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Diablo/SMAC; a novel biomarker of pollutant exposure in European flounder (*Platichthys flesus*).

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Abstract

Diablo (or SMAC) is a protein released from mitochondria following apoptotic stimuli and inhibits the actions of Inhibitors of Apoptosis (IAP) proteins. IAPs regulate the activity of caspases and NFkB, the primary executioners of apoptosis and of inflammation respectively. Thus, Diablo is important for the regulation of cellular responses to damage. In Northern Europe, statutory governmental marine monitoring programs measure various biomarkers in flounder to indicate biological effects of pollutant exposure. More recently transcriptomic techniques have been applied in flounder to gain a more comprehensive understanding of pollutant effects, and to discover novel biomarkers. In most of these studies utilising flounder, Diablo was amongst the most highly increased transcripts identified. The aim of this study was to further examine piscine Diablo, at the gene level and mRNA level, after exposure to prototypical pollutants, and in flounder caught from polluted environments. The results show that two genes encoding Diablo exist in fish species, and in flounder one of these genes is increased in liver after exposure to polyaromatic hydrocarbons and polychlorinated biphenyls, and also in livers from fish living on contaminated estuarine sediments. Therefore, Diablo measurement has potential as a biomarker of pollutant exposure, and could indicate damaging effects of chemical contaminants.

Keywords: Diablo, SMAC, pollutant, environment, biomarker, flounder, Platichthys flesus

1. Introduction

The assessment of chemical pollutant status in coastal waters and sediments is an important aspect of environmental protection. Various government agencies have embarked upon the development of methodologies for setting limits for individual priority contaminants, these limits being set on the basis of results from laboratory toxicological tests on test species (Lyons et al., 2010). Examples of this approach include those used by OSPAR in Europe (Thain et al., 2008) and NOAA in North America (Long et al., 1998) whereby Environmental Assessment Criteria (EAC) and Sediment Quality Guidelines (SQG) have been in continuous development over a number of years. Thus, priority substance-specific EACs and SQGs are defined as a level of chemical contamination in the environment below which it is unlikely that unexpected or unacceptable biological effects will occur in marine species. However, this approach, based on data from single chemical acute laboratory exposures on a limited number of compounds and test species, may not accurately predict pollutant-related biological effects in the field, where much broader ranges of species are often exposed long-term to multiple pollutants. For this reason many monitoring programs also include a variety of biological effects measures on sentinel species such as European flounder (*Platichthys flesus*), and can include measurements of vitellogenin (which is responsive to endocrine disrupting chemicals), cytochrome P401A (CYP1A, responsive to polyaromatic and polyhalogenated aromatic hydrocarbons), and liver histopathology among others. One of the advantages of using these techniques is that they can indicate links between contaminant exposure and biological effects, as well as detecting the impact of substances (or combination of substances) that may not be analysed as part of routine chemical monitoring programmes (van der Oost et al., 2003; Thain et al., 2008).

More recently the measurement of global gene expression profiles using *P. flesus* high density DNA microarrays has been tested as a means of inferring a more comprehensive assessment of fish health in coastal environments, and also as an unbiased method for discovering novel biomarkers of pollutant exposure (Williams et al., 2008; Falciani et al., 2008; Leaver et al., 2010). The deployment of these microarrays in *P. flesus* and in other species has not always generated the gene expression profiles that might be predicted from knowledge of contaminants, their acute toxic effects, and their associated biomarkers. The reasons for this may include the likelihood that global gene expression profiles may be site-specific, varying in response to natural environmental variables such as salinity, food preference, temperature and genetics, all effects which could mask pollutant signals or confound analyses. In addition, in the wild, organisms are usually exposed to complex mixtures of pollutants over longer periods than the laboratory studies of single chemical exposures which have been the main method for the discovery of biomarkers. However, at least three separate studies utilising a cDNA microarray on *P. flesus* exposed to sediments containing complex mixtures of pollutants have shown that the mRNA for a pro-apoptotic gene, Diablo, is amongst the most significantly increased features (Williams et al., 2008; Falciani et al., 2008; Williams et al., 2011). In addition, in a long-term mesocosm experiment, designed to eliminate confounding environmental variables during exposure to multiply-polluted sediments, Diablo was the most highly increased transcript of all microarray features (Leaver et al., 2010) despite a lack of response in other “traditional” biomarker genes.

Diablo (direct IAP binding protein with low pI), also known as SMAC (second mitochondria-derived activator of caspase), is one of several proteins released into the cytoplasm from mitochondria following an apoptotic stimulus. Diablo/SMAC binds to and blocks the activity of members of the IAP (inhibitor of apoptosis proteins) family of proteins (Du et al., 2000; Verhagen and Vaux, 2002; Verhagen et al., 2000). IAPs inhibit the primary effectors of apoptosis, caspase 3 and caspase 7, as well as initiator caspases, such as caspase 9 (Salvesen and Duckett, 2002). Thus, the effect of an increase in cytoplasmic Diablo/SMAC is to potentiate or sensitize cells to apoptotic stimuli by removing the inhibiting effect of IAPs (Hunter et al., 2007). IAPs also influence other cellular processes, in particular pathways leading to the activation of nuclear factor κB (NFκB) transcription factors, which in turn drive the expression of genes important for inflammation, immunity, cell migration and cell survival (Gyrd-Hansen and Meier, 2010). Both apoptotic and NFkB-dependent cellular processes are frequently deregulated in cancer and contribute directly or indirectly to disease initiation, and tumour maintenance and progression (Hunter et al., 2007). The importance of Diablo/SMAC and IAPs in carcinogenic processes had led to the development of compounds which are structural mimics of Diablo/SMAC. These have been shown to increase the sensitivity of cancer cells to drug treatment and are showing considerable promise as anticancer drugs (Flygare and Fairbrother, 2010).

The objective of this study was to extend the transcriptomic studies described above by assessing the potential of Diablo as a biomarker of pollutant exposure in *P. flesus*. This was achieved by identifying and comparing Diablo genes in *P. flesus* and other fish species, and by measuring mRNA expression in *P. flesus* tissues, in experimentally treated fish and in *P. flesus* caught in clean and polluted UK estuaries.

2. Materials and methods

2.1. Tissues, experimental treatments and environmental samples

For experimental procedures, gonadally immature male *P. flesus* (mass, 96-115g; length 20-23cm) were obtained by trawl from an unpolluted area of the Scottish East coast and maintained in filtered seawater in 1.5 m^2 fibreglass tanks for 3 months at 10^oC prior to use. During this period fish were fed with with commercial pellets (Bio-Optimal START 1.5mm, Biomar, 1% of body weight/day). For experimental treatments with prototypical pollutants fish were injected intraperitoneally with 1 mL kg^{-1} olive oil containing 100 mg ml^{-1} perfluorooctanoic acid (PFOA; n=4), 50 mg ml^{-1} Arochlor 1254 (ARO; n=3), 5 mg ml^{-1} Lindane (LIND; n=3), or 25 mg ml^{-1} 3-methylcholanthrene (3MC; n=3). Control fish were treated with 1 mL kg^{-1} olive oil alone (CON, vehicle control; n=3). These amounts and numbers of animals were chosen based on previous reports indicating minimum numbers of animals and doses required to establish the effects of prototypical pollutants (Williams et al., 2008). Injected fish were transferred to aerated static 0.5m^2 tanks, (3-5 fish per tank) containing filtered seawater (10^oC) which was changed daily for three days. The flounder were not fed during this period. After three days animals were killed by a blow to the head and a set of organs was dissected to evaluate the expression profile of investigated genes: liver, kidney, intestine, gill, heart, spleen, muscle and brain. Tissues were homogenised in 10 volumes of TriReagent (Sigma, UK) before storage at -80^oC until required for RNA preparation. These procedures were performed under license to, and in accordance with United Kingdom

Home Office regulations governing animal experimentation, and following oversight by an institutional ethics review committee.

For environmental samples, livers were collected from male *P. flesus* (mass, 41-241g; length 15-30cm) living in four different UK locations of differing pollutant status (Tyne, Morecambe Bay, Alde, and Mersey). Fish were selected for analysis on the basis of being indentifiably male, but with undeveloped (or resorbed) testes. Sampling was from two sites in the Irish Sea; the Mersey estuary, at Eastham Sands, Liverpool (lat 53^{o}19N, long 2^{o}55W) and Morecambe Bay (lat 54^{o}10N, long 2^{o}58W), and two sites In the North Sea; the Alde estuary, Suffolk (lat 52^{o}95N, long 01^{o}33E) and the Tyne estuary at Howdon, Tyne and Wear (lat 54^{o}57N, long 01^{o}38W). Fish were caught using beam trawls during statutory monitoring programs carried out by the UK Centre for Environment, Fisheries and Aquaculture Science (CEFAS) in April 2006. Liver samples (100 mg) from 10 male *P. flesus* from each site were immediately dissected and flash frozen in liquid nitrogen, then stored at -80°C until required for RNA preparation.

2.2 Liver Histopathology

Flounders were examined for external lesions, liver gross appearance and parasite infection. Sections of liver tissue were removed, placed into individual histological cassettes, transferred to 10% neutral buffered formalin and processed for histopathology as described previously Stentiford et al, 2003. Liver pathology was assessed according to the criteria of Feist et al., 2004.

2.3. Total RNA extraction and synthesis of P. flesus cDNA

Total RNA extraction was performed for all the samples using TriReagent RNA extraction buffer (Sigma UK) according to the manufacturers protocol. Final RNA concentrations were measured using ND-1000 Nanodrop spectrophotometer (Labtech Int., UK) and integrity checked (ratio of 28S:18S rRNA banding intensity) by electrophoretic separation and visualization of 1 μg of total RNA from each sample using an agarose gel.

2.4. Synthesis of P. flesus Diablo cDNAs

By sequence similarity with mammalian Diablo/SMAC, several candidate European *P. flesus* ESTs (EC377887, DV567420, DV566149, DV566726, AJ580508, DV567320), and ESTs from related Pleuronectid flatfish (eg. EU412105) were identified in Genbank/EMBL databases. Using these flatfish sequences, oligonucleotide primers were designed to amplify *P. flesus* cDNAs.

Total *P. flesus* cDNA was generated from 1μg of intestine total RNA using the SMART 3' and 5' RACE kit according to the manufacturers instructions (Clontech). The resulting 3' and 5' RACE cDNA was then subjected to PCR using primers (Diablo1FullF1, Table 1), designed to target the 5' untranslated region predicted from a *P. flesus* EST, or designed from a halibut EST (*Hippoglossus hippoglossus*, hhDiablo2R, EU412105), each paired with the Universal Primer Mix supplied with the SMART RACE kit. The thermocycling program consisted of 5 cycles at 95°C for 20 sec, 72°C for 2 min followed by 5 cycles at 95°C for 20 sec, 70°C for 30 sec, 72°C for 2 min, followed by 25 cycles at 95°C for 20 sec, 63°C for 30 sec, 2 min at 72°C with 5 min of final extension at 72°C. Following sequencing of products, further amplicons

were generated on 3' and 5' SMART cDNA templates using primers QflDiablo2F and QflDiablo2R respectively, each paired with the Nested Universal Primer supplied with the SMART RACE kit.

Following purification, amplicons were ligated into pCR 2.1 (Invitrogen, UK) and sequenced using a Beckman 8800 autosequencer (Beckmann Coulter, UK) and contigs assembled using Lasergene SEQman software (DNASTAR, USA).

Table 1. Primers and gene IDs used for cDNA isolation and QPCR.

PRIMER	SEQUENCE (5' – 3')	TARGET mRNA	REACTION TYPE
Diablo1FullF1	TATCCACCAGCAGACAGAAAG	DV567420	3' SMART RACE
QflDiablo1F	ATGTCGCTGCTGCCGAGCAG	JN686640	qPCR
QflDiablo1R	GCAGCGCTGGTCCATTCCCC	JN686640	qPCR
hhDiablo2R	TTAGTCTTCTCTCAGATAAGCTTCAGG	EU412105	5' SMART RACE
QflDiablo2F	GCGGTGGGCTGTGTGCTGTAC	JN686641	3' SMART RACE, qPCR
QflDiablo2R	ACCTCTGCCCGCTGGCCAAT	JN686641	5' SMART RACE, qPCR
b-ActinF	GACCACTGGGATGACATGG	AF135499	qPCR
b-ActinR	GCGTACAGGGACAGCACAGC	AF135499	qPCR
Cyp1AF	CAACCATGATCCAGAGCTGTG	AJ132353	qPCR
Cyp1AR	GATTATTCTTCCTCCACTGACTCT	AJ132353	qPCR
Ugt1BF	CCTTCCCGCAGAGAGTCATA	AM746199	qPCR
Ugt1BR	AGAGAGCCCCATGACTGAGA	AM746199	qPCR
18SF	CTGCCCTATCAACTTTCGATGGTACT	DV566337	qPCR
18SR	AAAGTGTACTCATTCCAATTACAGGG	DV566337	qPCR

2.5. Phylogenetic analysis

TBLASTN in the Ensembl genome browser (http://www.ensembl.org/index.htmLl) was used to identify genes homologous to *P. flesus* Diablo in the genome sequences of medaka (*Oryzias latipes,*), pufferfish (*Tetraodon nigroviridis*), zebrafish (*Danio rerio*), and stickleback (*Gasterosteus aculeatus*), as well as in human (*Homo sapiens*), frog (*Xenopus tropicalis*) and bird (*Taeniopygia guttata*). The predicted proteins encoded by areas of genomic sequence with high homology to *P. flesus* Diablo were then derived using Wise2 (www.ebi.ac.uk/Wise2/). The resulting Diablo polypeptide sequences were aligned using ClustalW (Chenna et al., 2003), and phylogenetic relationships between Diablo from different organisms were inferred from the similarities of all pairwise protein alignments by the Neighbour Joining method (Saitou and Nei, 1987), as implemented by ClustalW. Confidence in tree topology was estimated by bootstrapping permutated data through 1000 iterations.

2.6. Quantitative RT-PCR (qPCR)

Complementary DNA was prepared by first denaturing 1 µg of total RNA at 70°C for 5 min and then adding, in a reaction volume of 20 µL, 300 ng of random hexamers, 125 ng of anchored oligo-dT, 0.5 mM dNTPs, 4 µL ImProm-II™ 5X Reaction Buffer and 1µL of ImProm-II™ Reverse Transcriptase (Promega). The synthesis reaction was carried out at 42^{o}C for 1 h and then stopped by heating at 75°C for 10 min. Finally, all the cDNA reactions were diluted

to 200 µL total volume (dilution 1:10) with nuclease free water and stored at -20°C until required for qPCR. All primers used for qPCR are listed in Table 1. Quantitative RT-PCR was performed on 2 µL of each diluted experimental and environmental cDNA sample (equivalent to 10 ng of input RNA) in reaction volumes of 20 µL, containing 10 µL of SYBR Green mastermix (FastStart Universal SYBR Green Master, Roche, Germany), and 300 nM each of gene-specific primer. β-actin was employed as a reference gene for both experimental treatments and environmental samples, and 18S rRNA for tissue expression profiles (as in Leaver et al., 2007). Reactions were initiated by heating to 95°C for 15 min followed by 45 cycles of 95°C for 15s and annealing at appropriate temperatures (Table 1) for 15 secs, followed by 72^{o}C for 1min at which point flourescence data was collected. After 45 cycles, a melt curve was generated by measuring sample fluorescence during heating from 75 to 95°C. The specificity of reactions was checked by inspecting melting curve profiles and by sequencing of amplicons from a random selection of samples. Determination of amplification efficiencies was measured using a dilution series of a pool of cDNA samples. All amplification efficiencies were over 95%. Relative expression was calculated by applying the 'delta Ct' or the "delta-delta Ct" method (Pfaffl, 2001) with the reference gene, β-actin, and using control samples as indicated in the results sections below.

3. Results

3.1. Sequence and phylogenetic analysis of P. flesus Diablo/SMAC

In silico assemblies of all *P. flesus* cDNA fragments clearly indicated the existence of two distinct genes for Diablo in this species. The first of these, hereafter pfDiablo1, possessed a complete reading frame encoding a polypeptide of 258 amino acids. The second, hereafter pfDiablo2, was assembled from overlapping 3' and 5' RACE products and contained an open reading frame encoding a polypeptide of 235 amino acids. Alignment of these proteins with the single Diablo protein from human demonstrates extensive similarity (Figure 1).

```
pfDiablo1     MQVVRQCSASASRAAGGFLRNPTDMSLLPSRRGAVCSRDLSLESSPLSSRKFGVQKSGEWTSAAHMSIAS
pfDiablo2     MAAVR.........................RGAAYFFRSSAR.VLFNCKNTAVHKPRKWTNVLYTSLAS
hsDiablo      MAALKSWLS.....................RSVTSFFRYRQCLCVPVVANFKKRCFSELIRPWHKTVT.

pfDiablo1     LSVARGLFT...QQVET...LTHDSLIRRAVSVVTDSSSTFLSQITLALIDALTHYSKAVHTRIAVQRRY
pfDiablo2     LAVGGGLCAVPFKQVEQ...LSHDSLIRRAASLVTDSSTTFLSQATLALIDAIDEYSKAVHILNALQRKY
hsDiablo      IGFGVTLCAVPIAQKSEPHSLSSEALMRRAVSLVTDSTSTFLSQTTYALIEAITEYTKAVYTLTSLYRQY
                      $$$$
pfDiablo1     LASVGKLTSFEEDSHKQAINAMRAEVTYRLDDCKRFESSWINAVNLCKMAAEAANTSGAEQASISVKTNI
pfDiablo2     LTSLGKLTKDEEDSIWQVIIGQRAEVNDRQDECKRFESTWVSAVKMCEMAADAAYTSGADHASITMNSNL
hsDiablo      TSLLGKMNSEEEDEVWQVIIGARAEMTSKHQEYLKLETTWMTAVGLSEMAAEAAYQTGADQASITARNHI

pfDiablo1     QVAQSQVEEARRVSADAEKKLAETKVEEIQRMAEYAASFDDE.EHEVHEAYLRED
pfDiablo2     EVALSQVEKAQKLSTEADKKLAETKVMEVQRMAQHSATVQNNDEEEMPEAYLRED
hsDiablo      QLVKLQVEEVHQLSRKAETKLAEAQIEELRQKTQEEG...EERAESEQEAYLRED
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Figure 1. Diablo protein alignment.

Residues identical in two of the three proteins are shaded. The IAP binding motif is underscored with $. hs, human; pf, *P. flesus*

Searches of the existing fish genome sequences (zebrafish, pufferfish and medaka) showed the existence of homologous genes of both pfDiablo1 and pfDIablo2. In the genomes of the tetrapods, *X. laevis*, *T. guttata* and *H. sapiens* only one Diablo gene is present. Phylogenetic reconstruction (Figure 2) of the relationships between fish and Tetrapod Diablo genes is not

entirely unambiguous. Whereas all Acanthopterygian fish (*P. flesus* , pufferfish, medaka and stickleback) have two well supported distinct clusters representing Diablo1 and Diablo2 which are in turn distinct from the sole Tetrapod Diablo, the Cyprinid (zebrafish) Diablo genes are not so clearly separated. Examination of the chromosomal positions and the adjacent genes to Diablo in Acanthopterygians and zebrafish (not shown) indicate that all Acanthopterygians (ie medaka, pufferfish, stickleback) have duplicated Diablo genes side by side on a single chromosome with extensive synteny in this region. In contrast the zebrafish has two genes on seperate chromosomes which exhibit synteny with each other and with the Acanthopterygian species.

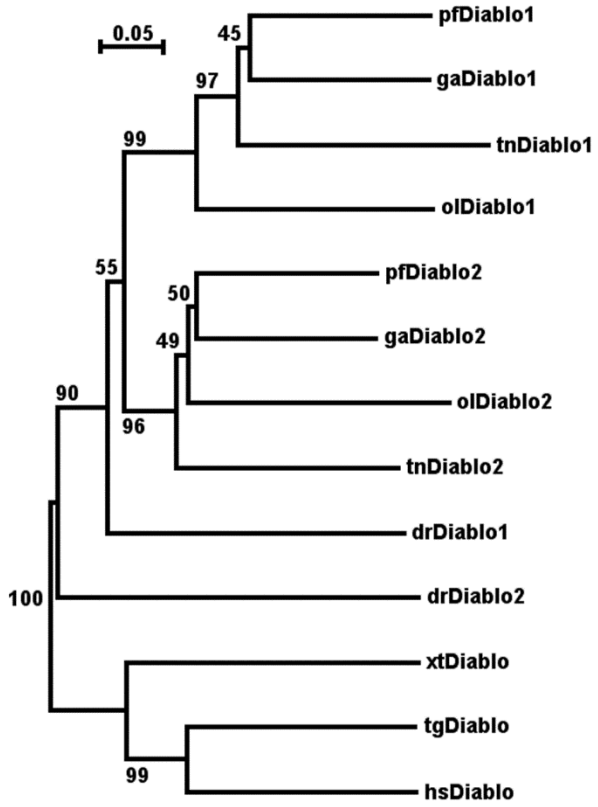

Figure 2. Phylogenetic comparison of vertebrate Diablo proteins.

The tree was constructed from a Clustal multiple alignment of the deduced polypeptide sequences of Diablo coding sequences. Coding sequences were taken from Ensembl genomic databases. *T. nigroviridis* sequences have not been previously annotated and were predicted from the gene sequences by homology with other fish proteins. Numbers represent the percentage of times the tree topology was returned after computing trees from 1000 random samplings of the alignment. hs, human; dr, zebrafish; tn, pufferfish; ol, medaka; ga, stickleback; pf, *P. flesus* ; xt, frog; tg, bird. Diablo1 and 2 are two different isoforms in fish.

3.2. Tissue expression of P. flesus Diablo genes

The mRNA expression of each Diablo gene was measured in several different tissues from 4 individual male *P. flesus* maintained in filtered seawater for 3 months (Figure 3). Expression levels of both pfDiablo1 and pfDiablo2 between individuals tended to be quite variable, and levels of Diablo1 varied greatly across tissues. However it was clear that the highest expression level of any gene was that of pfDiablo1 in liver. Kidney, heart and spleen also showed measureable Diablo1 expression, although less than liver. In contrast, pfDiablo2 was

expressed at lowest level in liver, and showed a higher and similar expression in all other tissues tested. In tissues other than liver Diablo2 tended to be expressed at higher levels than Diablo1, although in any particular tissue this difference was not statistically significant (T-test, $p<0.05$).

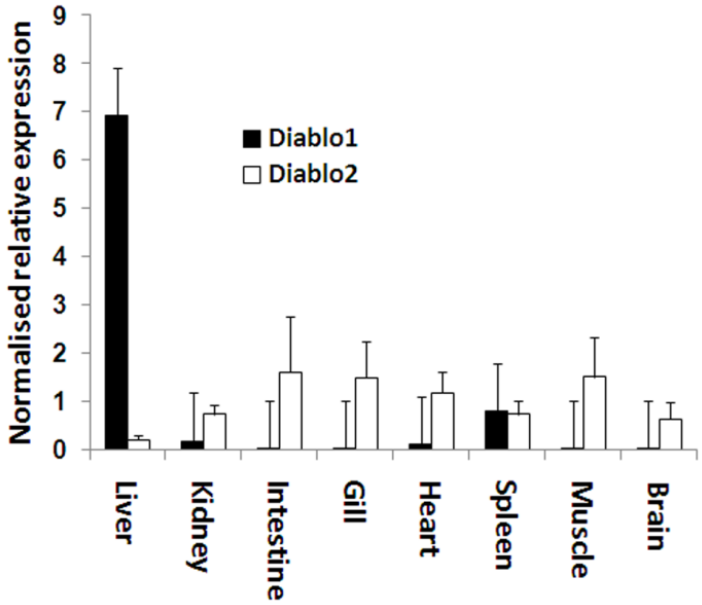

Figure 3. Tissue expression of *P. flesus* Diablo 1and 2.
Generated from qPCR as described in the text. Diablo mRNA values are normalized to 18S rRNA. Arbitrary expression levels are calculated according to the "delta Ct" method. Error bars represent standard deviations of the mean (n=4 fish) normalized to the median expression level across tissues for each individual.

3.3. Gene expression in experimental treatments and environmental samples

Expression of the two pfDiablo mRNAs in liver after intra-peritoneal injection of different chemical contaminants is shown in Figure 4. Data were also compared with CYP1A and UGT1B mRNA expression values. PfDiablo1 gene expression after treatments was not significantly different from the control group in all the pollutants investigated. In contrast pfDiablo2 mRNA was significantly elevated after Arochlor1254 and 3MC treatments, approximately 8- and 3-fold respectively. No significant effects on pfDiablo2 were observed after lindane or PFOA treatment. As expected, the expression of CYP1A, and UGT1B, biomarkers of polyaromatic and polyhalogenated hydrocarbon exposure, were increased after 3MC (CYP1A) and Arochlor (UGT1B) treatment. The increase in CYP1A expression in the Arochlor treatment, although not statistically significant ($p = 0.06$), is likely to be biologically significant given the magnitude of effect and previous evidence (Williams et al, 2008).

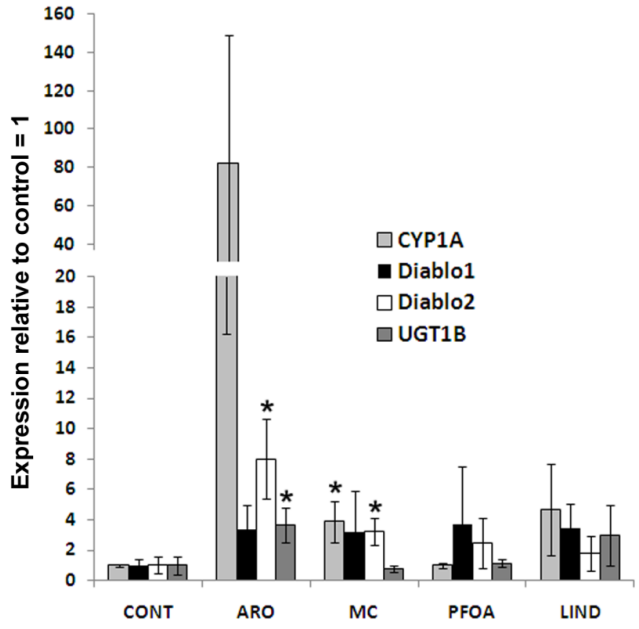

Figure 4. Expression of *P. flesus* Cyp1A, Diablo 1 and 2 mRNA after treatment with prototypical chemical pollutants.

Figure is generated from qPCR data as described in the text. Relative expression level is calculated using the "delta-delta Ct" method using expression of β-actin as a reference gene. Values are plotted relative to control set to 1. Effects of i.p.-injection of, Arochlor 1254 (ARO, n = 3 individuals), 3-methycholanthrene (3MC, n = 3 individuals,) perflurooctanoic acid (PFOA, n = 4 individuals) or lindane (LIND, n = 3 individuals). Vehicle control is olive oil (CON, n = 3 individuals). Asterisks indicate significant difference from control ($p < 0.05$, Student's t-test).

Expression of CYP1A and both pfDiablo mRNAs in flounder livers from UK estuaries of differing pollutant status is shown in Figure 6. The locations known to be the most polluted, Tyne, Mersey, and Morecambe Bay were compared with Alde, the least polluted. At the time of fish sampling levels of a variety of sediment contaminants were also determined by Cefas UK. These results have been reported previously (Williams et al., 2011) and are summarized for the major contaminants in Figure 5. The most contaminated sites are the Mersey and Tyne, both industrialized estuaries, and levels of lead, polyaromatic hydrocarbons and polychlorinated biphenyls exceed the effects range low (ERL) threshold for biological effects (Long et al., 1998). Pathology and histopathology results showed that high numbers of fish (80% of each group) in Alde, Morcambe Bay and Mersey were infected by *Lepeophtheirus* and *Acanthochondria* copepod parasites, whereas at the Tyne site these parasites were much lower (20%). Gross liver appearance showed no abnormalities and no tumours were observed. Liver histopathology showed a variety of possible early neoplastic lesions, foci of cellular alteration and non-specific inflammatory lesions. In total 80% of Tyne fish showed abnormality in at least one of these categories, 30% at the Alde site and 20% at each of the Morcambe Bay and Mersey sites. The majority of these lesions were categorised as fibrillar inclusions and melanomacrophage centres.

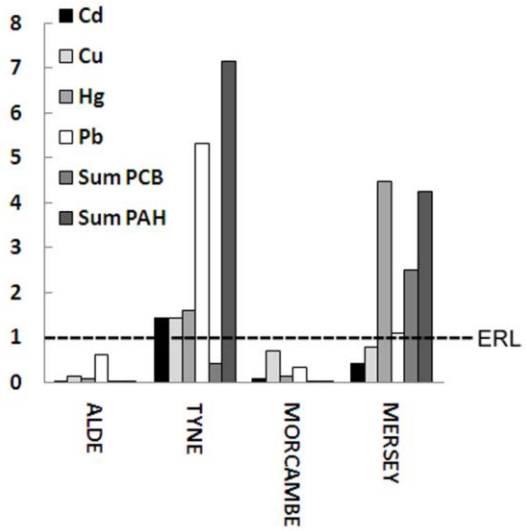

Figure 5. Relative levels of sediment contaminants at flounder sampling sites.
Data are taken and adapted from Williams et al.,2011, and plotted relative to the Effects Range Low (ERL) sediment quality criteria (Long et al., 1998). Sum PAH is the sum of concentrations of 16 priority PAH compounds (USEPA, 1993). Sum PCB is the sum of concentrations of 7 priority chlorinated biphenyls (ICES, 2003). Actual concentrations (per Kg dry weight sediment) at the Tyne site were: Cd, 1.72 mg/Kg; Cu, 48.75mg/Kg; Hg, 0.24mg/Kg; Pb 248.20 mg/Kg, Sum PCB, 9.41 mg/Kg; Sum PAH 23562.3 mg/Kg.

In the case of Tyne, CYP1A and pfDiablo2 mRNA expression values were found to highly significantly different to control group ($p<0.01$) and were increased approximately 9-fold for CYP1A and 2-fold for pfDiablo2. No significant differences in pfDiablo1 were observed and the expression of this gene was highly variable within groups. Similarly in the Mersey location, CYP1A was increased up to 3-fold and pfDiablo2 up to 2-fold. At the Morecambe Bay site the results showed no significant differences to Alde in any of the genes analyzed.

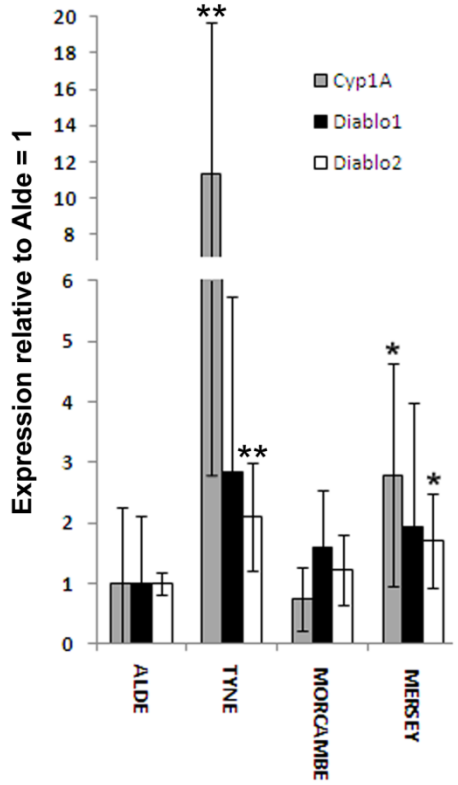

Figure 6. Expression of Cyp1A and Diablo mRNAs in different UK estuaries of differing pollutant status.

Figure is generated from qPCR as described in the text. Expression levels are calculated according to the "delta-delta Ct" method using expression of β-actin as a reference. Values at sites (Mersey, n=10 individuals; Tyne, n=10 individuals; and Morecambe Bay n=10 individuals) are plotted relative to Alde (n=10 individuals). Asterisks indicate significant difference from Alde (*,$P < 0.05$; **, $P<0.01$; Student's t-test).

4. Discussion

Examination of fish genome and cDNA sequence databases clearly shows that fish species have two genes with high similarity to the single Diablo/SMAC of humans, rodents (Du et al., 2000; Verhagen et al., 2000) and amphibians (Montesanti et al., 2007) . The major difference between the two fish genes is in the structure of the N-terminal region. In mammals this region contains a mitochondrial targeting sequence, which, following mitochondrial import, is cleaved and the mature protein sequestered in mitochondria. Apoptotic stimuli cause the release of mature SMAC, along with several other apoptotic proteins from the mitochondrion. The N-terminal amino acid residues of the mature mammalian Diablo/SMAC comprise an IAP binding motif, which enable complexation with cytosolic IAP proteins in turn suppressing the inhibitive effect of IAP on caspases (Du et al., 2000; Verhagen et al., 2000). Although several reports have indicated that IAP binding is dependent on the presence of an IAP binding motif (IBM) comprising four N-terminal amino acid residues (AVPF), recent evidence suggests that IAP binding extends beyond the four N-terminal IBM residues and that a truncated Diablo/SMAC lacking the IBM is also able to bind IAP and to potentiate apoptosis (Burke and Smith, 2010). Furthermore the identification and characterisation of Smac β, a cytosolic Smac/DIABLO splice variant that lacks the mitochondrial targeting sequence and IAP-binding domain (Roberts et al., 2001) has shown that proapoptotic activity can occur independently of IAP-binding. The activity of cytosolic Diablo/SMAC is also dependent on dimerisation which may be regulated by post-translational modification of a dimerisation interface (Burke and Smith, 2010; Chai et al., 2000).

One of the fish Diablo/SMAC forms, here termed Diablo2, is very similar to the mammalian protein, having an N-terminal mitochondrial targeting sequence, immediately before an IBM and, based on this structural similarity, can be inferred to function in the same way as the mammalian Diablo/SMAC. However the second fish form, here termed Diablo1, contains a longer N-terminal region which in most fish species does not contain a recognisable IBM. It also appears from the phylogenetic tree topology that the duplication of Diablo genes in Acanthopterygian fish post-dates the evolutionary split from Tetrapods. However in the Cyprinid, zebrafish, it appears that one form of Diablo clusters more closely with the Tetrapod sequences, while the other is an early branch of the Acanthopterygian cluster, suggesting an alternative evolutionary scenario where Tetrapods have lost a Diablo gene retained by Cyprinids. This scenario is supported by the chromosomal evidence in sequenced fish genomes. The presence of two Diablo genes on separate chromosomes with synteny, at least in the region of Diablo, suggest that it is likely that zebrafish have retained two Diablo genes which probably arose from a whole genome duplication at the base of the teleosts (Meyer and Peer, 2005). The intrachromosomal duplication of Diablo in Acanthopterygians suggest that they have lost one of these basal teleost chromosomal duplicates, but have then gained a second Diablo by a segmental duplication within the

other chromosome. It is also notable that both of the zebrafish Diablo forms have retained a recognizable IBM (not shown), whilst only Diablo2 of the Acanthopterygians has an IBM.

As discussed above, four human forms of Diablo/SMAC are expressed by alternative splicing and at least three of these forms can promote apoptosis to some extent, despite lacking, depending on form, IBMs, dimerization interfaces, or mitochondrial targeting sequences (Burke and Smith, 2010; Roberts et al., 2001; Fu et al., 2003). Importantly, all vertebrate Diablo/SMAC proteins have a conserved C-terminal domain. The C-terminal domain of Diablo/SMAC is functionally less well characterised than the N-terminal IAP-interacting domain, but the high degree of structural conservation of the C-terminal region of all Diablo/SMAC forms across all vertebrates suggests that an IAP-independent proapoptotic function is a common and important feature of this region of the protein.

The two *P. flesus* Diablo genes show considerable differences in expression across tissues in untreated fish. pfDiablo2, like human Diablo/SMAC (Du et al., 2000) has a broad tissue expression profile, whilst pfDiablo1 was restricted to liver, kidney, heart and spleen. In most tissues, pfDiablo2 mRNA is expressed at higher level than pfDiablo1. The exception is liver where pfDiablo1 is predominant. However, there was a relatively high level of interindividual variability in expression, particularly of pfDiablo1 in non-hepatic tissues, which meant that, other than in liver, it was not possible to conclude that expression levels of pfDiablo1 and pfDiablo2 were statistically different within or between tissues. In this study no alternatively spliced forms of *P. flesus* Diablo2 were detected, although there was some evidence for alternative *P. flesus* Diablo1 transcripts (not shown). The PCR primers used to determine expression of pfDiablo1 would not have amplified the alternative form, and we cannot rule out the presence of other alternatively spliced Diablo, and potentially differentially expressed transcripts giving rise to multiple protein products, as observed in humans.

In the livers of *P. flesus* treated with model environmental contaminants there were clear differences in mRNA expression levels of pfDiablo1 and pfDiablo2. Message for both genes was increased by Arochlor 1254 and 3MC, but not by PFOA or lindane. As positive controls we also measured the effects of these compounds on CYP1A and UGT1B, genes which are well known to be induced in flounder by planar polyaromatic and polyhalogenated hydrocarbons (Leaver et al., 2007; Leaver et al., 1993). As expected, these mRNAs were also increased after Arochlor and 3MC, but not by PFOA or lindane treatment. This suggests the possibility that pfDiablo may be part of the Ah gene battery. The Ah receptor is a vertebrate transcription factor which binds and is thus activated by planar polyaromatic and polyhalogenated hydrocarbons, resulting in the transcriptional up-regulation of genes containing Ah-responsive promoters, CYP1A being the prototypical example (Beischlag et al., 2008). However, there was no correlation between CYP1A expression and either pfDiablo gene (not shown), which would argue against a direct Ah receptor-mediated mechanism for *P. flesus* Diablo1 or Diablo2 transcription.

Although there was a response of pfDiablo1 and pfDiablo2 to acute challenge by certain model pollutants, the main aim of this study was to determine response in wild *P. flesus* chronically exposed at multiply polluted field sites. Variables such feeding status, sex, age, temperature, season etc. may influence gene expression and this is clearly recognised in biomonitoring studies of the type described here (Thain et al, 2008). We were careful to

control those variables over which we had influence. Accordingly, both the experimental treatments and fish sampling were carried out at the same time of year, and, as in standard montoring exercises usin flounder, only male fish which were either sexually immature, or had resorbed testes were used. Firthermore, in order to minimise any possible population genetic effects, we selected pairs of clean and polluted sites in the same geographical regions; Irish Sea (Mersey and Morcambe Bay) and southern North Sea (Tyne and Alde). Despite the large interindividual variability in fish collected from field sites, there was a significant increase in pfDiablo2 at the most polluted sites (Mersey and Tyne), compared to the least polluted (Alde and Morecambe Bay). The polluted sites each had multiple contaminants present at levels exceeding theoretical threshold limits for effects. In addition, at these polluted sites, and also at the relatively less polluted Morcambe Bay site, a higher level of hepato-toxicopathological lesions compared the Alde site was observed. Interestingly the prevalence of parasitic copepods was lower at the Tyne site. This pattern of pathology and parasite infection is consistent to that previously reported in other flounder taken from the same sites. (Stentiford et al., 2003; Williams et al., 2011).

Notably, whilst both the Tyne and Mersey sites had raised PCB and PAH, only the Tyne site showed higher CYP1A, whereas an effect on Diablo2 was observed at both polluted sites, indicating that pfDiablo2 may be more specific than CYP1A for detecting pollutant effects. However the response of CYP1A was greater than that of pfDiablo2, indicating that CYP1A may be more sensitive in some situations. The reasons for the lack of significant response of CYP1A in the Mersey site, despite similar PAH and PCB sediment levels to Tyne is not clear, but in a mesocosm experiment a similar lack of CYP1A response and an increase in Diablo1 was observed despite higher levels of PAH and PCB in contaminated sediment (Leaver et al., 2010). In this mesocosm experiment mRNA levels were measured by microarray, and only pfDiablo1 was represented on the array, but, given the sequence similarities between regions of pfDiablo1 and pfDiablo2, the possibility of cross-hybridisation between pfDiablo forms cannot be excluded.

Taken together the results indicate that exposure to certain pollutants, and possibly mixtures of pollutants, cause an increase in the mRNA of pfDiablo2 in *P. flesus*. It is also possible from previous results (Leaver et al., 2010) that pfDiablo1 is similarly increased, although in the results reported here the inter-individual variability in the expression of this gene was too large to come to a conclusion that it was responsive to pollutants.

In most mammalian studies involving Diablo/SMAC, measurements are made of cytosolic protein following release from mitochondria after apoptotic stimuli, and we have not yet assessed the significance of *P. flesus* Diablo mRNA as regards protein levels. There have been some experimental studies where mammalian Diablo/SMAC mRNA and gene expression have been measured. Diablo/SMAC is deregulated in cancer (Martinez-Ruiz et al., 2008) and treatment with combinations of drugs increases mRNA expression in some cancerous cells (Lu et al., 2010). More direct evidence of transcriptional responses comes from analyses of the human Diablo/SMAC promoter which required transcriptional up-regulation via the cAMP/PKA/CREB pathway in order for cellular apoptosis to take place (Martinez-Velazquez et al., 2007). Diablo/SMAC has also been reported to be transcriptionally regulated by the transcription factor E2F1 and to potentiate apoptosis induced by 4-hydroxytamoxifen (Xie et al., 2006) Thus, there is mounting evidence that

cellular levels of Diablo/SMAC can be modulated transcriptionally and that this alters the cellular response to apoptotic stimuli.

5. Conclusions

Based on the results reported here and previous knowledge of mammalian Diablo/SMAC, it seems that *P. flesus* exposed to chemical pollutants, both acutely and chronically in the environment, increase the expression of Diablo in response to cellular damage. This increase in Diablo would likely have consequences for the regulation of apoptosis and of inflammatory processes, sensitising cells to programmed cell death and also causing changes in expression of inflammatory genes. In this regard it has also been observed that, along with Diablo, a group of genes involved in inflammation are upregulated in flounder liver following chronic exposure to sediments contaminated with mutiple pollutants (Leaver et al., 2010).

Furthermore the increase in pfDiablo mRNA following pollutant exposure may provide a novel and useful biomonitoring tool which integrates multiple pathways of chemical damage and toxicity at the level of apoptosis and inflammation. However, several questions remain to be addressed, including the IAP-interactions and apoptotic functions of the two fish Diablo forms, their mitochondrial and cytosolic expression patterns, as well as the mechanisms by which chemical pollutants cause increases in Diablo mRNAs.

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