising that comparing gene expression in different populations of fish from different locations is not straightforward. Estuaries in particular are highly variable environments and it is possible that pollutant responses are masked by large changes in expression of other genes that may be responsive to variables such as salinity, temperature, feeding, and, as shown with *F. heteroclitus*, population differences. It is also possible that the long-term response to living on multiply-polluted sediments differs from the response to acute, short-term, single-chemical exposures which have been used to identify many of the current commonly used molecular and biochemical biomarkers. The aim of the study reported here was to compare the transcriptomic effects of chronic exposure to a real polluted estuarine sediment with a real 'clean' sediment in a single population of flounder. This was achieved by chronic exposure in a controlled mesocosm study designed to eliminate many of the variables present in different estuaries, followed by microarray and quantitative PCR measurement of gene expression in isolated hepatocytes from exposed and

unexposed fish. The use of isolated hepatocytes provided a means to directly compare the transcriptomic effects of acute exposure to prototypical pollutants with long-term chronic exposure to sediments, whilst minimizing the effects of natural genetic and environmental variation in outbred wild populations of fish.

2. Methods

2.1. Experimental animals

Juvenile (0-group) flounder, were collected by beam trawl at low tide in May-June from the Ythan estuary (NE Scotland). Benthic invertebrates (mostly *Tellina* and *Macoma* bivalves, together with some polychaete worms) were collected by wet sieving (4 mm) from clean sediment at the Ythan estuary, Loch Ewe (NW Scotland), and at Balmerino on the Firth of Tay estuary (E Scotland).

2.2. Mesocosm Establishment

The mesocosms consisted of four 1.5 m tanks contained 4-5 cm of test sediment over ~3 cm of filter sand, covering ~5 cm of filter gravel, on top of perforated, corrugated plastic sitting on the base of the tank. Four air-riser pipes per tank ensured a circulation of water down through the sediment and up the air-riser in order to prevent anoxia of the sediment, reduce sediment resuspension, and lessen water turbidity. Each mesocosm was supplied with ~100 L per hour of carbon-filtered 50% seawater (salinity = 17gL⁻¹).

Three bulk sediments were collected for use in the mesocosm experiments. Two contaminated sediments were obtained from dredged material from the estuary of the River Tyne, Riverside Quay, Jarrow, and also sediment collected by van Veen grab from the Firth of Forth, adjacent to the Grangemouth petrochemical complex. A clean reference sediment was manually collected from the estuary of the River Ythan, in North East Scotland, adjacent to the site of flounder collection. Each of the sediments was thoroughly mixed, and stored as large (~75 kg) aliquots that were held in plastic packing crates within a commercial frozen storage facility until required.

Prior to introducing flounder to the tanks, the bulk Forth and Tyne sediments were combined (FT). This sediment and the reference Ythan (Y) sediment were each thoroughly mixed (75:25) with filter sand, and aliquoted/stored as described above. The filter sand was added to equalize the grain size of all sediments and to reduce the likelihood of the sediment becoming anoxic during the test. The final test sediments, ie mixed and with filter sand added, are hereafter referred to as 'clean' and 'polluted'.

The test sediments were added to duplicate tanks in May and benthic invertebrates were added 30 days later. After a further 30 days 60 0-group flounder (3.1±1.1 g, 56±6 mm) were added to each tank. Benthic *in situ* invertebrate prey species were supplemented daily with frozen mysid shrimp (at a rate of ~1% of body weight/day) and once a week with commercial pellets (Bio-Optimal START 1.5mm, Biomar, 1% of body weight/week). Water temperature and salinity were recorded weekly. After three months, an additional 1-2 cm of test sediment was added to each mesocosm and the mesocosm-exposed fish were sampled after 7 months of exposure to the sediments.

Five aliquots from each of the 'clean' and 'polluted' sediments were collected for chemical analysis at the point of first addition to the tanks.

Tanks were regularly inspected for mortalities and dead fish removed. After 7 months, surviving fish were counted and taken for biometric, chemical and biological analyses. Salinity over the course of the experiment was 19.8±1.5‰ (mean of weekly measurements from each of the 4 tanks) and temperature was 12.3±2.7°C (mean of weekly

measurements from each of the 4 tanks), ranging from a maximum of 16°C to a minimum of 8°C, in accordance with the normal environmental variation over the period.

2.3. Sediment chemistry

Unless stated otherwise, chemical analyses were accredited to ISO/IEC 17025:2005 by the UK Accreditation Service.

2.3.1. Determination of sediment characteristics

The particle size distributions of freeze dried test sediments were determined using a laser granulometer, after initial sieving to 2 mm. The concentrations of Total, Inorganic, and Organic Carbon (TC, TIC, TOC) in freeze dried sediments, were determined using an elemental analyser. Finely ground sediment samples were analysed either without acidification (TC), or following the addition of hydrochloric acid (TOC). TIC was determined as the difference between the two.

2.3.2. Determination of trace metal concentrations

Freeze dried samples of test sediment were sieved (2 mm) and the fine material ball-milled before being digested in a closed vessel microwave using concentrated nitric acid. Fish samples were homogenised and similarly digested. Sample digests were diluted, internal standards added, and trace element concentrations determined by Inductively Coupled Plasma Mass Spectrometry (ICPMS). Concentrations of Cr, Ni, Cu, Zn, As, Cd, Hg, and Pb were determined in sediments and flounder muscle and liver; additionally Mn, Co and Se were also determined in flounder muscle and liver.

2.3.3. Determination of polycyclic aromatic hydrocarbons (PAHs)

Hydrocarbons, including PAHs, were extracted from wet test sediment by sonication in dichloromethane and methanol, and from flounder muscle and liver by saponification. The extracts were purified and the PAHs separated from the aliphatic hydrocarbons using high performance liquid chromatography (HPLC). Quantitative analysis of 40 two- to six-ring (parent and branched) PAHs was carried out by gas chromatography with mass selective detection (GC-MSD, (Webster et al., 2009) (Webster et al., 2004). Results are presented as total naphthalenes (naphthalene + 1-methyl naphthalene + 2-methyl naphthalene + C2,C3,C4 naphthalenes), total of the 40 PAH compounds, or as total benzo[a]pyrene-equivalent PAH concentrations (derived from the function of individual compound concentration and the Toxic Equivalency Factor (TEF), for the 16 PAH compounds for which TEFs are available.

2.3.4. Determination of chlorinated biphenyls (CBs) and organochlorine pesticides (OCPs)

CBs and OCPs were extracted from freeze dried sediments and dehydrated samples by either soxhlet extraction using methyl-tert-butyl ether (MTBE) as the solvent or by Pressurised Liquid Extraction (PLE) using iso-hexane and with 5% deactivated alumina in the PLE cells as a fat retainer to partially clean-up the samples. Concentrated extracts were further cleaned-up and OCPs and CBs separated with deactivated silica and the concentrations of 28 CB congeners and 20 OCPs determined by gas chromatography with electron capture detection (GC-ECD; (Webster et al., 2009)). The determined OCPs were o,p' DDE, p,p' DDE, o,p' DDD, p,p' DDD, o,p' DDT, p,p' DDT, α -chlordane, γ -chlordane, oxychlordane, α -chlordane, γ -chlordane, total heptachlor, heptachlor epoxide, trans-nonachlor, dieldrin, aldrin, endrin, α -HCH, γ -HCH, and hexachlorobenzene (HCB). Concentrations of individual CBs were determined and the reported results also included the total concentration of all the CBs monitored and the total of the "ICES7" CBs (sum of CBs

28, 52, 101, 118, 138, 153 and 180). Individual OCP concentrations were often below detection limits; data for those compounds most-often detected are presented (the sum of DDT and its metabolites, HCB) together with the sum of all OCP compounds.

2.3.5. Determination of organotin compounds

Freeze dried test sediment samples were sieved (2 mm) and the fine material ball-milled before organotin compounds were extracted using acetic acid and methanol. Extracted organotins were derivatised using sodium tetraethylborate and the ethylated organotins simultaneously re-extracted into iso-hexane. The iso-hexane extracts were cleaned-up with deactivated silica and the concentrations of ethylated di-, and tri-butyltin were determined by GC-MSD.

2.3.6. Determination of polybrominated diphenylethers (PBDEs)

PBDEs were extracted from freeze dried test sediments and dehydrated samples by PLE using iso-hexane as solvent. The concentrated extracts were cleaned-up and the concentrations of 17 (tri- to deca-brominated) PBDE congeners determined by GC-MSD (Webster et al., 2009).

2.4. Hepatocyte preparation and treatment

The overall experimental scheme is shown in Figure 1. Four male fish and two female flounder from each test group ('clean' and 'polluted') were selected randomly, by netting all fish into a holding tank and sampling the first four males and two females to be sub-sampled. These fish had lengths between 12 and 16.3cm. The skin of the fish was swabbed with 10% FAM disinfectant before removing livers. A sub-sample of each liver was frozen in liquid N₂ for individual analyses. The remainder of each liver was finely chopped and hepatocytes prepared as described previously (Leaver et al., 2007). Following hepatocyte preparation washed cell pellets were resuspended in 10ml of DMEM, 10%FBS, PSK and cell concentration determined in each preparation using a hemocytometer. Two pools were then made containing equal numbers of cells originating from each of the six flounder from each 'clean' or 'polluted' group. These pools were then distributed to six-well polystyrene culture

dishes to provide 10⁶ cells in 2ml in each well. Culture dishes were incubated for 2 hours at 12°C, 4% CO₂ before adding 5µl of DMSO or 5µl DMSO containing 20mM benzo(a)pyrene (BAP), 0.4mM estradiol (E2) or 4mM copper sulphate (Cu), to give final exposure concentrations of 50µM BAP, 1µM E2 and 10µM Cu. Hepatocytes were also treated with a mixture of all three compounds (MIX), each at the concentrations stated above. All treatments were performed as six replicates. After 40 hours of exposure at 12°C, 4% CO₂, three replicates of each treatment were harvested by aspiration and cells pelleted at 1000g for 3 mins. Cell pellets were immediately resuspended with vigorous vortex mixing in 200µl of RNA extraction solution (TriReagent, Sigma). Cells in the remaining culture dish wells were assessed for viability by counting number of cells excluding Trypan Blue using a hemocytometer and microscope. Viability was between 72 and 81% in all treatments after 40 hours and did not significantly vary between treatments.

2.5. Microarray Hybridisation

Total RNA was prepared from pooled hepatocyte cell pellets using TriReagent RNA extraction buffer (Sigma) according to the manufacturers protocol. Each hepatocyte

treatment consisted of three experimental replicates for microarray hybridisation. Total RNA from individual livers was prepared by homogenising liver fragments in 8 ml/g methanol, 2.5ml/g water. One hundred microlitres of the aqueous phase was then processed for RNA purification using binding to/elution from an anion exchange mini-column (RNeasy Midi Kit, Qiagen). Final RNA concentrations were measured using a Nanodrop spectrophotometer and integrity checked by assessing the relative proportions of 28S and 18S RNA using agarose gel electrophoresis. All samples showed approximately 2 fold more ethidium bromide staining in 28S band than in 18S band, indicating that the samples were not significantly degraded.

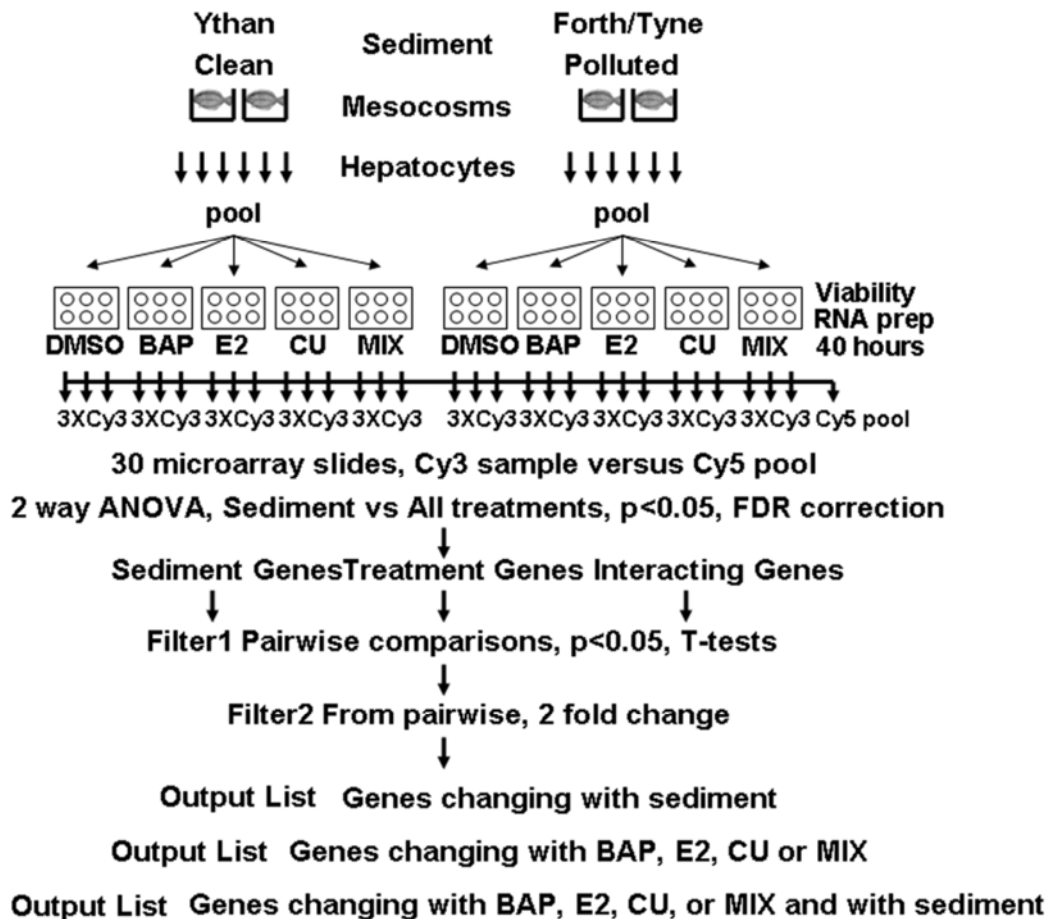


Figure 1. Schematic of experimental design and procedures.

Five hundred nanograms of each total RNA sample was amplified using a commercial amplification and amino-allyl labelling kit (Amino Allyl MessageAmp II aRNA Amplification kit, Ambion). Amplified RNA yield was quantified using a Nanodrop spectrophotometer and 20µg was incubated with mono reactive NHS ester Cy3 dye (GE Healthcare). Labelled, amplified RNA probe was purified according to commercial kit instructions. A pool containing equal amounts of total RNA from each sample was labelled with Cy5 dye using the same method. Yields of labelled, amplified RNAs were quantified using a Nanodrop spectrophotometer.

The construction and validation of the GENIPOL European flounder 16K cDNA microarray used here has been described in detail previously (Williams et al., 2006) (Diab et al., 2008). The array consisted of 27648 elements, which, after contig clustering, resolve into 3336 genes. The array design with accession numbers is available through EMBL ArrayExpress, accession number A-MAXD-12 (<http://www.ebi.ac.uk/microarray>)

as/aer/lob?name=adss&id=1308203622). A universal pooled reference design was used to plan microarray hybridisations (Fig1). Thus, each slide received 80pmol of Cy3 labelled, amplified RNA and 80pmol of Cy5 labelled pooled reference amplified RNA. Hybridisation procedures and solutions have been described previously (Williams et al., 2006).

Table 1. Primers and gene IDs used for QPCR.

Gene	Forward (5'-3')	Reverse (5'-3')	GenBank
β-actin	GACCAACTGGGATGACATGG	GCGTACAGGGACAGCACAGC	AF135499
α-tubulin	CACAGCCTCACTTCGTTTTG	AGATGACAGGGGCATAGGTG	AJ291985
EF1A	GTTGAGCGCAAGGAGGCTAA	CGAGCATACCGGGCTTAATG	EC378684
Cyp1A	CAACCATGATCCAGAGCTGTG	GATTATTCTTCTCCACTGACTCT	AJ132353
UGT1B	CCTTCCCGCAGAGAGTCATA	AGAGAGCCCCATGACTGAGA	AM746199
Vitellogenin	CTGACCTTCGTGGATATTGAG	ATCTGAGCCTCGGCATTG	AJ416327
Metallothionein	CTTGCGAATGCTCCAAGACTG	TGGTGTGCGCATGTCTTCCT	DV565672
Glutathione reductase	GGCTTCTTGCGTGAATGTTGG	CGAGGTTGCTACGATAAATGCG	AJ578037
Hepcidin	TCACCAGCAGAGTCAAAGAACT	ATGCTCAGCAGCTGCATTGT	DV569429
Diablo	CCTCATTCGAGGAGGATTCG	ACTGGGCCACCTGAATGTTT	EC377887
Transthyretin	ACTCCGAGCTGATGATTTGG	CCAAGACCCTCCCAGTATGA	DV566569
Complement C9	GCTCATGCCCTCAAACAGGT	GTTGGACATGCCTGGTGCTA	DV569533
Glutathione S-transferase A	TCACGCTCACCCAGAAAATG	GAAAACCGACACATCAGCCA	DV567811
CuZn superoxide dismutase	TGGAGACAACACAAACGGG	CATTGAGGGTGAGCATCTTG	AJ291980

Following hybridisation, microarray slides were washed, dried and scanned with a Perkin Elmer ScanArray Express microarray slide scanner. Scanned images were first analysed by Bluefuse software (BlueGnome Ltd, UK) which was used to identify features, fuse replicates and quantify Cy3 and Cy5 fluorescent signals from each feature. Bluefuse data was then analysed in Genespring GX7.3.1. (Agilent). To normalise data, all features with less than 0.01 fluorescence units were first discarded, then a Lowess per spot, per chip normalisation was carried out. Features whose sequence data formed contigs (Williams et al., 2006) were treated as on-chip replicates in Genespring.

The entire data set was first analysed by 2 Way ANOVA, implemented in Genespring, using Sediment ('clean' and 'polluted') and Treatment (BAP, EST, CU and MIX) as factors. This provided a list of features whose expression was significantly (with Bonferroni and Hochberg False Discovery Rate correction, FDR $p < 0.05$) dependent on sediment, on treatment, or which exhibited interaction between sediment and treatment. To identify features which were changed by individual treatments or which showed interaction with individual treatment and sediment, the 2 way ANOVA output list were filtered first by pairwise t-tests ($p < 0.05$) and second by 2 fold up- or down-expression.

The probability that a particular biological process GO term was enriched in an output GO list (all GO annotations associated with the experimentally altered gene list) compared to the input GO list (All GO annotations associated with the array) was calculated

using a hypergeometric distribution model as implemented by GeneSpring GX 7.3.1. The GO annotation of the flounder microarray has been described (Williams et al., 2006).

MIAME-compliant microarray data (Brazma et al., 2001) were submitted to ArrayExpress at EMBL-EBI (<http://www.ebi.ac.uk/microarray-as/ae/>) using MaxLoad2 software (Hancock et al., 2005) and have been assigned accession number E-MAXD-55.

2.6. Quantitative real time PCR (QPCR)

QPCR was performed using the treated hepatocyte RNA extracts, or using RNA from the individual hepatocyte source fish. Complementary DNA (cDNA) was synthesized from total RNA using a commercially available kit according to the manufacturers instructions (Reverse-iT Max 1st strand synthesis kit, ABgene). Briefly each reaction of 20 μ L contained 1.0 μ g of total RNA, 300ng of random hexamers and 125ng of anchored oligo-dT. Following cDNA synthesis at 42°C for 1 hour reactions were stopped by heating at 75°C for 10 min and cDNA diluted to 500 μ L total volume with water. QPCR was performed using a Quantica machine (Techne). QPCR analysis for each gene was performed in triplicate in a total volume of 20 μ L containing 5 μ L cDNA (equivalent to 10ng of input RNA), 100nM of each primer and 10 μ L of Absolute QPCR SYBR Green Mix 2X (ABgene). For each of target genes, forward and reverse primers were chosen from the available EST sequences by using Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Target genes were selected by consideration of microarray data and by BLAST comparison of flounder ESTs deposited in Genbank with sequences of mammalian and other fish genes of interest. Primer pairs and EST information are provided in Table 1. Thermal cycling was initiated with incubation at 95°C for 15 min in order to activate the DNA polymerase present in the mix. After this initial step, forty-five cycles of PCR were performed. Each PCR cycle consisted of heating for 15 s at 95°C for denaturing, and then for 15 s at 60°C and 30 s at 72°C for annealing and extension. Cycle threshold (C_T) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit.

Melting curve analysis was performed to indicate the production of a single product in these reactions. Standard curves were established for each gene by plotting C_T values against the log₁₀ of five different dilutions (in triplicate) of cDNA sample solutions. In addition a subset of randomly selected samples generated by each primer pair was analysed by agarose gel electrophoresis and sequencing to confirm the identity of amplicons. Real-time efficiency was determined for each gene from the slopes given by Quantsoft (Techne) software, applying the equation $E=10^{(-1/\text{slope})}$. The calculated relative expression ratio of each gene was based on the PCR efficiency (E) and C_T of sample compared with control, and expressed in comparison to the reference genes, β -actin, α -tubulin and eflA. These genes were not changed in any of the treatments, as observed after microarray analysis. Statistically significant differences in gene expression between the ‘clean’ vehicle (DMSO) treated hepatocytes or fish and all other possible treatment X sediment conditions were evaluated in group means by randomization tests (Pfaffl et al., 2002) using REST© software. Five thousand random allocations were applied and differences were considered to be significant at $P\leq 0.05$.

3. Results

3.1. Flounder Biometry

Forty eight fish in the ‘clean’ mesocosms (40% mortality rate) and twenty-nine fish in the ‘polluted’ mesocosms (24% mortality) died during the experiment. Chi-squared

analysis indicated that mortality of fish on the “clean” sediment was higher than expected (24%) and mortality of fish on the “polluted” sediment was lower than expected (Fisher’s exact χ^2 test, $p=0.012$). There was a tank effect (Pearson χ^2 test, $p<0.001$); whilst tank 1 (‘clean’; 20% mortality) and tank 4 (‘polluted’; 45%) had mortalities not significantly different to those expected (32%), mortalities in tank 3 (‘clean’; 60% mortality) were higher than expected and in tank 2 (‘polluted’; 3.3%) were lower than expected.

In the 7 months between introduction to the mesocosm and sampling, the flounder grew in length and weight, although they were in poorer condition (based on comparison of body wt / L³ x 100, not shown). There were no treatment-related differences between length, weight, or condition (not shown). All were classed as unsexed when introduced, and there were no significant variations from the expected sex 50:50 ratio for either treatment after 7 months. There were no significant treatment-related differences (t-tests) in the gonadosomatic index (GSI; gonad weight / total weight x 100) of fish classed as either male or female after 7 months (not shown). There were no external signs of disease (fin rot, skin lesions or abnormal pigmentation) in fish after 7 months from either treatment.

3.2. Sediment chemistry

The contaminant profiles of each sediment are presented in Table 2. Several different priority chemical pollutants were measured in order to establish the extent to which the contaminated mesocosm represented a typical multiply-polluted estuarine sediment. Despite mixing filter sand with both Tyne/Forth and Ythan sediments, there was still a significant difference in grain size distribution between the final ‘clean’ and ‘polluted’ test sediments in the mesocosms. A difference in total organic carbon (TOC) content between the two sediments was also apparent and therefore results for organic and metal contaminants are presented as absolute values, and as values normalised to either TOC or grain size. Irrespective of normalisation, the ‘polluted’ mesocosm sediment showed significantly higher values for most contaminants than the ‘clean’ sediment. The exception was that the ‘clean’ sediment was more contaminated with “Total OCPs” than the ‘polluted’ sediment, and that normalised concentrations of Cd and some PAHs (total “NOAA n=14 PAH” were not significantly different between the two sediments. PAH ratios (not shown) indicated that the PAH contamination of both sediments was predominantly pyrolytic in origin (Webster et al., 2004).

In several cases sediment contaminant concentrations exceeded environmental quality standards. OSPAR Background Assessment Concentrations (BACs; (OSPAR, 2008)) were exceeded in both sediments for several PAHs (naphthalene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene and benzo[a]pyrene) and five CB congeners, whilst the concentrations of two further CBs and the PAH benzoperylene also exceeded the BAC in the ‘polluted’ sediment. CB concentrations did not exceed OSPAR Environmental Assessment Criteria (EACs; (OSPAR, 2008)) in either sediment. Similarly, the ‘clean’ sediment did not exceed the NOAA Effects Range Low (ERL; (NOAA, 1999)) for any compounds; however, the ‘polluted’ sediment exceeded the NOAA ERLs for Σ DDTs, naphthalene, phenanthrene, As, Hg, Ni, and Pb. A single sample of sediment was taken from each tank when the fish were removed. Clean and polluted sediments were each pooled and analysed to provide a single measurement of PAH and CB in each sediment 8 months after setting up the mesocosms. There was no apparent decrease in total PAHs or total CBs; the measured levels fell within the 95% confidence intervals for the initial measurements.

Table 2. Summarised absolute and normalised contaminant concentration data.

		ABSOLUTE				NORMALIZED			
		Ythan		Forth/Tyne		Ythan		Forth/Tyne	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Grain size	%<20 µm	10.5	0.74	<u>25.2</u>	0.70	Metals normalised to grain size Organics normalised to carbon			
	%<63 µm	44.6	1.29	<u>54.4</u>	0.77				
Carbon	%TOC	1.15	0.21	<u>2.77</u>	0.26				
OCPs	DDT & metabolites	1.09	0.19	<u>1.72</u>	0.48	2.39	0.20	1.57	0.52
	HCB	0.18	0.003	<u>1.33</u>	0.06	0.40	0.07	<u>1.21</u>	0.16
	TOTAL OCPs	2.25	0.19	<u>4.07</u>	0.53	4.97	0.51	<u>3.71</u>	0.73
CBs	ICES 7 CBs	1.21	0.24	<u>4.43</u>	0.71	2.67	0.66	<u>4.06</u>	1.00
	TOTAL CBs	1.56	0.45	<u>8.95</u>	1.81	3.52	1.34	<u>8.22</u>	2.36
PAH	Naphthalenes	103	29	<u>2080</u>	718	228	37	<u>1903</u>	757
	NOAA (n=14) PAH	372	59	<u>1527</u>	531	792	143	1300	520
	TOTAL PAH	1104	209	<u>7267</u>	2402	2444	505	<u>6649</u>	2626
	B[a]P-eq PAH	59	9	<u>174</u>	65	129	17	159	68
Organotins	Tributyltin	<6	-	<u>48.8</u>	8.8	<4.5	-	<u>89.7</u>	16.0
	Dibutyltin	<6	-	<u>9.2</u>	0.6	<2.9	-	<u>16.9</u>	1.1
Metals (mg/kg)	As	4.00	0.18	<u>9.09</u>	0.18	8.98	0.51	<u>16.7</u>	0.16
	Cd	<0.24		0.201	0.075	<0.27		0.37	0.13
	Cr	21.5	0.7	<u>33.8</u>	2.5	48.2	1.80	<u>62.1</u>	4.57
	Cu	7.50	0.32	<u>24.1</u>	1.5	16.8	0.81	<u>44.2</u>	2.20
	Hg	<0.063	-	<u>0.310</u>	0.024	<0.07	-	<u>0.57</u>	0.05
	Ni	14.0	0.5	<u>21.4</u>	0.5	31.4	1.38	<u>39.4</u>	0.67
	Pb	11.7	0.3	<u>51.4</u>	1.5	26.1	0.87	<u>94.4</u>	3.19
	Zn	39.8	1.1	<u>98.1</u>	1.2	89.3	3.36	<u>180.2</u>	1.87

Data is mean and SD of five replicate subsamples for each of the two test sediments (clean, Ythan and polluted, Forth/Tyne) prior to addition to the mesocosm aquaria (µg/kg dry wt, unless indicated otherwise). Normalised to a TOC content of 2.5% (for organic contaminants) or to a sediment grain size of 100% <63 µm (metals and organotins). “Less than” values indicate results below analytical detection limits. TOC = total organic carbon; OCP = Organochlorine pesticides (HCB = hexachlorobenzene; total = sum concentration of 20 different compounds, see methods section); CBs = chlorinated biphenyls (see methods); PAH = polycyclic aromatic hydrocarbons (NOAA compounds = sum of those PAH compounds for which NOAA Sediment Quality Guideline ER values are available (see Table 1); total = sum of 33 PAH compounds; B[a]P-equivalent concentration based upon the toxic equivalency factors for the “USEPA 16” (USEPA, 1993), see methods for details). Tyne/Forth concentrations are boldened and underlined where they are significantly different (p<0.05) from Ythan concentrations. “NOAA” = US National Oceanic and Atmospheric Administration.

3.3. Comparison of individual fish livers with hepatocyte pools

To determine whether the levels of gene expression in the hepatocyte pools were representative of gene expression in the populations of fish exposed to each type of sediment, the expression of selected genes which showed differences in microarray analysis were tested. Thus, quantitative rtPCR (QPCR) measurement of the expression levels of five genes which were changed in hepatocytes from fish from ‘polluted’ sediment compared to those from ‘clean’ sediment, showed that these genes were also changed in the same direction and by similar amounts when measured in each of the individual source fish from which the hepatocytes were prepared (Fig2).

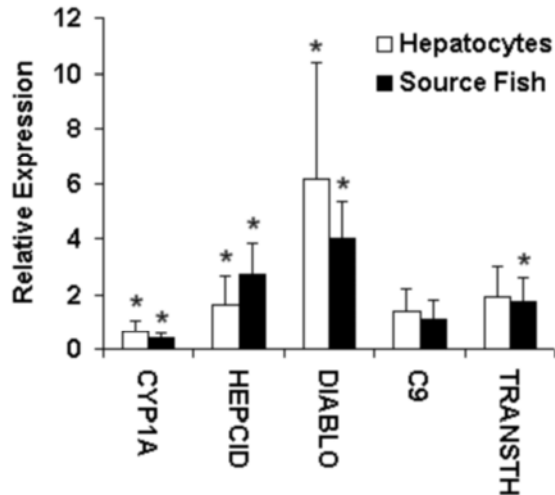


Figure 2. QPCR determination of gene expression in pooled hepatocytes and individual hepatocyte source fish.

The open bars represent the expression level of selected mRNAs in hepatocytes (n=3 replicate pools) from fish exposed to 'polluted' sediments relative to expression in hepatocytes (n=3 replicate pools) from fish exposed to 'clean' sediment. Closed bars represent the expression level of the selected mRNAs in individual flounder (n=6) from 'polluted' mesocosms relative to flounder (n=6) from 'clean' mesocosms. Error bars represent standard deviations of the mean and asterisks indicate a significant difference ($p < 0.05$, REST 2008) from the controls groups.

3.4. Genes affected by sediment exposure or treatment

Initially an overall exploratory analysis of all the genes affected in hepatocytes was conducted by first applying 2-way ANOVA to generate a list of significantly changed genes in any treatment or sediment group. This list was then analysed by clustering methods, to search for parameters which may be predictive of gene expression or of treatment. Thus, application of 2-way ANOVA, with false discovery rate correction (FDR $p < 0.05$), to compare all combined treatments (DMSO, BAP, CU, E2 or MIX) with sediment ('clean' or 'polluted') returned a list genes which were significantly changed by sediment, by treatment or by both factors. Nine hundred and seventy eight of these genes were associated with sediment, 951 with treatment and 206 showed interaction between sediment and treatment. Hierarchical clustering (Fig 3) of all of these significantly altered genes based on experimental condition, showed that neither sediment origin, nor subsequent acute treatment could predict the gene expression profile. Thus, vehicle-treated hepatocytes from fish from the 'clean' sediment mesocosms clustered separately from all other groups, whilst Cu and E2 treated hepatocytes clustered according to sediment type rather than treatment. There was some indication that BAP and MIX treatments clustered together, irrespective of sediment type. Clustering by gene was performed hierarchically and by supervised K-means clustering for five groups. These two methods produced similar clusters (Fig 3), and examination of the K-means clusters in more detail indicated that the majority of genes in four of the groups could be interpreted as: increased by polluted sediment (Group 2); decreased by polluted sediment (Group 3); increased by BAP or MIX (Group 1); decreased by BAP or MIX (Group 4). The fifth group did not correspond to any particular treatment, but contained the majority of genes which were present in the interaction group output from the initial 2-way ANOVA test.

Further filtering of the gene list associated with sediment was performed to generate a final output list. The filtering process first eliminated genes from the total sediment 2 way ANOVA output, which showed a p value of ≤ 0.05 after a comparison of 'clean' DMSO-treated hepatocytes with 'polluted' DMSO-treated hepatocytes. Secondly, the T-test output list was filtered to exclude genes which were less than 2 fold up or down-regulated after comparing 'clean' DMSO-treated with 'polluted' DMSO-treated. This resulted in a final output list of 57 genes whose expression was considered to be changed by exposure to polluted sediment (Table 3). The biological process GO terms associated with these genes were compared with the total GO list for the microarray, and tested for significant ($p \leq 0.05$) over-representation as implemented in GeneSpring GX 7.3.1 (Agilent). Only GO categories

represented by more than one gene from the final gene list were considered significant and are reported in Table 4. Genes whose expression changed after each treatment (BAP, CU, E2 or MIX) were defined by the same methods. First the total 2-way ANOVA output list which was associated with treatment was filtered by T-test p-value using comparisons between BAP, CU, E2 and MIX treated hepatocytes, each with DMSO-treated hepatocytes. Second each of these output lists were filtered by 2-fold up or down in expression level (Supplementary Tables 1-4)

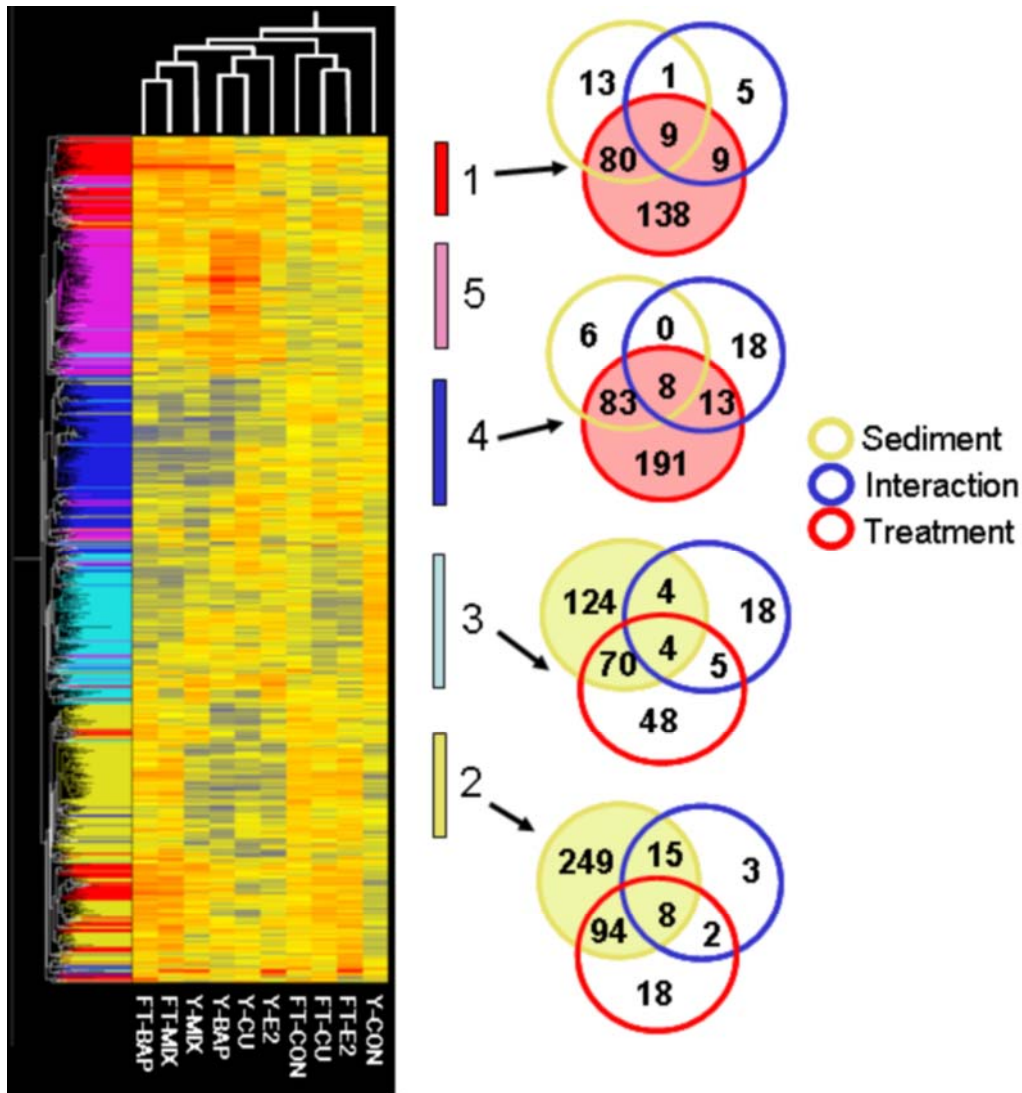


Figure 3. Clustering of 2-way ANOVA hepatocyte output list by condition and by gene.

All genes from hepatocytes which were changed according to 2-way ANOVA (Sediment X Treatment, $p < 0.05$, FDR correction) were clustered hierarchically by condition (Y-CON, Ythan, untreated; Y-BAP, Ythan, benzo(a)pyrene treated; Y-E2, Ythan, estradiol treated, Y-CU, Ythan copper sulphate treated; Y-MIX, Ythan BAP+E2+Cu treated; FT-CON, Forth/Tyne, untreated; FT-BAP, Forth/Tyne, benzo(a)pyrene treated; FT-E2, Forth/Tyne, estradiol treated, FT-CU, Forth/Tyne copper sulphate treated; FT-MIX, Forth/Tyne BAP+E2+Cu treated) and by gene (not annotated). The relative expression levels of the genes are indicated in heat map form, with red representing high and blue, low. Genes were also clustered using K-means supervised for 5 groups. Genes from the five K-means clusters are mapped onto the hierarchical gene tree using colours. The similarly coloured bars to the right of the heat map correspond to congruent clusters of genes produced by K-means and hierarchical methods. The numbers of genes in each cluster which belong to Sediment, Treatment or Interaction between sediment and treatment groups from 2-way ANOVA are indicated using Venn diagrams.

Table 3. Genes whose expression changed in pooled hepatocytes from fish exposed to ‘polluted’ sediments compared to hepatocytes from fish exposed to ‘clean’ sediments.

Clone	Fold	p-Value	Blast Hit	GenBank ID
<u>APOPTOSIS</u>				
Contig122	2.449	0.00938	Diablo	DV566149
PfIL279C03	3.073	0.0141	Diablo homolog	DV568917
Contig265	2.171	0.00313	similar direct IAP binding protein with low PI	aj580508
PfIL227E10	2.563	0.00462	similar direct IAP binding protein with low PI	DV566712
PfIL273F12	2.146	0.0434	deoxyribonuclease II, lysosomal	EC378848
<u>IMMUNE</u>				
Contig439	4.64	3.12E-05	Hepcidin precursor	DV566834
Contig468	2.409	0.000314	hepcidin precursor	aj578016
PfIL201C01	2.411	0.0305	hepcidin precursor	DV565788
PfIL201H05	0.48	0.00143	interleukin 8	DV565814
Contig59	2.049	0.00277	interleukin 8 precursor	DV565292
Contig812	0.464	0.0077	interleukin-8 variant	DV565810
PfIL020F12	2.401	0.00012	similar to small inducible cytokine B6	DV565696
PfFMO2-II0	2.891	0.00375	inter-alpha-trypsin inhibitor heavy chain 2	aj310420
Contig533	2.473	0.0125	complement component C9	DV565432
PfIL008C04	3.012	0.0392	complement component C9	DV565523
PfIL292H03	2.215	0.000754	C-type lectin 2-1 (poor seq)	na
(likely C-type lectin 2.1)	2.082	0.000823	(likely C-type lectin 2.1)	na
PfIL228H02	2.018	0.0157	integrin beta 1 isoform 1A precursor	DV566763
Contig413	2.176	0.000753	MASP protease / haptoglobin	DV566657
Contig146	2.117	0.000898	Phospholipase A2	DV566640
PfIL223G08	2.476	0.00528	similar to transthyretin (4L369)	DV566569
<u>DETOXIFICATION</u>				
PfPARA-o2	2.272	0.00881	Paraoxonase 2	aj292086
PfIL210E01	2.548	0.0188	hydroxyacid oxidase 1	DV566184
<u>AA METABOLISM</u>				
Contig483	2.157	0.00243	betaine-homocysteine methyltransferase	DV567657
PfIL218B01	2.568	0.000927	betaine-homocysteine methyltransferase	DV566389
<u>MEMBRANE TRAFFICKING</u>				
PfIL001B04	2.125	0.0005	Reticulon-1	DV565298
<u>RNA PROCESS</u>				
PfPARP-11	2.194	0.00999	RNA binding motif protein 25	aj310714
<u>GROWTH</u>				
PfIL244E12	2.691	0.00199	Insulin-like growth factor IA precursor (IGF-IA)	DV567425
<u>PROTEOLYSIS</u>				
Contig685	0.49	4.03E-05	High choriolytic enzyme 1 precursor	DV567267
<u>ENERGY GENERATION</u>				
PfIL298D09	0.494	0.000176	NADH dehydrogenase subunit 2	DV569603
<u>NUCLEAR TRANSPORT</u>				
PfIL249C07	0.392	0.0023	nucleoporin 214kDa	DV567616
<u>OXYGEN TRANSPORT</u>				
PfIL288E08	0.4	0.000476	alpha-type globin	EC379099
<u>RIBOSOME</u>				
PfIL214A05	0.407	0.00235	18S ribosomal RNA	DV566284
PfIL230E06	2.107	0.0311	18S ribosomal RNA (poor seq)	na
PfIL209H10	2.325	0.00476	18S rRNA gene	EC377855
PfIL217F03	0.46	0.031	60S ribosomal protein L10a	EC377989

Derived by determining which genes from 2-way ANOVA output list “sediment genes” were significantly changed (T-test $p < 0.05$ and > 2 -fold up or down as indicated in table) in vehicle-treated ‘polluted’ pooled hepatocytes versus vehicle-treated ‘clean’ pooled hepatocytes (T-test, $p < 0.05$, indicated in table). Clone is contig name and GenBank ID gives a representative accession number for the contig. Blast hit is best match of contig sequence to non-redundant protein database by BLASTX (maximum e value $1.0e^{-6}$).

Table 4. Over-represented biological process GO categories in pooled hepatocytes from fish exposed to ‘polluted’ sediments compared to hepatocytes from fish exposed to ‘clean’ sediments.

Category	In Background	In List	p-Value
GO:6955: immune response	113	10	2.78E-05
GO:6959: humoral immune response	30	3	0.0193
GO:6956: complement activation	23	3	0.00925
GO:8219: cell death	91	5	0.0291
GO:6917: induction of apoptosis	20	3	0.0062
GO:43065: positive regulation of apoptosis	22	3	0.00815
GO:6919: caspase activation	9	3	0.000528
GO:8635: caspase activation via cytochrome c	4	2	0.00218
GO:19835: cytolysis	12	2	0.0217
GO:42221: response to chemical stimulus	115	7	0.00537
GO:9410: response to xenobiotic stimulus	34	4	0.0037
GO:6805: xenobiotic metabolism	34	4	0.0037
GO:6935: chemotaxis	23	3	0.00925

‘In Background’, the number of genes present in the indicated GO category present on the total microarray. ‘In List’, the number of genes in the indicated GO category present in the output list (polluted versus clean sediment). The p-Value indicates the probability that the GO category is over-represented in the output list compared to the background list.

From these microarray output lists it is apparent that BAP and E2 have the expected inducing effects on, for example induction of CYP1A and vitellogenin mRNA respectively. These effects were confirmed by QPCR (Fig 4), along with measurements on other genes which were associated with sediment type (Diablo, transthyretin, hepcidin) and genes which are regularly reported in biomarker studies (UGT1B1, CuZnSOD, glutathione reductase, GSTA) Together these measurements demonstrate that ‘clean’ and ‘polluted’ hepatocytes respond similarly, although not identically when treated with the BAP or E2 or Cu. Thus, although BAP treatment caused large increases in both CYP1A and UGT1B, both previously shown to be responsive to BAP in flounder (Leaver et al., 2007), it is apparent that BAP and Cu also increased vitellogenin mRNA levels in ‘clean’ hepatocytes, but not in ‘polluted’ hepatocytes. Co-administration of BAP, E2 and Cu caused a similar level of increase in CYP1A and UGT1B as BAP alone, but levels of vitellogenin mRNA were much reduced in comparison to levels seen with E2 alone. Also apparent is that both UGT1B1, glutathione reductase and glutathione S-transferase show a very similar treatment-response profile to that of CYP1A. Furthermore the responses of Diablo and transthyretin, which were associated with sediment treatment in microarray analysis are also associated with sediment in QPCR profile, being higher in all polluted sediment hepatocytes than in clean, irrespective of subsequent acute treatment.

4. Discussion

The experiment described here was designed to discover how flounder are affected after relatively long-term association with multiply polluted estuarine sediments. This was achieved by categorising genes in flounder whose expression was changed in response to chronic exposure for 7 months to contaminated sediment. In order to reduce the variability which may be associated with site-specific parameters such as salinity, temperature, or feeding behaviour, or to avoid effects of genetic differences between flounder populations,

the experiment was conducted in mesocosms using a single population of flounders taken from a “clean” site, the Ythan estuary. These fish were then exposed to a sediment derived from their native Ythan estuary, or a mixture of sediments taken from two contaminated sites in the UK, the Tyne and Forth estuaries. After 7 months of exposure there were no differences in sex ratios or condition indexes between fish from the ‘clean’ and ‘polluted’ tanks. There was evidence of a tank-related difference in mortality rate. One of the ‘clean’ replicate mesocosms had an unusually high mortality rate and one of the ‘polluted’ mesocosms, an unusually low rate. Such variability in survival in mesocosms, either for flounder, (Vethaak et al., 1996) or more generally for many organisms has been observed before. Whilst this variability is a problem for the assessment of effects on community structure and other higher level ecosystem variables, inter-individual variation in organisms, within or between groups is much lower (Caquet et al., 2000). Furthermore, since the higher mortality occurred in one of the clean mesocosms, it is clear that the pollutant status of the sediments is unlikely to be the cause.

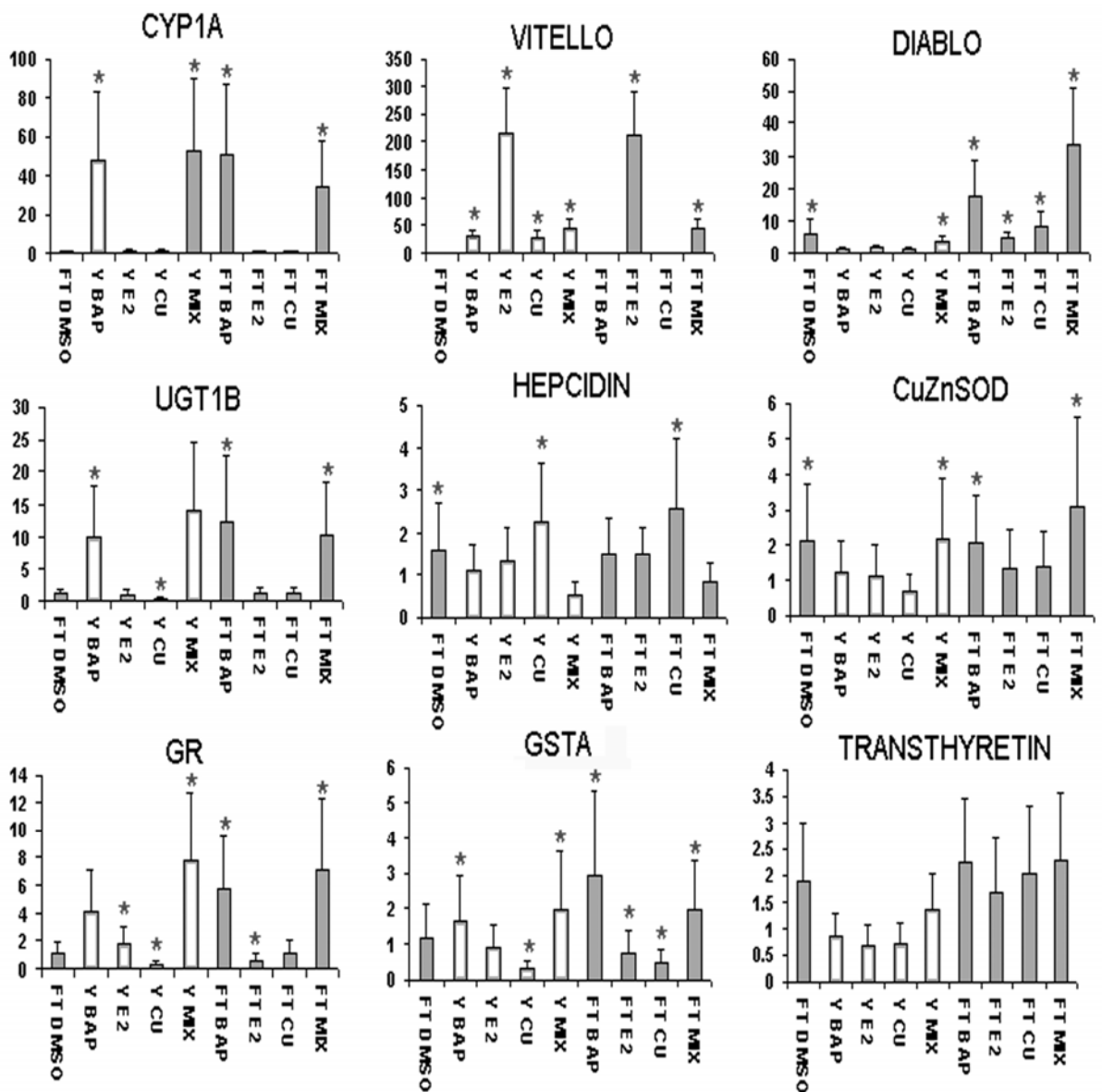


Figure 4. QPCR determination of mRNA levels for selected genes in pooled hepatocytes
 Expression level in treated hepatocytes is represented relative to expression in pooled hepatocytes from fish exposed to ‘clean’ (Ythan) sediment. Error bars represent lower bound standard deviations of the mean and

asterisks indicate a significant difference ($p < 0.05$, REST 2008) from the 'clean' group. Genes measured are CYP1A, cytochrome P4501A; UGT1B, UGT-glucuronosyltransferase 1B; GR, glutathione reductase; GSTA, glutathione S-transferase A; Vitello, vitellogenin; CuZnSOD, copper-zinc-superoxide dismutase. Treatments are: Y-BAP, Ythan, benzo(a)pyrene treated; Y-E2, Ythan, estradiol treated, Y-CU, Ythan copper sulphate treated; Y-MIX, Ythan BAP+E2+Cu treated; FT-CON, Forth/Tyne, untreated; FT-BAP, Forth/Tyne, benzo(a)pyrene treated; FT-E2, Forth/Tyne, estradiol treated, FT-CU, Forth/Tyne copper sulphate treated; FT-MIX, Forth/Tyne BAP+E2+Cu treated. To aid clarity the open bars represent measurements made in hepatocytes for fish exposed to the clean Ythan (Y) sediment and shaded bars to measurements made in hepatocytes from the polluted Forth/Tyne (FT) sediment.

Chemical analysis of the sediments indicated that the 'polluted' test sediment contained significantly higher levels of a range of contaminants than the 'clean' sediment and were higher than recognised background concentrations. In several cases contaminant concentrations exceeded internationally recognised environmental quality thresholds, indicating a potential to cause biological effects in exposed organisms. We interpret these results as strong evidence that the mesocosms accurately reflected the natural situation of long-term, chronic contaminant exposure that flounder would be subject to in a typical industrialized, Northern temperate estuary. Despite the clear elevation of contaminant levels in the polluted sediment, there was only limited evidence of an increase in flounder tissue contaminant levels after 7 months of exposure. Of the metals only lead showed a significant elevation in flounder muscle tissue and none of the organic contaminants showed elevations (not shown). However, there was a significant elevation in bile 3-hydroxypyrene in fish from the 'polluted' mesocosms (not shown), indicating that although PAHs were not accumulating, they were being taken up, metabolised and excreted to a greater extent than in fish maintained on the 'clean' sediment.

In order to compensate for the natural variability in gene expression amongst individual flounder we analysed the transcriptome in pooled hepatocytes derived from multiple fish exposed either to polluted or to clean sediment. In addition, hepatocytes were further treated with prototypical environmental pollutants. This provided a means to directly compare chronic and acute chemical effects in hepatocytes from fish exposed to clean or polluted sediments.

Validation of the transcriptomic results consisted of a pre- and post microarray analysis. The pre-microarray analysis consisted of QPCR measurement of a representative selection of genes in the original 6 fish from 'clean' and 6 fish from 'polluted' sediment. The purpose of this test was to determine whether the expression levels of these genes in the individuals used to make the hepatocyte pools were representative of populations of fish exposed to each type of sediment, in turn indicating the validity of the pooling process. Five genes whose mRNA levels were increased in pooled hepatocytes from fish exposed to polluted sediments were selected. If these genes were also statistically significantly elevated in the individuals from which the pooled hepatocytes were derived, then the pooling procedure is experimentally valid. The results of this analysis demonstrated good agreement between the pooled hepatocytes and the individual source fish. There were no 'outliers' in the individual source fish and it was possible to show statistical significance of differences in gene expression, similar to hepatocyte replicates. We thus conclude that the gene expression measured in pooled hepatocytes was a reasonable representation of the hepatic gene expression population means of the fish in the mesocosms.

After clustering all changed genes and conditions by unsupervised hierarchical method or by a supervised clustering method, it was apparent that sediment of origin had a large effect on gene expression, irrespective of subsequent acute treatment. Treatment with Cu or E2 had little effect on overall gene expression compared to sediment of origin. In contrast, treatment of hepatocytes with BAP or MIX did have large effects. This overview of gene expression indicates that exposure to the multiply-polluted sediment caused clear

changes in gene expression, and that the effects of acute exposures to single chemicals were in some cases small in comparison (Cu and E2), and in other cases clearly different (BAP and MIX), showing one reason why it has been difficult to associate acute laboratory-derived transcriptomic profiles with field-derived profiles.

To derive more biological meaning from global profiles, the list of genes changed by sediment after 2 Way ANOVA was categorized by filtering using pairwise T-tests and fold expression in 'polluted' versus 'clean' sediment hepatocytes. Several over-represented gene ontology categories could be derived from this list. These included; immune response, particularly complement activation; apoptotic induction and caspase activation and xenobiotic metabolism. Consideration of the individual genes making up these GO categories, along with other genes on the output list which were not annotated, indicates that a large proportion of the contribution to the immune response and a number of other genes could also be classified as acute phase reactants (Heinrich et al., 1990). Although it is not possible to predict the effect of these liver gene expression changes on blood protein levels, the level of increase (2 to 3 fold) of acute phase reactant mRNA expression is indicative of low grade inflammation, typically caused by low level chronic infection, metabolic dysfunction, or chemical exposure (Forrester and Bick-Forrester, 2005) (Mullenix et al., 2005) (Yudkin et al., 2000). It is also notable that this list of genes changed by exposure to polluted sediment does not include typical biomarker genes. To further investigate, CYP1A, a gene which characteristically increases in expression following acute exposure to PAH, dioxin and planar CBs, and is thus a very widely used exposure biomarker (van der Oost et al., 2003) was measured by QPCR in both the hepatocytes and in the individual source fish. Contrary to expectation, CYP1A mRNA was decreased in flounder exposed to 'polluted' sediments for 6 months. Many studies have reported increases in CYP1A in flounder exposed to contaminated sediments (van der Oost et al., 2003). However, other studies on the effects of chronic exposure to multiply-polluted sediments have also reported a lack of expected response by hepatic CYP1A in flounder and in other species (Eggens et al., 1996) (Reynolds et al., 2003). Notably, despite a lack of CYP1A response, flounder held long-term in mesocosms containing contaminated sediment did exhibit an increased incidence of hepatic lesions (Vethaak et al., 1996). In the experiment reported here there was no indication of any reduction in either PAH or CB levels in the sediments after 7 months. It is possible that flounder in the mesocosms simply did not accumulate sufficient contaminant to induce CYP1A, or that induction of CYP1A in specific tissues is dependent on route of exposure (VanVeld et al., 1997). Alternatively, CYP1A may become unresponsive after long-term exposure to PAH, as has been reported in flounder fed PAH-spiked food which demonstrated increases in CYP1A after 1 month, but no effect after 6 months of feeding (Reynolds et al., 2003).

The effects of hepatocyte treatments with prototypical pollutants were very similar to hepatic expression profiles from previous studies on acutely exposed whole flounder (Williams et al., 2008); supplementary tables 1-4). Importantly, it was clear that the expected genes, CYP1A, UGT1B for BAP and vitellogenin, choriogenin for E2 were highly elevated (Fig 4). In addition glutathione reductase and GSTA showed very similar treatment-response profiles to CYP1A, indicating that in flounder these genes are likely to be regulated by the same mechanism as CYP1A, by the aryl hydrocarbon receptor (Beischlag et al., 2008). This demonstrates that although these genes were not induced by sediment exposure at environmentally relevant concentrations, the systems which regulate them in response to contaminants were intact in the isolated hepatocytes. Some interesting interactions between sediment and treatments were also apparent, from the microarray data and also confirmed by QPCR. For example, whilst vitellogenin mRNA is highly induced by E2 in hepatocytes from both polluted and clean fish, it is also induced, albeit to a lesser extent, by BAP and by CU

in the 'clean' hepatocytes, but not in 'polluted' hepatocytes. The reasons for this are not obvious, although both BAP and its metabolites, and more recently metals, including Cu, have been shown to mimic estrogen when present at sufficient concentration (Darbre, 2006) (Fertuck et al., 2001). The fact that this response is clearly different depending on hepatocyte source, suggest that these effects may extend to fish living in chronically polluted and clean environments, and should perhaps be investigated further given the relevance for environmental monitoring programs.

Also apparent are effects of the mixture on hepatocytes. Although this treatment induced CYP1A, and UGT1B to the same extent as BAP alone, vitellogenin expression was much reduced compared to E2 alone. These types of interactions between pathways which depend on the aryl hydrocarbon and estrogen receptors have been previously observed in mammals (Safe and Wormke, 2003) (Beischlag and Perdew, 2005). If the same effects are seen in fish, as seems evident from the results reported here, then the results of CYP1A and vitellogenin biomarker measurements in multiply-polluted environments should be treated with caution.

In addition to biomarker genes associated with acute chemical exposure, we also measured by QPCR some of the genes which were associated with clean or polluted sediment. As indicated by global clustering of the microarray data, some of these genes (Diablo and transthyretin) showed a pattern of expression which was largely dependent on sediment of origin, rather than acute treatment. However QPCR results showed that these sediment-related genes may also be further modulated by acute exposure. An interesting example is Diablo. The expression of this gene was higher in all polluted samples than in clean, but following mixture treatment, expression in polluted hepatocytes further increases to levels 30 fold higher than in clean vehicle-treated hepatocytes. Diablo (or Smac) is a protein released from mitochondria which inhibits the activity of a repressor of caspase 3, a major apoptotic executioner (Du et al., 2000). An increase in Diablo might thus be an indication that under cell death stimuli, apoptosis is highly favoured. The signals which promote increases in Diablo are not known, but as a component of the mitochondrion it is likely associated with the intrinsic apoptotic pathway, responding to cellular damage (Shiozaki and Shi, 2004), and Diablo mimetics have recently been shown to increase the susceptibility of diseased cells to apoptosis-inducing drugs (Fulda et al., 2002) (Bank et al., 2008). The pattern and magnitude of expression changes in flounder liver indicate that Diablo, and the pathways to which it belongs are worthy of further investigation, with the prospect of developing more relevant environmental biomarkers.

5. Conclusions

In summary and conclusion we have shown that long-term, chronic exposure of flounder to multiply-polluted estuarine sediments causes increases in the hepatic expression of genes involved in innate immunity, inflammation and apoptotic pathways. Genes commonly employed as environmental contaminant exposure biomarkers (eg CYP1A, vitellogenin, metallothionein) were not affected, or were changed contrary to expectation in this experimental system, suggesting that they may be most useful in monitoring acute spill, or point-source contaminant inputs to the environment. The results of this experiment show that, during long-term exposure to diffuse, multiple contaminants there may be other, more useful biomarkers of exposure (or effect) which indicate changes in chronic inflammatory and apoptotic pathways.

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Appendix A

Supplementary data detailing the genes whose expression is changed in hepatocytes from flounder exposed to clean or polluted sediments are included in Supplementary Tables 1 to 4.

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Supplementary Table 1. Hepatocyte genes whose expression changed in response to benzo(a)pyrene treatment

List and associated numbers were generated by pairwise T-test of gene expression from treated 'clean' or 'polluted' hepatocytes versus untreated 'clean' hepatocytes and untreated 'polluted' hepatocytes respectively, using an input list from the total list of genes shown to change in response to treatment by 2 way ANOVA (FDR $p < 0.05$). The fold increase in hepatocytes from fish held on 'clean' sediment is under the heading 'CleanFold' and from polluted sediment 'PollFold'. P values of each pairwise test are also indicated. Only genes which were >2 fold up or down, $p < 0.05$ in either hepatocyte group are included. Shading indicates where the response was >2 fold up or down, and where $p > 0.05$. Genes without any associated annotation have been omitted from this list.

Array Contig	CleanFold	PollFold	CleanPval	PollPval	BLAST
Contig504	14.91	20.37	0.0169	0.0298	CYP1A Cytochrome P450 1A
PfIL262B03	8.516	1.05	0.0201	0.871	microsatellite DNA, locus StPfI016
PfIL303G04	6.596	1.057	0.0111	0.794	ReO_6 protein
PfIL289G08	6.413	1.755	0.00655	0.474	Myeloid/lymphoid or mixed-lineage leukemia protein 4
Contig297	5.168	1.89	0.00655	0.0754	similar to transient receptor potential cation channel
Contig525	4.394	4.214	0.0313	0.0261	UDP-glucuronosyltransferase (UGT1B gene)
Contig765	4.17	0.999	0.00657	0.994	JFMIP1b pseudogene premature mRNA for MIP1beta
PfIL305H02	3.986	1.79	0.012	0.441	G protein-coupled receptor kinase-interactor 2 isoform 1
PfIL262G01	3.42	1.871	0.00657	0.0511	Trans-golgi network protein 2
PfIL248A04	3.356	1.304	0.0208	0.162	Vacuolar protein sorting-associated protein 45
PfIL232F01	3.265	1.973	0.00734	0.0298	ATPase family, AAA domain containing 4
PfIL295F02	3.162	1.829	0.00643	0.0477	GTP cyclohydrolase I feedback regulatory protein
PfIL285F04	3.08	1.001	0.00657	0.994	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2, 8kDa
PfIL259G06	2.897	1.37	0.00704	0.168	KH-type splicing regulatory protein
PfIL258B11	2.885	1.532	0.00657	0.0468	Inhibitor of DNA binding 2
PfIL229D06	2.664	1.428	0.0283	0.0631	anion exchanger 2
Contig405	2.643	1.498	0.0117	0.053	cDNA clone down072 (sim to pre-acrosome localisation protein?)
PfIL320H03	2.591	1.425	0.0195	0.042	pleurocidin-like peptide WFYT (ple5, ple6), WF1L (ple7) genes, and ple10
Contig27	2.56	0.989	0.028	0.99	chemokine (C-X-C motif) ligand 12a
Contig413	2.546	1.898	0.0092	0.0137	MASP protease / haptoglobin
Contig423	2.52	1.511	0.00643	0.0259	calreticulin
PfIL213G03	2.358	0.947	0.0474	0.892	MGC84339 protein
PfIL295C01	2.329	0.411	0.0642	0.0226	hypothetical protein LOC552925
PfIL011C04	2.325	1.384	0.011	0.155	splicing factor arginine/serine-rich 3

PfIL227C08	2.304	0.822	0.00643	0.252	Butyrate response factor 2
PfIL237C04	2.291	1.929	0.00657	0.034	p24 delta1 putative cargo receptor
Contig281	2.262	1.279	0.00834	0.17	Heterogeneous nuclear ribonucleoprotein A/B
PfIL279B01	2.251	1.953	0.0387	0.0298	ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A, like
Contig870	2.247	1.506	0.0441	0.0396	small ubiquitin-like modifier protein 3; SMT3
Contig148	2.236	1.368	0.00793	0.185	RAB6A, member RAS oncogene family isoform a
PfIL292C12	2.205	1.623	0.0277	0.0298	transcription elongation factor B (SIII) polypeptide 2
PfIL259D04	2.203	1.83	0.00643	0.0298	signal peptidase complex subunit 3 homolog
Contig291	2.176	1.347	0.00643	0.0871	ER-resident chaperone calreticulin
PfIL261B02	2.174	1.93	0.00643	0.00619	Probable diphthine synthase
PfIL227B02	2.165	1.164	0.028	0.355	guanine nucleotide binding protein (G protein), beta polypeptide 1 (gnb1)
PfIL246G07	2.141	1.465	0.0117	0.034	Phosphomannomutase 2
Contig273	2.134	1.203	0.00823	0.0963	sister of P-glycoprotein
PfIL303B09	2.13	1.074	0.0148	0.722	cDNA clone CDA45-H11.y1d-s sim to ubiquitin specific protease 9
PfIL234A06	2.116	1.739	0.0161	0.116	Karyopherin alpha 5 (importin alpha 6)
PfIL315E01	2.106	1.458	0.013	0.112	B-lymphocyte activation marker BLAST-1 precursor
PfIL300G11	2.093	1.805	0.00643	0.034	similar to programmed cell death 6
PfIL304D07	2.018	1.228	0.0195	0.463	Ring finger protein 10
PfIL257A11	2.016	2.255	0.0821	0.017	Siah-interacting protein (Sip-prov)
PfIL232H02	1.959	2.044	0.00823	0.0163	lipocalin-type prostaglandin D synthase-like protein
PfIL309A08	1.952	2.221	0.0362	0.0259	P-glycoprotein type A pEXI-homologous region
PfIL301B12	1.884	2.217	0.0596	0.0298	microsomal stress70 protein ATPase core precursor
Contig597	1.839	2.263	0.0118	0.0137	alpha-1-microglobulin/bikunin precursor
PfIL273D07	1.765	2.007	0.00768	0.0234	cDNA clone Pf_IL_302B12 5' similar to Acyl-CoA-binding protein
PfIL278F06	1.76	2.309	0.00835	0.0303	hypothetical protein LOC553776
PfIL298C06	1.584	2.42	0.121	0.0262	Rb1-inducible coiled coil protein 1
PfIL254F08	1.554	2.773	0.0751	0.00956	Nf-kappa B activator (TANK)
Pp002	1.441	3.761	0.199	0.0303	3' untranslated region of GSTA1
PfIL238F01	1.396	2.06	0.028	0.00992	glutathione S-transferase
PfIL289E03	1.082	2.543	0.569	0.00619	archipelago CG15010-PC, isoform C
PfIL308C06	0.7	0.46	0.0415	0.0234	Transmembrane 4 superfamily member 7
PfIL271G09	0.696	0.308	0.318	0.0378	Ubiquitin specific protease 16
PfIL243B02	0.586	0.28	0.117	0.0163	immunoglobulin D heavy chain

Contig864	0.54	0.442	0.00793	0.0331	Phospholipase A2 precursor
PfIL250G09	0.536	0.476	0.0642	0.0176	carboxypeptidase A1 (poor seq)
Contig1106	0.534	0.486	0.00657	0.00992	actin related protein 2/3 complex subunit 5, 16 kDa
Contig527	0.52	0.435	0.0116	0.00422	type II keratin
Contig633	0.505	0.477	0.0276	0.0153	Chymotrypsin B precursor
PfIL209E08	0.5	0.378	0.047	0.0345	6-pyruvoyl tetrahydrobiopterin synthase
PfIL236A07	0.499	0.739	0.00734	0.0975	DnaJ (Hsp40) homolog, subfamily C, member 1
Contig316	0.497	0.606	0.00657	0.0137	chemotaxin
PfIL223D11	0.497	0.621	0.0302	0.053	eukaryotic translation initiation factor 4E binding protein 1
Contig681	0.495	0.483	0.0177	0.0298	Elastase 1
PfIL276A07	0.493	0.482	0.0354	0.0348	phosphatidate phosphohydrolase type 2a
Contig686	0.491	0.832	0.0208	0.173	40S ribosomal protein S2
Contig914	0.489	0.492	0.0171	0.033	trypsinogen 2 precursor
Contig718	0.484	0.676	0.0161	0.0381	anticoagulant protein C precursor (PROC)
PfIL251H03	0.482	0.536	0.0309	0.0303	unnamed protein product CAG01129
PfIL315H04	0.481	0.833	0.0121	0.425	Delta3,5-delta2,4-dienoyl-CoA isomerase, mitochondrial precursor
PfRHO-o1	0.48	0.611	0.0133	0.0298	Rho
PfIL298B07	0.474	0.743	0.0119	0.145	apolipoprotein H precursor
Contig1074	0.474	0.665	0.0157	0.0137	Coronin 1A
Contig473	0.474	0.73	0.00704	0.109	C-type lectin 1
Contig738	0.474	0.397	0.00657	0.0298	High choriolytic enzyme 1 precursor
PfIL227F02	0.473	0.492	0.00657	0.0259	complement component 1 q subcomponent
PfIL006C07	0.472	0.593	0.0239	0.0346	clone sp006sk 5' similar to Coronin, actin binding protein, 1A
Contig1079	0.467	0.417	0.00835	0.0298	trypsinogen 2 precursor
PfIL241D10	0.464	0.62	0.0123	0.00618	arginase, type I; A-I
Contig648	0.462	0.459	0.00957	0.0334	carboxypeptidase A1
Contig78	0.461	0.248	0.0389	0.148	immunoglobulin light chain L2
PfIL298D09	0.46	0.405	0.00614	0.0137	NADH dehydrogenase subunit 2
PfIL291C08	0.458	0.979	0.013	0.93	Glutathione S-transferase, theta 3
PfIL273H09	0.454	0.698	0.00823	0.0629	Hsc70-interacting protein (Hip)
PfIL012H08	0.451	0.785	0.0116	0.551	inhibitor of differentiation 1C; Id 6-2
PfIL217A03	0.45	0.743	0.0123	0.0561	Chaperonin containing TCP1, subunit 3 (Gamma
PfIL209A06	0.44	0.68	0.0156	0.0648	Asparaginyl-tRNA synthetase

Contig774	0.44	0.552	0.0106	0.02	MHC II invariant chain
PfIL241E06	0.435	0.471	0.025	0.151	Golgi transport 1 homolog A
Contig975	0.435	0.627	0.00614	0.0572	trypsin III
Contig629	0.432	0.583	0.0126	0.038	prothrombin precursor
Contig624	0.427	0.458	0.0116	0.0754	Sulfatase modifying factor 2 precursor
Pp007	0.426	0.676	0.00657	0.0508	UDP - glucose phosphatase
Contig919	0.419	0.482	0.00682	0.034	Chymotrypsinogen 1
Contig330	0.418	0.625	0.00823	0.0645	Asparaginyl-tRNA synthetase
Contig474	0.417	0.684	0.00657	0.0362	Perforin 1 precursor (P1) (Lymphocyte pore forming protein) (Cytolysin)
PfIL209A08	0.412	0.579	0.0106	0.0298	ATP binding protein associated with cell differentiation
Contig986	0.41	0.441	0.00657	0.0181	bile salt-activated lipase (BAL)
PfIL202H02	0.408	0.588	0.00695	0.0724	chaperonin containing TCP-1 delta
PfSHA2F7	0.407	0.492	0.00614	0.0568	Antifreeze glycoprotein precursor
PfIL279B04	0.407	0.501	0.0116	0.0212	Phospholipase A2
PfIL253F11	0.404	0.487	0.0127	0.00992	NADH dehydrogenase subunit 3
Pa016	0.403	0.643	0.0116	0.0352	Cytochrome P450 2X
Contig693	0.401	0.443	0.0258	0.0396	selenoprotein M
Contig418	0.396	0.372	0.013	0.0298	Alpha amylase
Pp005	0.394	0.615	0.0111	0.0378	Fatty acid binding protein
Contig885	0.393	0.522	0.00614	0.034	Perforin
Contig1060	0.388	0.434	0.00657	0.0511	trypsin
Contig447	0.388	0.551	0.0149	0.0226	unnamed protein product CAG03272
Contig346	0.383	0.571	0.00657	0.307	unnamed protein product CAF90585
Contig804	0.382	0.266	0.00643	0.00619	immunoglobulin light chain precursor
PfIL263A03	0.38	0.692	0.00834	0.145	poly A binding protein, cytoplasmic 1
PfIL243F11	0.379	0.732	0.0357	0.474	Kruppel-like factor 2a klf2a
Contig1049	0.37	0.485	0.00669	0.0298	Chymotrypsinogen 2
PfFMO2-II0	0.37	0.44	0.0116	0.0303	inter-alpha-trypsin inhibitor heavy chain 2
Contig744	0.367	0.608	0.00657	0.0286	cDNA clone CR729877 sim to fibrinogen, alpha chain isoform alpha-E
PfILCOMA06	0.367	0.627	0.00643	0.0856	perforin
Contig467	0.364	0.504	0.00643	0.0303	Deoxyribonuclease I precursor (DNase I)
Contig568	0.355	0.438	0.00682	0.0298	Carboxypeptidase A2
PfIL218F05	0.329	0.561	0.00797	0.0298	P18 splicing regulatory protein

PfIL307D04	0.326	0.669	0.0146	0.127	irn70ks / 18S rRNA (poor seq)
Contig998	0.321	0.356	0.00643	0.0259	Elastase 3
Contig1091	0.315	0.428	0.00657	0.0298	elastase A precursor
Contig1108	0.312	0.363	0.0119	0.0176	elastase 2-like protein
Contig903	0.292	0.564	0.0116	0.0259	ribosomal RNA
PfIL007A06	0.289	0.433	0.0106	0.0298	carboxypeptidase B
PfIL306D01	0.288	0.98	0.00677	0.914	18S ribosomal RNA
PfIL214A05	0.285	0.633	0.00643	0.0677	18S ribosomal RNA
PfIL245C07	0.282	0.327	0.00643	0.0107	Integrin alpha5
PfIL259C04	0.281	0.329	0.00614	0.0298	elastase 2 precursor
Contig22	0.216	0.593	0.00643	0.0298	metallothionein
PfIL288C01	0.181	0.178	0.00366	0.0164	type I keratin S8

Supplementary Table 1. Hepatocyte genes whose expression changed in response to estradiol treatment

List and associated numbers were generated by pairwise T-test of gene expression from treated 'clean' or 'polluted' hepatocytes versus untreated 'clean' hepatocytes and untreated 'polluted' hepatocytes respectively, using an input list from the total list of genes shown to change in response to treatment by 2 way ANOVA (FDR $p < 0.05$). The fold increase in hepatocytes from fish held on 'clean' sediment is under the heading 'CleanFold' and from polluted sediment 'PollFold'. P values of each pairwise test are also indicated. Only genes which were >2 fold up or down, $p < 0.05$ in either hepatocyte group are included. Shading indicates where the response was >2 fold up or down, and where $p > 0.05$. Genes without any associated annotation have been omitted from this list.

Array Contig	CleanFold	PollFold	CleanPval	PollPval	BLAST
PfIL257D11	63.06	45.17	0.00257	0.0367	Vitellogenin
Contig458	50.74	37.97	0.00215	0.0187	Vitellogenin
Contig398	16.68	17.12	0.00257	3.62E-05	choriogenin L
Contig450	10.67	11.02	0.00215	0.0169	Vitellogenin
Contig403	3.987	3.094	0.0215	0.0169	IgM heavy chain constant region
PfIL304D06	3.035	2.756	0.0387	0.0338	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4
Contig457	2.716	1.494	0.0178	0.0466	CDA08-B12.y1d-s SHGC-CDA sim to hippocalcin-like 1
PfIL013A02	2.147	1.652	0.0163	0.0169	Oligosaccharyl transferase 48 kDa subunit
PfIL238C10	2.057	1.736	0.0234	0.0377	Hypothetical protein zgc:77713
PfIL237C04	2.049	1.788	0.0274	0.00544	p24 delta1 putative cargo receptor
PfIL255E12	2.028	1.548	0.0169	0.0169	heat shock protein gp96
PfIL262G01	1.878	2.135	0.185	0.0338	Trans-golgi network protein 2
PfIL271G09	0.668	0.32	0.12	0.0378	Ubiquitin specific protease 16
Contig750	0.656	0.494	0.12	0.0378	Tetraspan 1
PfIL307D04	0.582	0.472	0.283	0.0275	im70ks / 18S rRNA (poor seq)
PfIL218F05	0.498	0.369	0.0169	0.0161	P18 splicing regulatory protein
PfIL263E08	0.447	0.63	0.0485	0.0157	DnaJ (Hsp40) homolog, subfamily B, member 1
Contig903	0.398	0.47	0.0113	0.0152	ribosomal RNA
PfIL245C07	0.372	0.523	0.00685	0.0169	Integrin alpha5
PfIL214A05	0.353	0.484	0.0169	0.0378	18S ribosomal RNA
Contig22	0.348	0.316	0.00676	0.00766	metallothionein
PfIL302H09	0.291	0.527	0.0115	0.0356	RAP1B, member of RAS oncogene family

Supplementary Table 1. Hepatocyte genes whose expression changed in response to copper treatment

List and associated numbers were generated by pairwise T-test of gene expression from treated 'clean' or 'polluted' hepatocytes versus untreated 'clean' hepatocytes and untreated 'polluted' hepatocytes respectively, using an input list from the total list of genes shown to change in response to treatment by 2 way ANOVA (FDR $p < 0.05$). The fold increase in hepatocytes from fish held on 'clean' sediment is under the heading 'CleanFold' and from polluted sediment 'PollFold'. P values of each pairwise test are also indicated. Only genes which were >2 fold up or down, $p < 0.05$ in either hepatocyte group are included. Shading indicates where the response was >2 fold up or down, and where $p > 0.05$. Genes without any associated annotation have been omitted from this list.

Array Contig	CleanFold	PollFold	CleanPval	PollPval	BLAST
PfIL303G04	3.892	0.892	0.0348	0.276	ReO_6 protein
PfIL289G08	3.635	1.065	0.0348	0.703	Myeloid/lymphoid or mixed-lineage leukemia protein 4
PfIL304D06	3.488	1.665	0.0378	0.215	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4
Contig403	3.13	2.709	0.0349	0.0835	IgM heavy chain constant region
Contig297	3.057	1.45	0.0401	0.435	similar to transient receptor potential cation channel
PfIL303B09	2.987	1.638	0.0332	0.336	cDNA clone CDA45-H11.y1d-s sim to ubiquitin specific protease 9
Contig870	2.872	1.686	0.0218	0.222	small ubiquitin-like modifier protein 3; SMT3
PfIL213G03	2.794	2.603	0.025	0.151	MGC84339 protein
PfIL259G06	2.75	2.149	0.0428	0.162	KH-type splicing regulatory protein
PfIL295C01	2.725	0.705	0.0473	0.476	hypothetical protein LOC552925
PfIL227B02	2.706	2.012	0.0226	0.117	guanine nucleotide binding protein (G protein), beta polypeptide 1 (gnb1)
Contig765	2.654	0.952	0.0401	0.438	JFMIP1b pseudogene premature mRNA for MIP1beta
PfIL229C08	2.607	1.08	0.0348	0.556	clone uogacrs-8c7-1
PfIL262G01	2.552	1.387	0.0348	0.322	Trans-golgi network protein 2
PfIL233G09	2.482	4.638	0.0332	0.118	formin-binding protein 17 (rapostlin)
PfIL303A02	2.356	3.072	0.0463	0.17	cDNA clone 1RT138K09_A_F05 sim to taurine transporter (poor seq)
PfIL013A02	2.318	1.696	0.025	0.0745	Oligosaccharyl transferase 48 kDa subunit
PfIL227C08	2.313	1.316	0.00993	0.31	Butyrate response factor 2
Contig281	2.263	1.722	0.0332	0.15	Heterogeneous nuclear ribonucleoprotein A/B
PfIL246G07	2.169	1.459	0.0417	0.151	Phosphomannomutase 2
Contig1052	2.096	1.131	0.0348	0.636	cDNA clone CIWU128 similar to keratin associated protein 4-12
PfIL292C12	2.079	1.967	0.0378	0.0669	transcription elongation factor B (SIII) polypeptide 2
PfIL011C04	2.077	1.709	0.025	0.151	splicing factor arginine/serine-rich 3

PfIL243B02	2.061	0.739	0.0409	0.549	immunoglobulin D heavy chain
PfIL001E05	1.962	2.035	0.0515	0.00677	RAP1A, member of RAS oncogene family
PfIL278D10	0.497	0.422	0.0348	0.159	NADH dehydrogenase (ubiquinone) Fe-S protein 4
PfFMO2-II0	0.491	0.464	0.0348	0.151	inter-alpha-trypsin inhibitor heavy chain 2
Contig1049	0.484	0.729	0.0348	0.14	Chymotrypsinogen 2
PfIL306D01	0.46	0.575	0.0348	0.157	18S ribosomal RNA
PfIL265A10	0.454	0.738	0.0463	0.133	Hypothetical protein MGC40107
Contig1060	0.45	0.712	0.0348	0.363	trypsin
Contig903	0.434	0.506	0.0382	0.14	ribosomal RNA
Contig424	0.426	0.651	0.0348	0.0213	low molecular weight heat shock protein Hsp30B
Contig1091	0.417	0.73	0.0348	0.211	elastase A precursor
Contig998	0.415	0.675	0.0453	0.14	Elastase 3
PfIL307D04	0.407	0.63	0.0348	0.266	irn70ks / 18S rRNA (poor seq)
PfIL298D09	0.405	0.409	0.0348	0.0745	NADH dehydrogenase subunit 2
SSH control DNA	0.361	1.493	0.0347	0.565	SSH control DNA
PfH70-g1	0.333	0.586	0.0473	0.246	heat shock protein 70
PfIL313F10	0.273	0.306	0.0349	0.207	Rho GTPase activating protein 19
PfIL218F05	0.268	0.478	0.025	0.0669	P18 splicing regulatory protein
Contig22	0.176	0.448	0.022	0.0745	metallothionein

Supplementary Table 1. Hepatocyte genes whose expression changed in response to mixture treatment

List and associated numbers were generated by pairwise T-test of gene expression from treated 'clean' or 'polluted' hepatocytes versus untreated 'clean' hepatocytes and untreated 'polluted' hepatocytes respectively, using an input list from the total list of genes shown to change in response to treatment by 2 way ANOVA (FDR $p < 0.05$). The fold increase in hepatocytes from fish held on 'clean' sediment is under the heading 'CleanFold' and from polluted sediment 'PollFold'. P values of each pairwise test are also indicated. Only genes which were >2 fold up or down, $p < 0.05$ in either hepatocyte group are included. Shading indicates where the response was >2 fold up or down, and where $p > 0.05$. Genes without any associated annotation have been omitted from this list.

Array Contig	CleanFold	PollFold	CleanPval	PollPval	BLAST
Contig504	16.7	19.55	0.0174	0.00264	CYP1A Cytochrome P450 1A
Contig458	11.15	6.777	0.0137	0.121	Vitellogenin
PfIL257D11	9.263	6.244	0.006	0.0577	Vitellogenin
Contig525	6.841	4.533	0.0201	0.118	UDP-glucuronosyltransferase (UGT1B gene)
Contig398	6.476	6.184	0.0131	0.0155	choriogenin L
PfIL309A08	4.122	1.74	0.00564	0.0479	P-glycoprotein type A pEXI-homologous region
PfIL301B12	2.773	2.806	0.019	0.00607	microsomal stress70 protein ATPase core precursor
PfIL263A08	2.693	1.623	0.0222	0.0412	clone cmcon186 microsatellite sequence
PfIL257A11	2.659	2.091	0.00771	0.156	Siah-interacting protein (Sip-prov)
Contig413	2.455	1.771	0.0144	0.0758	MASP protease / haptoglobin
Contig492	2.442	2.067	0.00796	0.0155	c-Fos
PfIL261A12	2.324	1.234	0.0139	0.511	Cytochrome b5
PfIL273D07	2.311	1.675	0.0186	0.0168	cDNA clone Pf_IL_302B12 5' similar to Acyl-CoA-binding protein
Contig969	2.238	1.355	0.0133	0.0199	similar to HIG1 domain family, member 2A
PfIL257G07	2.221	1.009	0.0179	0.876	cDNA clone up050 similar to PTPRJ
PfIL308C03	2.187	1.738	0.0119	0.0601	Annexin A11b
PfIL011H10	2.164	2.136	0.0137	0.043	DnaJ-like subfamily A member 4 (Hsp40A4)
PfIL237C04	2.156	2.032	0.00771	0.0457	p24 delta1 putative cargo receptor
PfIL278F06	2.151	2.314	0.00835	0.0178	hypothetical protein LOC553776
PfIL223G08	2.13	2.495	0.006	0.0622	similar to transthyretin (4L369)
Contig678	2.116	1.174	0.006	0.656	NADPH oxidase flavocytochrome b small subunit p22phox
PfIL261B02	2.097	1.428	0.0274	0.15	Probable diphthine synthase
Contig405	2.092	2.181	0.0247	0.016	cDNA clone down072 (sim to pre-acrosome localisation protein?)

PfIL260G05	2.08	0.909	0.0464	0.417	14 kDa apolipoprotein
PfIL300G11	2.072	1.632	0.00564	0.171	similar to programmed cell death 6
Contig952	2.07	1.851	0.0202	0.157	18S ribosomal RNA gene
Contig57	2.04	1.69	0.0139	0.016	phenylalanine hydroxylase
Contig752	2.023	1.631	0.0264	0.0636	Liver-expressed antimicrobial peptide 2A
PfIL259D04	2.012	1.622	0.011	0.0734	signal peptidase complex subunit 3 homolog
Contig459	2.006	1.909	0.0242	0.0772	Peroxiredoxin (Thioredoxin peroxidase) (NKeF)
PfIL285E11	2.004	1.623	0.0107	0.0182	Transmembrane protein 4 TMEM4
PfIL298C06	1.784	2.103	0.0632	0.0161	Rb1-inducible coiled coil protein 1
PfIL241D10	1.694	2.001	0.0152	0.00264	arginase, type I; A-I
PfIL254F08	1.518	2.367	0.203	0.0088	Nf-kappa B activator (TANK)
PfIL320H03	1.496	2.063	0.128	0.0109	pleurocidin-like peptide WFYT (ple5, ple6), WF1L (ple7) genes, and ple10
Contig357	1.328	2.017	0.183	0.0088	Cystathionine-beta-synthase
PfH70-g1	1.196	2.725	0.692	0.0088	heat shock protein 70
PfIL259A02	1.102	2.119	0.431	0.0173	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 2
PfIL012C12	0.785	0.411	0.373	0.00823	S100-like calcium binding protein
PfIL309H12	0.782	0.448	0.152	0.0276	MHC class II alpha
Contig801	0.779	0.399	0.155	0.0199	Protein phosphatase 1, regulatory (inhibitor) subunit 3B
PfIL295C01	0.768	0.492	0.495	0.00823	hypothetical protein LOC552925
PfIL217D10	0.761	0.382	0.0573	0.0199	unnamed protein product CAF91835 (EST OD-118)
PfIL224F02	0.755	0.455	0.359	0.0179	ZPAX
Contig495	0.719	0.462	0.245	0.0268	Calcium binding protein, glucose regulated protein GRP170 precursor
Contig885	0.697	0.47	0.0315	0.0199	Perforin
PfIL050E09	0.689	0.465	0.0311	0.0199	bad sequence
Contig44	0.683	0.441	0.0455	0.00607	chemotaxin
Contig925	0.676	0.473	0.0264	0.00636	unnamed protein product CAG03272
Contig1049	0.648	0.468	0.0294	0.0183	Chymotrypsinogen 2
Contig403	0.617	0.345	0.0258	0.027	IgM heavy chain constant region
PfIL236A07	0.608	0.435	0.0215	0.0161	DnaJ (Hsp40) homolog, subfamily C, member 1
Contig316	0.601	0.43	0.0392	0.00607	chemotaxin
Contig441	0.579	0.46	0.0208	0.00607	hepcidin precursor
Contig693	0.573	0.396	0.0597	0.0216	selenoprotein M

Contig447	0.569	0.46	0.0133	0.00607	unnamed protein product CAG03272
Contig397	0.538	0.317	0.006	0.00264	unnamed protein product CAG03272
Contig470	0.526	0.471	0.0281	0.0232	hepcidin-like precursor
Contig1108	0.51	0.48	0.006	0.0355	elastase 2-like protein
PfIL319D08	0.51	0.435	0.0432	0.0162	kelch-like 9
PfIL217A03	0.496	0.709	0.00837	0.026	Chaperonin containing TCP1, subunit 3 (Gamma
Contig418	0.495	0.585	0.00564	0.0381	Alpha amylase
PfIL263G01	0.489	0.368	0.00837	0.0199	T-lymphocyte antigen receptor beta-chain constant region 2
Contig1074	0.488	0.594	0.0292	0.0332	Coronin 1A
Contig205	0.486	0.431	0.00564	0.0083	NADPH oxidase cytosolic protein p67phox
PfRHO-o1	0.486	0.557	0.0242	0.0176	Rho
PfIL007A06	0.485	0.454	0.0475	0.0199	carboxypeptidase B
Contig998	0.483	0.428	0.0142	0.0183	Elastase 3
Contig824	0.48	0.689	0.0179	0.195	Immunoglobulin lambda-like polypeptide 1 precursor
PfIL226G02	0.479	0.803	0.0258	0.362	carboxypeptidase N, polypeptide 1, 50kD precursor
PfIL298D09	0.477	1.113	0.0102	0.343	NADH dehydrogenase subunit 2
Contig427	0.476	0.403	0.00946	0.00607	hepcidin-like precursor
PfIL285A07	0.476	0.465	0.0292	0.0622	Lysozyme c (poor seq)
PfIL308C06	0.475	0.376	0.006	0.0126	Transmembrane 4 superfamily member 7
PfIL259C04	0.468	0.442	0.00837	0.026	elastase 2 precursor
Contig633	0.467	0.521	0.00697	0.0819	Chymotrypsin B precursor
PfIL318A02	0.467	0.736	0.0137	0.0933	Hypothetical protein zgc:73259
PfIL318D02	0.466	0.703	0.0412	0.223	Elastase 1
PfIL260F08	0.466	0.549	0.0113	0.0381	hypothetical protein LOC406709
Contig738	0.461	0.567	0.0139	0.028	High choriolytic enzyme 1 precursor
PfIL223G10 (likely lysozyme c)	0.458	0.622	0.0208	0.07	Leucine-rich alpha-2-glycoprotein 1
Contig181	0.447	0.516	0.0258	0.0115	(likely lysozyme c)
Contig1060	0.445	0.488	0.00796	0.0573	gelatinase, matrix metalloproteinase 9
Contig1106	0.441	0.443	0.00835	0.0199	trypsin
Contig747	0.44	0.625	0.0264	0.0355	actin related protein 2/3 complex subunit 5, 16 kDa
					Myo-inositol monophosphatase

Contig41	0.439	0.566	0.00771	0.00636	Lysozyme c
Contig994	0.429	0.477	0.0179	0.00888	glyceraldehyde 3-phosphate dehydrogenase, GAPDH
Contig527	0.423	0.419	0.00564	0.0332	type II keratin
PfIL211F10	0.419	0.833	0.006	0.531	importin 9
Contig429	0.418	0.389	0.0144	0.00607	hepcidin precursor
Contig774	0.416	0.53	0.0208	0.0216	MHC II invariant chain
PfIL243B02	0.412	0.234	0.0242	0.0126	immunoglobulin D heavy chain
Contig685	0.408	0.472	0.0119	0.0276	High choriolytic enzyme 1 precursor
Pp005	0.405	0.512	0.0294	0.0479	Fatty acid binding protein
PfIL277H02	0.405	0.484	0.0452	0.028	Ubiquitin-protein ligase RMA1
PfIL290E09	0.401	0.54	0.0311	0.0126	Myeloid-associated differentiation marker
PfIL276A07	0.396	0.481	0.006	0.0293	phosphatidate phosphohydrolase type 2a
PfIL202H02	0.394	0.564	0.0186	0.0879	chaperonin containing TCP-1 delta
PfIL251H03	0.369	0.417	0.0345	0.0176	unnamed protein product CAG01129
Contig346	0.365	0.461	0.019	0.188	unnamed protein product CAF90585
Contig624	0.35	0.648	0.0322	0.224	Sulfatase modifying factor 2 precursor
PfIL227F02	0.343	0.496	0.0345	0.0758	complement component 1 q subcomponent
PfIL305B08	0.338	0.896	0.0133	0.536	GATA-binding protein 6
PfIL245C07	0.324	0.33	0.00606	0.0126	Integrin alpha5
PfIL249C07	0.311	0.609	0.00564	0.212	nucleoporin 214kDa
PfIL313F10	0.301	0.204	0.0397	0.0844	Rho GTPase activating protein 19
PfIL214A05	0.296	0.705	0.006	0.376	18S ribosomal RNA
Contig804	0.269	0.212	0.00796	0.000757	immunoglobulin light chain precursor
PfIL288C01	0.131	0.116	0.006	0.00607	type I keratin S8