Title of research project: Assessment of environmental contamination in water and cultured fish in San Pablo lakes and toads in Laguna Lake, Philippines

Names of project members

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Purposes

This study generally aims to examine the presence and effects of environmental pollutants in the freshwater fishes in San Pablo and other non-target organisms such as cane toads in Laguna Lake and identify the potential risk factors contributing to contamination. Specifically, this study determined the potential effects of environmental pollutants in the expression of selected cancer-linked genes.

Methods

Sample collection

Water samples from San Pablo lakes were collected in 2019-2020. Water was collected at the surface (1m depth) and then transferred to 1L amber bottles, which had been washed with ethanol and deionized water. The water samples were then transferred to the laboratory and stored at 0–4 °C. Selected organochlorine pesticides were analyzed by mass spectrometry. Nitrate and phosphate analysis were determined using a spectrophotometer and commercially available test kits.

Cultured tilapia fish from San Pablo lakes and cane toads around the riparian zones of Laguna Lake were collected. Tissues were removed and transported to Ecotoxicology Laboratory, CMES, Ehime University, Japan, and stored at -80°C for further analysis.

Quantitative PCR

To quantify mRNA expression levels of different genes as biomarkers for pollution, two step real-time RT-PCR was performed. Total RNA was isolated from the liver samples and then treated with DNase. Reverse transcription by a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was done and ran for amplification with GeneAmp PCR System 9700 (Applied Biosystems). The cDNA was then subjected to real-time PCR using TB Green ® Premix Ex Taq II (Tli RNaseH Plus) Master Mix and analyzed using StepOnePlus Real-time PCR System (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 normalization was performed using endogenous genes.

Results

The seven tropical maar lakes in San Pablo City, and the Laguna Lake are freshwater lakes found in Laguna, Philippines and are under the monitoring and

administration by the Laguna Lake Development Authority (LLDA). Both lakes offer several ecosystem services to the neighboring communities. Fish pens and floating cages are common features and increased anthropogenic activities near these lakes may have contributed to degrading water quality and lake pollution. In Lakes Palakpakin, Sampaloc, and Pandin, emerging organic contaminants (EOCs) were found such as pesticides, pharmaceutical compounds, organophosphate-based fire retardants and plasticizers, artificial sweeteners, and surfactants (Dimzon et al, 2018). A study conducted in Bunot Lake found that the lake was contaminated with organic pollutants, such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), which are associated with industrial and urban pollution (Salvacion et al., 2016). The study found that the PAH and PCB concentrations were highest near the industrial and urban areas surrounding the lake.

The presence of persistent pollutants affects the water quality and may have adverse effects on the organisms in the lake as well as on human health. Exposure to environmental pollutants has been linked to an increased risk of developing cancer. Environmental pollutants can damage DNA and cause mutations in cancer-linked genes, leading to an increased risk of cancer development. Exposure to chemicals such as polychlorinated biphenyls (PCBs) and dioxins has been shown to affect the expression of genes involved in cell proliferation, differentiation, and apoptosis, leading to alterations in cell behavior that may promote cancer development. Understanding the link between environmental pollutants and cancer-linked genes is crucial for developing effective strategies to identify potential biomarkers of exposure and effects that could be used in future monitoring efforts.

Expression of cancer-linked genes were assessed in the liver of toads from Laguna Lake. The primers used in amplification and expression using quantitative real-time PCR are shown in Table 1.

Table 1. Primers for qRT-PCR of cancer-linked genes in *Rhinella marina.*

Gene	**Primer**	**Sequence (5' - 3')**	**Length (bp)**	**Tm**	**GC%**
anxa2	Forward	GTCTCACAGTAGGCGGCCAG	20	62.56	65
	Reverse	CATTGCTGTGTCCCCGATGC	20	62	60
smarca2	Forward	TGGTCAAAAGGTGGCACTGA	20	59.74	50
	Reverse	GGTCCCATTCCTGTGTGAGG	20	60.04	60
p53	Forward	ATTGCACAAAGAAACGGGGC	20	59.97	50
	Reverse	TAGAGGCCCTCCCTTTACCC	20	60.03	60

angptl7	Forward	GCCTGACCTCCTTCAACCGA	20	61.83	60
	Reverse	ATTCTGCGTGCCGTACACCT	20	62.17	55
itgb1	Forward	CAGACCTGCGTTGGTGTTTG	20	59.97	55
	Reverse	GTCACGGCGCTCTTGTAGAT	20	60.18	55
ptp4a3	Forward	CATGTGCTCAGGGAAAGCAG	20	59.19	55
	Reverse	TCGAACGGCCAGTCCATTAC	20	60.11	55
e2f4	Forward	GGGCTACTCACCAGCAAGTT	20	59.96	55
	Reverse	TTCTGGAGGTGGAACAGGGA	20	60.1	55
mmp1	Forward	GGCTGCTAACACCACAGACT	20	59.96	55
	Reverse	ATGAGCAACAGTCGCTGTCA	20	59.97	50
*gapdh**	Forward	TGGGCTCTCGTGAGTCTTCT	20	60.25	55
	Reverse	TCTTGGAAGACGGTGACTGC	20	59.97	55

*Reference gene

Annexins are a family of calcium-dependent phospholipid-binding proteins that are involved in a variety of cellular processes, including cell signaling, membrane trafficking, and cytoskeleton organization. Specifically, anxa2 modulates internalization of cell surface β1 integrin, such as integrin beta-1 (*itgb1*), required for forward cell movement (Rankin et al, 2013). Relative *itgb1* mRNA expression in the liver of cane toads from Laguna Lake showed higher expression in the western bay (Sta. Rosa), which is characterized as highly urbanized and industrialized area (Figure 1A).

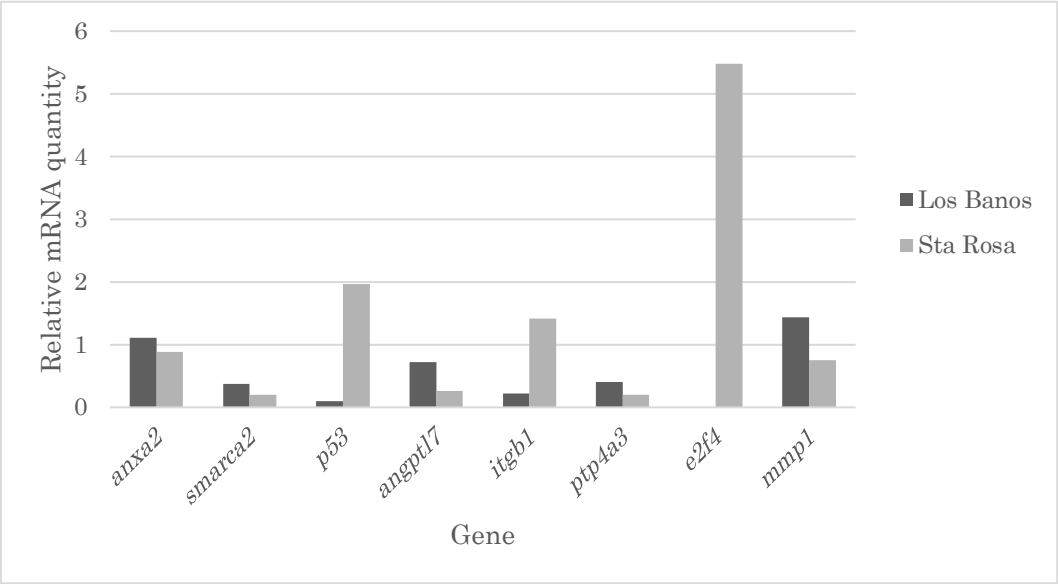

Figure 1. Relative mRNA expression of cancer-linked genes in *Rhinella marina*.

Some studies that have investigated the effects of environmental pollutants on *anxa* and *itgb1* gene expression showed that polycyclic aromatic hydrocarbons

(PAHs) exposure induced changes in the expression of several ANXA genes, including *anxa1*, *anxa2*, and *anxa6* in the liver of zebrafish (Wang et al., 2015). Meanwhile, Chen et al. (2017) investigated the effects of exposure to benzo[a]pyrene (BaP) on *itgb1* expression and function in human lung cells. They found that BaP exposure induced changes in ITGB1 expression and promoted cell migration, invasion, and metastasis.

Similar patterns of *smarca2* and *ptp4a3* gene expression was observed in the liver samples from the South Bay (Los Banos) and West Bay (Sta Rosa). *Smarca2* is a member of the SWI/SNF chromatin-remodeling complex, and alterations in its expression have been linked to various types of cancer. The *ptp4a3* gene encodes for the protein tyrosine phosphatase PRL-3, which has been implicated in various biological processes, including cell proliferation, migration, invasion, and metastasis. *ptp4a3* has been shown to be overexpressed in many types of cancer.

Like *ptp4a3, p53* expression or function have been linked to the development of various types of cancer. This gene is a tumor suppressor gene that is involved in the regulation of cell growth, DNA repair, and apoptosis. This is also associated with *e2f4* gene which is a member of the E2F family of transcription factors that play a key role in the regulation of cell cycle progression and DNA replication. In this study, there was an observed significant high expression in the liver samples from Sta Rosa (Figure 1).

On the contrary, significantly higher gene expression of *angptl7* and *mmp1* genes were observed in Los Banos, characterized as agro-industrialized area. *Angptl7* gene encodes a protein that is involved in the regulation of lipid metabolism and angiogenesis, while *mmp1* as a member of matrix metalloproteinases (MMPs) and its tissue inhibitors, have been implicated in the pathogenesis of liver diseases and cancer. Xu et al. (2019) examined the effects of exposure to benzo[a]pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH), on *mmp1* expression and function in human skin cells. They found that BaP exposure induced changes in *mmp1* expression and promoted cell migration and invasion, contributing to skin carcinogenesis. Guo et al. (2020) investigated that exposure to perfluorooctane sulfonate (PFOS) induced changes in *mmp1* expression and activity and promoted cell invasion and metastasis.

In summary, these findings suggest that environmental pollutants have potential impacts on organisms such as amphibians in and around lakes and other aquatic ecosystems. Exposure to environmental pollutants can affect the expression of cancer-linked genes, leading to changes in cellular processes that may contribute

to cancer development. Gene expression analysis showed valuable information on the mechanisms underlying the response of non-target organisms to environmental pollutants. It can also help to identify potential biomarkers of exposure and effects that could be used in future monitoring and conservation efforts, and for assessing the potential risks to aquatic organisms and on human health.

Future challenges

The potential impacts of environmental pollutants on the water quality and aquatic organisms in lakes, as well as the need for continued monitoring and management of pollution in the area needs to be done. Also, more research is needed to fully understand the implications of altered gene expressions in fishes and amphibians and to determine the potential long-term effects of exposure to environmental pollutants on health and populations. Selected toxic and biomarker genes in the cultured fish samples from San Pablo lakes are yet to be assessed for understanding the effects of environmental pollutants in these freshwater fishes. However, while more research is needed, reducing pollution is an important step in protecting aquatic organisms and maintaining healthy ecosystems.