Aims

This study aims to evaluate the influence of environmental pollutants to liver mRNA and protein expression of selected CYPs as biomarkers of pollution.

Procedure

Sample collection Nile tilapias were collected from February 22 – March 4, 2013 in Sta. Rosa and Los Banos, Laguna, and in Bilar, Bohol, Philippines. The fish samples were immediately dissected on board after measurement of biometry (body length, body weight, etc.). Organ (liver, spleen, muscle, and kidney) samples were removed and total organ weight was measured. The subsamples were frozen in liquid nitrogen, transported to Ehime University, Japan, and stored at -80°C.

Western Blotting

Equal amount liver microsomal proteins of each sample were loaded onto precast 4%-12% Bis-Tris gels (Invitrogen) and ran in MOPS buffer under reduced conditions. Electrophoresed proteins were then transferred to a nitrocellulose membrane using iBlot (Invitrogen) according to the standard protocol. The membranes were blocked with TBS-T containing 5% nonfat milk powder and 0.1% Tween 20 at 4°C overnight. CYP1A1 and CYP1B1 isoforms were detected using rabbit polyclonal anti-CYP1A1 (1:1000, Sta. Cruz Biotechnology), and rabbit polyclonal anti-CYP3A (1:1000, Sta. Cruz Biotechnology, respectively. Appropriate secondary antibody conjugated with horseradish peroxidase (HRP) was used to visualize the primary immune-recognitions using the ECL kit (ECL, Amersham Pharmacia Biotech) and imaging analyzer ChemiDoc (Bio-Rad).

Quantitative PCR To quantify mRNA expression levels of CYP1a1, two step real-time RT-PCR was performed. Total RNA was isolated from pooled samples in each sampling sites and then treated with DNase. After reverse transcription by a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), the cDNA was applied for a real-time PCR using Power SYBR® Green PCR Master Mix and StepOnePlusTM Software (Applied Biosystems). The optimization of the real-time PCR reaction was performed according to the manufacturer's instructions (Applied Biosystems). The PCR conditions were 10 min at 95 ◦C, 40 cycles of 15 s at 95 ◦C and 60 s at 95 ◦C. The calibration curves were generated by plotting Ct values against logarithmic value of template concentration. Each reaction was run in triplicate and inter-samples normalization was performed.

Results

CYP genes play an important role to regulate physiological functions. The induction of CYP1A in various fish species has been widely used as a biomarker of contamination in the aquatic environment. Meawhile, Nile tilapia has been used a sentinel species of aquatic biomonitoring.

In this study, CYP1A1 and CYP1B1 protein and CYP1A1 gene expression in Nile tilapia from the Philippines were determined. Protein expressions were done by Western blotting and densitometric analysis. Results of immunoblot analysis of proteins in liver microsomes of tilapia from two sites in Laguna: Los Banos (LB) and Sta. Rosa (SR), and a reference site Bilar, Bohol (BB) are shown in Fig. 1 and Fig 3.

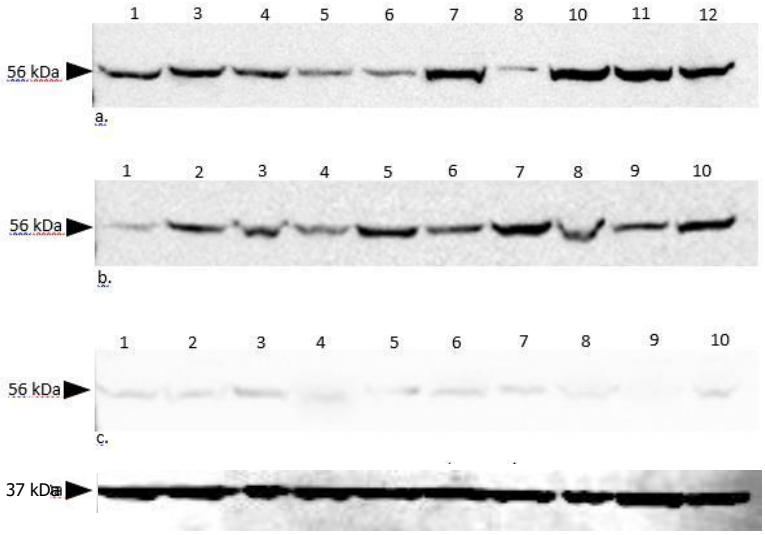

Figure 1. Western blots analysis of CYP1A1 proteins. Representative immunoblots of from Nile tilapia in (a) Los Banos (b) Sta Rosa and (c) Bilar, Bohol. Total protein (48 µg) was loaded in each lane. The levels of GAPDH (loading control) at 37kDa were analyzed to ensure samples' loading amount.

The immunoblot analysis of liver microsomes showed elevated contents of CYP1A1 in Los Banos and Sta Rosa fish compared to the content in reference site fish. There was an observed significant difference (*p<0.001*) in the CYP1A1 protein levels between the Laguna sites and the Bohol site (Fig 2). CYP1A1 in the fish samples from Los Baños and Sta Rosa was 2.4x-fold and 1.6x fold increase, respectively, than in Bohol.

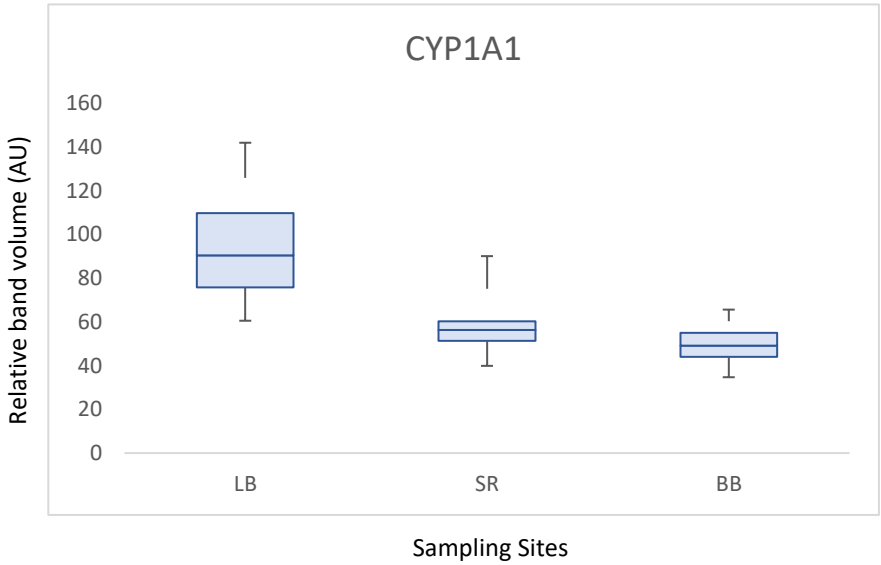

Figure 2. Boxplot of relative protein expression of Nile tilapia CYP1A1. Solid line within the boxes indicate the median. The box encompasses 25th and 75th percentiles. Mean

CYP1A1volume was 42.63 for LB, 39.9 for SR, and 34.7 for Bohol ($p = 0.000895$, One-way ANOVA).

For CYP1B1, immunoblot analysis of liver microsomes also showed elevated protein contents of in Los Banos and Sta Rosa fish compared to the content in reference site fish (Fig. 3). There was an observed significant difference ($p<0.001$) in the CYP1B1 protein levels between the Laguna sites and the Bohol site (Fig 4). Los Banos CYP1B1 was significantly higher ($p=3.7E-05$, Student t-test) than in Bohol; Sta Rosa was also significantly higher than Bohol CYP1B1 ($p=0.0005$, Student t-test). Further, between the two Laguna sites, CYP1B1 in the fish samples from Los Baños and Sta Rosa were significantly different ($p=0.0005$, Student t-test).

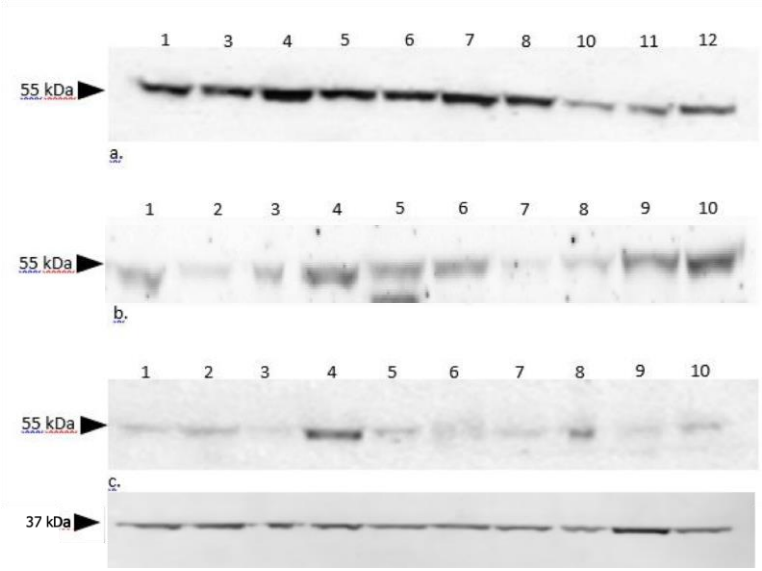

Figure 3. Western blots analysis of CYP1B1 proteins. Representative immunoblots of from Nile tilapia in (a) Los Banos (b) Sta Rosa and (c) Bilar, Bohol. Total protein (48 µg) was loaded in each lane. The levels of GAPDH (loading control) at 37kDa were analyzed to ensure samples' loading amount.

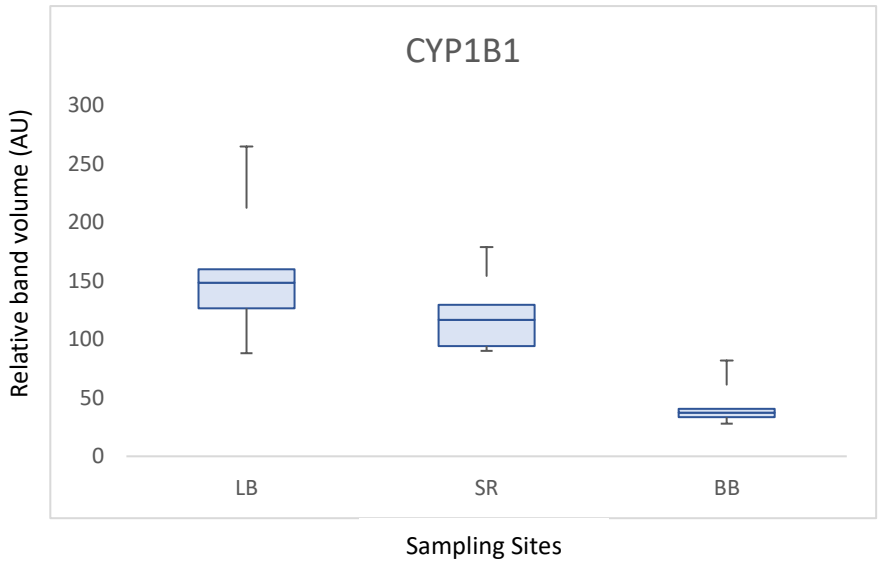

Figure 4. Boxplot of relative protein expression of cane toad CYP1B1Solid line within the boxes indicate the median. The box encompasses 25th and 75th percentiles.

Unlike CYP1A1, the CYP1B1 gene is expressed at low levels in the liver of fishes. The expression of CYP1B1 in rare minnow was high in the brain and low in the liver (Yuan et al, 2013). This may due to the different endogenous functions of CYP1A and CYP1B1. CYP1A has been proved to play a role in metabolizing endogenous AHR ligands, while CYP1B1 can catalyze the formation of retinoic acid and may play a role in retinoic acid mediated patterning during embryogenesis (Gao et al., 2011).

The observed increase in both CYP1A1 and 1B1 enzymes indicate that the Nile tilapia cell or tissue can form active AhR complexes and responds on a molecular genetic level to PCDDs, PCDFs and PCBs present in the lake environment. Though CYP1A gene of tilapia were identified in previous studies, the molecular characterization and basal expression and induced expression of other CYP1s, still needs to be investigated.

The quantitation of CYP1A mRNA levels as a direct indicator of CYP1A gene expression may help integrate the overall mechanisms of regulation specific for fish species. The transcripts of the Nile tilapia genes were determined using the following primers:

CYP1A	Forward	ATCTGTGGCACGTGCTTTGGCCGGCG
	Reverse	TGCCACTGATTGATGAAGACACAGG
Beta Actin	Forward	CACACAGTGCCCATCTACGA
	Reverse	TCCTTCTGCATCCTGTCAGC

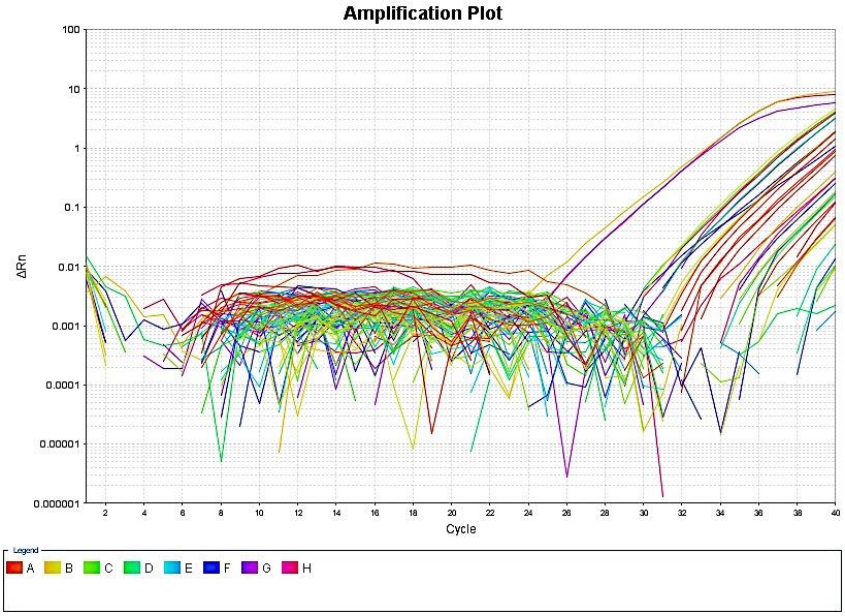

Figure 5. Amplification plot for Real Time-PCR reaction showing the fluorescence signal versus cycle number for an endogenous control (Beta actin) Nile tilapia genes.

Amplification of the B-actin gene to determine the CYP1A1 gene expression however did not yield transcript products. It is recommended to continue perform the gene expression experiments and check the amplification of Nile tilapia CYP1A1.

Previous studies have shown that CYP1A, 1B1, 1C1, 1C2, and 1D1 genes in fish were discovered and identified. Some studies show that the transcripts of CYP1A and CYP1B1 in mammals and other fishes were detected in muscle or skin (Yengi et al. 2003; Lee et al. 2005), whereas the transcripts of CYP1A and CYP1B1 in rare minnow were not detected. Based on the results of this study, further studies are therefore recommended to identify CYP 1B1, 1C1 and CYP2A genes and proteins in Nile tilapia and to characterize their basal expression pattern in different tissues such as gill, intestine, kidney, spleen, brain, skin, and muscle, which are involved with the uptake and metabolism of pollutants.

References:

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Yengi, L.G., Xiang, Q., Pan, J., Scatina, J.A., Kao, J., Ball, S.E., Fruncillo, R., Ferron, G., Roland Wolf, C., 2003. Quantitation of cytochrome P450 mRNA levels in human skin. Anal. Biochem. 316, 103–110.

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